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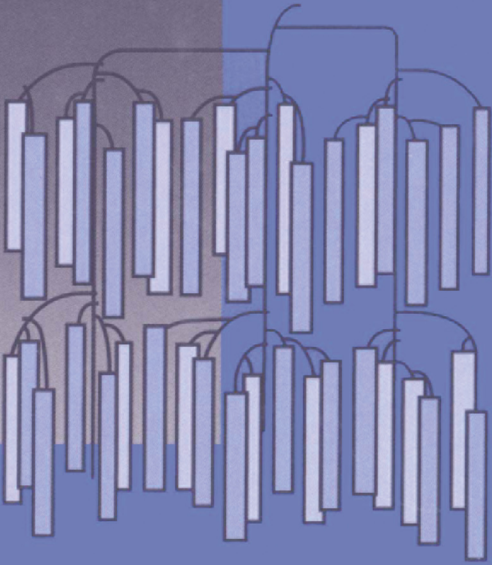

# starch
## ADVANCES IN STRUCTURE AND FUNCTION

*edited by* T.L. BARSBY,
A.M. DONALD *and* P.J. FRAZIER

# Starch
## Advances in Structure and Function

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# Preface

Starch is the primary component of most plant storage organs – tubers, cereal grains and legume seeds – and provides an essential food energy source for the global human population. Starch also contributes greatly to the structure (texture or viscosity) of a wide range of home-prepared and manufactured foods. Thus increased understanding of the granule synthesis and its behaviour in modern food processing is of vital importance to both manufacturers and consumers.

This book is the second in a series on Starch: Structure and Function and presents the proceedings of an international conference held at Churchill College, Cambridge from 27–29 March 2000. The meeting, organised by the Food Chemistry Group of the Royal Society of Chemistry, followed a highly successful meeting held in April 1996 (Starch: Structure and Functionality, 1997, Royal Society of Chemistry, Cambridge) and adopted a similar formula. Invited speakers provided key contributions on starch structure and characterisation, processing and ingredient functionality, and control of starch biosynthesis. Submitted research papers and posters delivered the latest information in various facets of these areas. The meeting was attended by biologists, chemists, food technologists, geneticists, nutritionists and physicists, so care was taken to ensure that lecture sessions were each made up of a range of topics to encourage inter-disciplinary discussion and promote wider understanding. However, for this book, chapters have been rearranged as far as possible to group similar topics together. The editors sincerely hope that this volume will provide a valuable reference compilation of the advances made in this field since 1996.

As before, the venue for the conference, Churchill College, was impressive and although the weather was not as kind as in 1996, the warmth of the friendships from across continents meeting again in Cambridge more than made up for the chill outside. Thanks to financial support from Biogemma UK Ltd and DuPont Cereals Innovation, the hospitality at both the poster sessions and the conference dinner was first class and Professor Derek Burke, CBE, past Chairman of the UK Advisory Committee on Novel Foods and Processes, gave an excellent after dinner talk to accompany the wine. Our thanks are also due to the Biotechnology and Biological Sciences Research Council who provided additional support for the meeting and to many individuals from the Cavendish Laboratory who assisted with the arrangements – most especially to Mrs Meg Staff who so adeptly managed all the administrative tasks involved with registering and accommodating over 160 delegates from around the world, not to mention preparation of all the conference papers and poster abstracts, *etc.* Success breeds success and by popular request we shall do it all again in 2004 when Churchill College has again been booked from 29–31 March.

Tina Barsby
Athene Donald
Peter Frazier

# Contents

# STARCH STRUCTURE / FUNCTION RELATIONSHIPS: ACHIEVEMENTS AND CHALLENGES

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## 1 INTRODUCTION

The importance of starch, both biologically and technologically, is well known, as is its central role in the human diet. Many aspects of starch structure can be measured or detected by one or more chemical, physical, spectroscopic or microscopic methods. Functional performance of starches in technological applications can usually be assessed with appropriate end-use mimic tests, and underlying material properties can be measured using the arsenal of techniques available to the physical scientist. However, despite major progress in many aspects of starch structure / function relationships, there is still a significant gap in our ability to predict the functional properties of starch from a knowledge of structure. This is particularly relevant in the post-genomic era where it is, or soon will be, possible to select or modify plant sources of starch at the genetic level. There will therefore be a growing need to link structural features more precisely and predictably to defined functional performance. This review seeks to identify some of the key successes in describing and quantifying starch structure and function, as well as highlighting some of those areas where information or mechanistic understanding is lacking. Given the broad nature of the area, the choice of topics is necessarily subjective and will not include much in the way of introductory material.

## 2 LEVELS OF STRUCTURE IN STARCHES

Despite the textbook description as a ‘simple’ polymer of glucose, starch is one of Nature’s most complex materials. There are two fundamental factors behind this complexity. One is the existence of characteristic structures over a wide range of distance scales, and the second is the heterogeneity of structure at all of these different distance scales both within a single granule as well as the natural variation inherent in populations of granules.

At each level of structure, there have been significant recent advances in methodology and information. Progress in tackling the heterogeneity problem has been slower reflecting the greater challenges involved.

## 2.1 Molecular Structure

Methods for probing the molecular structure of starch polymers are well advanced. A combination of chemical[1], enzymic[2] and NMR[3] methods are available for describing the branching pattern and branch length profile of polymers. When coupled with prior chromatographic fractionation, a high information content is obtained. It is now accepted that there is a continuum of starch polymer structures from purely linear to extensively branched, and that for some starches there is a difficulty in describing constituent polymers as purely amylose (lightly branched, molecular weight $10^4$ - $10^6$ Da) or amylopectin (heavily branched, molecular weight $10^6$ - $10^8$ Da). Although branching levels and branch lengths can be readily determined, it is more difficult to define the pattern of branching points. It is well established that branch points have a tendency to cluster, but it is more difficult to determine the local molecular architecture around branch points. NMR methods[4] may help in this area in the future.

Molecular size is relatively easily determined for low or intermediate molecular weights, but sample preparation and handling requirements may make it difficult to identify true molecular weights for some large amylopectins. This is because above $10^8$ Da, molecules may be sufficiently large that either they are mechanically unstable in solution or they are more colloidal than molecular in dimensions and consequently cannot be chromatographed or measured accurately by light scattering.

It is now clear that it cannot be assumed that molecular structure features are distributed evenly throughout granules. This can be shown by differential iodine staining[5] or by sequential solubilisation from the outside of granules under defined chemical regimes[6]. It is not known whether this distributional heterogeneity has any impact on functional properties.

## 2.2 Helices and Crystalline Order

There are significant stretches of contiguous linear glucan in all starches. This leads to the potential to form the repetitive glycosidic conformations characteristic of helices. A range of single- and double-stranded helices have been identified and characterised from native and treated starches. Starch chains usually show a high proportion of local glycosidic conformations characteristic of V-type structures[7] that can be stabilised by complexation with iodine, fatty acids, monoglycerides etc. Endogenous starch granule lipid is present in such V-type complexes[8]. Double helical structures are found for part of the amylopectin component within granules, and are formed from both amylose and amylopectin after gelatinisation. A minimum length of ten glucose residues is required for double helix formation[9], although chains as short as six residues can co-crystallise[10]. It seems clear that the outer branch length of amylopectin is the major determinant of which double helical polymorph is found in native starch granules. Relatively short branches lead to A-type with longer branches giving B-type order[11]. A- and B-type polymorphs have very similar individual helical structures but differ in packing arrangements[12, 13]. *In vitro* studies suggest that the A-type polymorph is the kinetic product and B-type the thermodynamic product of amylopectin (branch) crystallisation[14]

From comparative $^{13}C$ NMR and X-ray diffraction measurements of double helical content and crystallinity extent respectively, it is found that typically about 40-50% be weight of starch chains are present as double helices in granules with approximately half of these helical chains present in crystallites large and perfect enough to diffract X-rays[15].

A significant recent advance has been the application of microfocused synchrotron X-ray beams to identify the relative extent and orientation of crystalline elements at defined positions within individual granules[16,17]. For larger granules such as from potato, a detailed picture of crystalline ultrastructure can be obtained. No such description of non-crystalline double helix (or single helix complex) location or orientation is possible yet.

### 2.3 Granule Ultrastructure and Morphology

A well-known characteristic of granular starch is the appearance of a 'Maltese cross' birefringence pattern under polarised light. This indicates a radial distribution of sub-micron elements within granules, and may be related to a structural motif with a repeating distance of ca. 9nm observed by small angle scattering[18]. This is ascribed to the regular repeating distance between adjacent clusters of branch points in the structure of amylopectin. Microfocus X-ray experiments[16] show that these repeating distances have a radial arrangement as is inferred for the longer distance scale (hundreds of nms) evident from light birefringence.

Little is known about the micron scale architecture of granule segments, although it is tempting to infer the presence of 'super-helices' from electron micrographs of granule shavings[19] and from detailed analysis of scattering and diffraction data[20]. A super-helical framework would provide an appropriate mechanism by which granule development could occur during biosynthesis, but remains unproven at this stage.

It can be inferred from very different swelling and leaching properties, that the local environments of amylose and amylopectin differ significantly between granules from different botanical sources. In particular, the ability of a fraction of amylose to be leached from many starches around their gelatinisation temperature shows that the forces that retain amylose in hydrated granules are overcome at 60-80°C. This rules out amylose/amylose double helices which are not expected to dissociate until above 100°C. Despite the fact that amylose double helices form more readily than amylopectin double helices from solutions, it is the latter that predominate in native granules. Nature has therefore found a way of kinetically trapping amylose as individual / separated molecules within granules. How this is achieved over the long time periods involved in granule biosynthesis is an intriguing question.

The size, shape and surface characteristics of starches are relatively well conserved for a given biological source, yet essentially nothing is known about the biological or physico-chemical factors that control these features. The surfaces of extracted starches frequently contain components (particularly proteins and lipids) characteristic of the matrix from which they were extracted e.g. cereal endosperms.

## 3 FUNCTIONALITY

From the above, it can be seen that starch structure has a series of characteristic length scales which all need to be taken into account when trying to derive mechanistic links to functional performance. To exemplify the challenge, consider a typical piece of e.g. food with ca. 2cm dimensions as an example of a functional application. If the length scale of an individual glucose residue (0.5nm) is compared with a person (1-2m), then 1-2cm is comparable to a global distance scale. This is illustrated below, including intermediate structural elements.

Relative Distance Scales

| Feature | Size | Relative Size | |
|---|---|---|---|
| glucose unit | 0.5nm | 1 |  |
| amylopectin branch | 10nm | 20 |  |
| granule | 20μm | 40,000 |  |
| foodstuff | 2cm | 40,000,000 |  |

It is apparent from this analogy that attempts to describe cm ('global') scale functionality based on nm ('person') scale structures is a major challenge. This is true for all types of materials, but is magnified for starches due to (a) the presence of characteristic features not only at the polymer length scale but also at the granule length scale, and (b) biological variation (heterogeneity) at every length scale.

In the context of this review, functionality is primarily concerned with rheological and structuring properties obtained after cooking. The approach will be to identify which of the various properties associated with starch are primarily due to molecular level effects, which are mostly derived from granule level behaviour, and which are a complex mixture of both. Over-simplification will be used to emphasise this distinction.

## 3.1 Molecular Level Functionality

*3.1.1 Gelatinisation Temperature.* As a first approximation, the length of double helices is proposed to be a determinant of gelatinisation temperature. Amylose double helices in e.g. gels probably occur over a length scale of 40-80 residues[21,22] and melt at approx. 150°C. Typical amylopectin-based double helices occur over 15-20 residues and melt at 60-80°C. Longer branch lengths found in so-called high amylose starches are proposed to be responsible for gelatinisation temperatures of 80-110°C. Lowest observed gelatinisation temperatures are around 50°C, probably corresponding to double helical lengths around the minimum of 10 residues[10]. Presumably shorter branch lengths (as found e.g. in glycogens) will not lead to stable granules. It seems unlikely that gelatinisation temperatures much lower than 50°C will be achieved / found for native starches.

*3.1.2 Solubilised Starch.* When sufficient energy has been applied to a starch system to completely erase all supra-molecular order, then the expectation is that the constituent polymers will behave as any other polymeric system. In several respects this is the case. One example is in phase separation behaviour. Despite the chemical similarity of amylose and amylopectin, mixtures of the two in solution show evidence of phase separation both with themselves and added polymers[23]. A second example is in the behaviour of depolymerised starches as modulators of freezing (at high moisture) and solidification or glass-like (at low moisture) properties[24]. In the absence of retrogradation, there is nothing unexpected in these properties of starch polymers based on the general physico-chemical principles of polymer behaviour.

*3.1.3 Retrogradation.* Retrogradation is a word invented and defined by the starch community[25]. In essence, however, the underlying mechanisms are analogous to those found for many other helix-forming polysaccharides[26]. This stems from the central role of double helix formation in either amylose or amylopectin retrogradation behaviour. For long amylose chains, gelation and related network properties are a direct result of multiple helix formation creating a meshwork of cross-links between chains in an exactly analogous mechanism to e.g. gelatin or agar. For amylopectin, the analogies are fewer due to the unusual clustering of relatively short branches. Nevertheless the factors affecting double helix formation in amylopectin and underlying e.g. bread staling are in general predictable based on the mechanism involved.

Complexity increases when mixtures of amylose and amylopectin are retrograding. Relevant questions here include the kinetics of phase separation compared with those for double helix formation and the possibility of helices linking amylose and amylopectin molecules. These factors are likely to be in a delicate balance, resulting in a richness of potential properties but also a difficulty in predicting the outcome. The effects of detailed amylose and amylopectin structure on mixtures of the two have not yet been put into a mechanistic framework.

## 3.2 Granule Level Functionality

*3.2.1 Swelling.* Following the molecular level melting induced by heating granules, a swelling process ensues. When observed microscopically, it is apparent that individual granules in any population go through the structural disorganisation phase of gelatinisation (typically monitored by optical birefringence) over a narrow temperature range followed by a characteristic swelling behaviour. Attempts to describe the gelatinisation of a collection of granules as a single process are therefore inappropriate. Although swelling behaviour is characteristic for botanical origin, there is no coherent explanation for observed differences. One factor which is certainly important for cereal starches such as maize and wheat is the amount and location of non-polysaccharide components. This is most graphically demonstrated by the consequences of removing surface lipids and proteins with sodium dodecyl sulphate (SDS) extraction. Whilst the swelling properties of starches such as waxy maize and tapioca (which naturally exhibit rapid and extensive swelling) are unaffected, wheat and maize starch swelling is dramatically altered. Following SDS extraction, these starches show similarly rapid and extensive swelling as waxy maize and tapioca[27]. This emphasises that in assessing the structural basis for starch functional properties, minor components as well as the major polysaccharides need to be taken into account.

*3.2.2 Why Don't Gelatinised Granules Dissolve?* Many biopolymers other than starch are used commercially starting from an ordered solid state (e.g. agar, gelatin). In order to solubilise these polymers, heating to above the relevant melting temperature is required. However, once this temperature is exceeded, the polymers readily dissociate from the 'granule' into solution. Why then does this not happen for starch? Two possible mechanisms can be put forward. One is that there are as yet unidentified restraining cross-links within granules that serve to limit granular swelling. Despite several studies, no evidence for this mechanism has been demonstrated[27]. A second possibility is that double helix (re-)formation occurs during the process of granule expansion, thereby preventing dissolution. In the absence of other evidence, this seems more likely as there are analogies with the behaviour of other gelling polysaccharides[28].

*3.2.3 Rheology of Swollen Granules.* For the simplified case of granules that swell on cooking without leaching polymeric material, the physics of deformable particles should apply. In rheological studies of chemically-crosslinked starches where this simplified case is applicable, this was indeed found to be the case[29]. In particular, the concentration dependence of the elastic modulus had a characteristic form also seen in other deformable particles such as beads of cross-linked dextran (as used in chromatography). The basis for this type of rheology is the volume occupancy of particles combined with repulsive forces between particles when they come into contact. However, none of a range of native starches showed this same class of rheology[29], indicating that this is too simplistic a view for unmodified starches. It is not yet clear why this is so. It is possible e.g. that leached polymers play a role or that the surfaces of swollen granules are not very distinct or that material distribution within swollen granules is significantly heterogeneous. Whatever the reason, it is an enduring challenge to adequately describe the physical mechanisms underlying cooked starch rheology. If swollen particles are critical, then micromechanical measurements of individual granules [30] should provide insights into the factors affecting bulk rheology.

### 3.3 Real World Functionality

As described above, it is possible to describe structuring and rheological properties for several well-defined model systems such as individual polymer types or chemically-crosslinked swollen granules. However, it cannot yet be claimed that there is true quantitative and mechanistic understanding for any real world application. This is not to say that there is no knowledge in the area; far from it. Producers and users have very successfully generated empirical 'rules' for selection and tailoring of starches to fit required uses. For many types of functional properties, there are directional guidelines for raw materials (e.g. more or less amylose, cross-linking etc.) and processes (e.g. time / temperature / shear regimes). The challenge now is to build on these guidelines and use the power of e.g. genetic diversity[31] and computational methods to tackle the complexity inherent in defining starch structure/function relationships in true scientific terms.

## 4 CONCLUDING REMARKS

The study of starch structure / function relationships continues to provide many challenges to physical, chemical and biological scientists. Complexity and heterogeneity superimposed on a hierarchy of structural elements at a range of distance scales provide plenty of opportunities for further definition of structural features. The interplay of kinetics and thermodynamics as drivers superimposed on structural diversity lead to many unanswered questions on the road to a full mechanism-based understanding of functional performance.

In the era of functional genomics, during which the biological activity of gene products and the factors that control gene expression will be elucidated, starch remains an excellent test case for our ability to make the connections illustrated below:-

macroscopic function ↔ macroscopic structure ↔ molecular structure ↔ biosynthetic genes / enzymes

Advances in understanding each of the elements above is required, but increasingly the focus should shift to integrated studies in which questions of function are addressed through control of structure. In turn this should eventually lead to molecular level specifications for desirable functional properties together with a toolbox of techniques for sourcing the specified structures.

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# MODELLING OF STARCH EXTRUSION AND DAMAGE IN INDUSTRIAL FORMING PROCESSES

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## 1 INTRODUCTION

Starchy foods represent the major source of carbohydrate in the human diet, estimated to comprise 80% of the global average calorie intake, and are available in various forms featuring different extents of pre-processing: (i) raw, e.g. potato or rice, featuring only post-harvest treatment and storage; (ii) as traditional foods, such as bread or pasta; (iii) as modern consumer foods, e.g. snack foods, featuring significant processing in order to achieve given textures, shapes and tastes. The latter category presents considerable challenges for manufacturers, as the rheology, chemistry and textural characteristics are often intimately related to the extent and nature of processing operations performed on the material. Gelatinisation of starch, for example, is affected by temperature, shear history and water content, all of which may vary during the manufacturing process and can be exploited to generate particular product forms or characteristics.

Extrusion is a frequently used forming technology in the food industry, where the food material may be mixed, wetted, melted, cooked and/or cooled, before being forced through a die in order to achieve a given product shape, and even texture (in the case of expanded corn snacks). Understanding the interactions between ingredients, process parameters and equipment (e.g. die) design and operation is therefore important in achieving product quality and developing new products. A significant body of work exists in the literature where relationships developed between process parameters and non-food starch characteristics have been used to improve process control, and provide insight into the effects of shear (predominantly in conjunction with temperature) on starch microstructures[1]. The physical and chemical complexity of many foodstuffs, however, means that such relationships can rarely be predicted in advance.

This work is concerned with the extrusion behaviour of a mixture of starchy solids used to make a snack food product. The key processing stage is ram extrusion at ambient temperatures, such that extrusion cooking does not occur. The composition of the starch dough is summarised in Table 1: it consisted of a wetted mixture of potato-derived materials, differing in their pre-processing history. Water is added to a dry mixture of the solid components and mixed to give a weakly cohesive dough, which is then charged to a

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ram extruder. The factory process involves a pre-compaction stage where the dough charge is de-aerated, prior to being extruded through a multi-holed die plate. The extrudate is cut as it leaves the die plate, cooked and conditioned. The objective of the work was to investigate how the processing step of extrusion affected the microstructure of the material, and vice versa, in order to explain certain phenomena experienced at the factory scale. Product properties directly determined by the microstructure were known to include the structural stability and cooking behaviour of the 'green' extrudate, and the appearance, taste and 'mouth feel' of the final snack. The results illustrate how complex microstructure-processing relationships can exist for a relatively simple food material.

**Table 1** *Summary of dough mixture ingredients*

| *Ingredients* | *% Content by Mass* |
|---|---|
| Potato Starch | 46 |
| Cooked Potato | 52 |
| (intact cells) | (41) |
| (ruptured cells) | (11) |
| Minor Ingredients | 2 |

## 1.1 Rheological Characterisation

The work reported here is concerned with axi-symmetric extrusion through concentric dies (of constant cross section), as shown schematically in Figure 1.

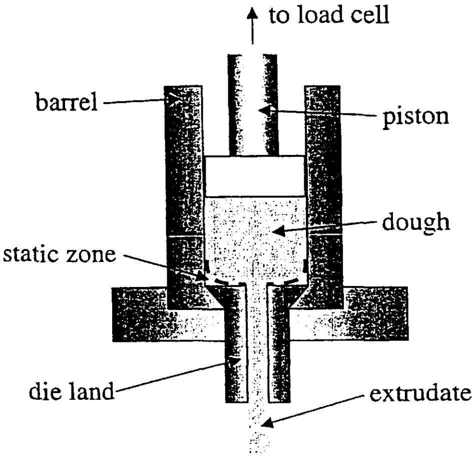

**Figure 1** *Schematic of the Dartec strain frame, modified for ram extrusion*

The material undergoes two different modes of deformation during the process: (*a*) mixed mode deformation at the die inlet, featuring significant elongation as the material changes shape, as well as any shear due to frictional contact at the equipment interfaces; (*b*) pure shear flow along the die land. A transition zone will exist at the die land entry where the shear flow is established; if the die land is relatively short, instabilities can arise which cause surface defects or even fracture[2]. The rheology of the material will therefore be determined by the response of the microstructure to this combination of deformation modes.

The dough studied in this work presents a challenge to conventional rheological classification, as it exists as a partially hydrated cohesive powder – similar to an unsaturated soil – which is compacted into a dense form before it undergoes yielding-type behaviour and deformation. The term 'dough' is used in a broad sense, as the material differs from bread doughs by containing no gluten and exhibiting little visco-elasticity. It is an example of a stiff 'paste', being a very dense suspension of solid particles, where the particles approach their maximum packing fraction. Such materials often exhibit non-linear shear rate dependency, and apparent yield stress behaviour.

Two studies of homogeneous mixtures of cooked potato particles[3,4] have been reported, over moisture content ranges of 35, 40 and 45 %wt at temperatures from ambient to 80°C and 50 and 60 %wt at ambient temperatures respectively. Reasonable agreement was found with Navier power law or Herschel-Bulkley models, viz.

$$\tau = K_b \dot{\gamma}^p \tag{1}$$

$$\tau = \tau_y + K_b \dot{\gamma}^p \tag{2}$$

Problems were reported modelling low water content materials at ambient temperatures in particular[3], where it was noted that there was difficulty in justifying use of analyses commonly applied to synthetic polymers. Under these conditions the rheology of doughs becomes more difficult to characterise successfully due to the increasing importance of solid-solid interactions, manifested in yield stress behaviour, and in the difficulty in performing reliable experiments. Slip behaviour and liquid phase redistribution effects can arise[5].

An alternative characterisation approach, which has been applied with reasonable success to the extrusion behaviour of 'stiff' pastes, is the quasi-plastic approach described by Benbow and Bridgwater[6]. This approach assumes that extensional deformation (at the die land entry) can be described in terms of plastic flow (modified for the strain rate), and that flow along the die land is dominated by wall slip. The force on a ram required to extrude the material in a system such as that in Figure 1, expressed as a mean extrusion stress *P*, is given by:

$$\begin{aligned} P = &\quad P_{deformation} \quad + \quad P_{friction} \\ &= \left(\sigma_o + \alpha V^m\right) \ln\left(\frac{A_o}{A}\right) + \left(\tau_o + \beta V^n\right) 4\frac{L}{D} \end{aligned} \tag{3}$$

where *V* is the extrudate plug flow velocity (i.e. pure slip). This approach has proved successful for describing the behaviour of related pastes and semi-solids such as ceramic pastes, soap, clays and fibre mixes at ambient temperature.

## 1.2 Microstructure

The microstucture of starch during processing has been extensively studied[7], but typically such work only considers the gelatinisation of pure starches at relatively high water contents (> 80 %wt). While it is well known that mechanical damage can be equally as significant as thermal degradation, there is relatively little literature examining the microstructural implications of mechanical energy input. Of this, almost all concerns the affect of screw extrusion on pure starches with plasticisers[1] where temperatures are typically 150 to 200 °C. As far as the authors are aware there have been no investigations of extrusion of mixes of native and gelatinised starch prior to this work.

Initial studies of both dough rheology and microstructure indicated that valuable insight could be obtained by examining the structure-deformation characteristics of each of the individual solid components. This information was then used to build up a composite qualitative model of the extrusion process.

## 2 EXPERIMENTAL

The starch dough was generated by dry mixing the solid components together in a Kenwood planetary mixer for 2 minutes before adding the water as a spray and mixing for a further 4 minutes. Doughs were kept at room temperature and used on the same day in order to minimise further liquid phase redistribution which was found to affect the extrusion behaviour.

The mixed dough resembled a cohesive powder rather than a saturated solid-liquid mixture. Water absorption isotherms indicated that the amount of water added (40 wt%) was insufficient to produce a fully saturated mixture, suggesting that the water content of each component was determined by competitive absorption during the mixing process. Hydration tests were therefore performed in order to gauge the relative rates of absorption of the solid components. Pre-weighed samples were contacted with excess water for prescribed periods, filtered to remove free liquid and weighed. The results are plotted in Figure 2, which shows significant differences in absorption behaviour between the individual components. The pre-gelatinised starch absorbed water significantly faster than the native starch, more water being absorbed by the unconfined fraction which was able to swell extensively.

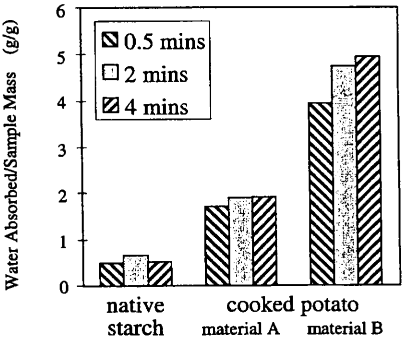

**Figure 2** *Water absorption rates of dough ingredients*

Compaction and extrusion experiments were performed using a Dartec A100 strain frame (Dartec, UK) configured to act as a batch ram extruder. Details of this device have been reported previously[2]. In the characterisation work, dough was extruded from a 25 mm i.d. by 190 mm long barrel through a square entry, concentric die of diameter $D$ = 1.0 to 3.0 mm and $L/D$ ratios of 2 to 16. Normal stresses were recorded by the crosshead load cell and pressure transducers located in the barrel and die sections. The device was usually operated in a controlled strain rate mode, with a displacement sensor accuracy of ±4 μm. For the confined compaction experiments, the die was replaced by a blank plate fitted with pressure transducers which recorded the transmitted normal stress. The dough was then compacted slowly (1 mm $s^{-1}$), initially removing air from the system, then compacting the solid matrix. The Coulombic friction coefficient, $\mu$, was obtained from the difference between applied and transmitted stresses by assuming that the Janssen-Walker analysis applied:

$$\frac{\sigma_{trans}}{\sigma_{app}} = \exp-\left(\frac{4\mu K\Psi z}{D_0}\right) \tag{4}$$

where $z$ is the height of the compact in the barrel of i.d. $D_o$. Figure 3 summarises the results obtained over a range of water contents, and shows that the dough behaved as a solid when compressed uniformly at stresses below the joining pressure. There was no evidence of cell or granule rupture during compaction until the joining pressure was exceeded (approximately 0.5 MPa), as observed for other particulate materials.

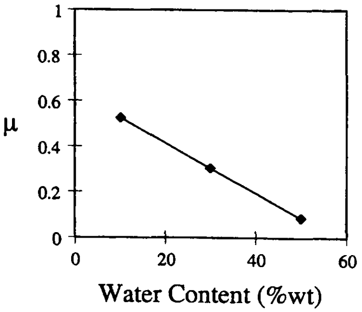

**Figure 3** *Variation of coefficient of friction with dough water content*

The friction coefficient obtained for the dough under study, c. 0.20 at 40 %wt moisture, confirmed that wall interactions play an important role in its extrusion behaviour. Wall friction in the barrel was found to be less significant at the higher speeds employed in extrusion experiments, where a dynamic phenomenon (wall slip) replaced the quasi-static Coulombic interaction.

The microstructure of native and extruded dough was examined using a number of techniques. SEM and ESEM analyses of extrudate structure were performed on a JEOL JSM 820 Scanning Electron Microscope (JEOL Ltd, Japan) and an ElectroScan Model 2010 Environmental SEM (ElectroScan Corporation, USA) respectively. Starch gelatinisation was investigated using DSC on a Perkin Elmer DSC7 (Perkin Elmer Corporation, USA). The following light microscopy techniques were used to explore particular features: cross-polarised (XP) light microscopy to detect undamaged starch granules; Differential Interference Contrast (DIC) to highlight edge boundaries; staining with Iodine vapour (IV), Lugols Iodine (LI) and Toluidene Blue (TB) for amylose, amylopectin and cell wall polysaccharides.

Starch crystallinity was studied on-line using a novel rheometer fitted with WAXS. The Cambridge Multipass Rheometer consists of a capillary (or slit) through which material passes from one of two sealed reservoirs. The inventory in each reservoir is controlled by a dedicated piston: the two pistons can be configured to run in a coupled, controlled strain mode to mimic a capillary rheometer, or in an oscillatory mode. Details of this device are given elsewhere[8]. In this work the device was operated as a slit rheometer, with doughs being extruded through a 1 by 10 mm slit fitted with beryllium windows, which are transparent to X-rays. WAXS data were generated using a Bruker X-Ray System (Bruker Analytical X-Ray Systems, USA).

## 3 RHEOLOGY AND MODELLING

### 3.1 Rheological Characterisation

Extrusion experiments were initially performed using a series of dies with $D = 3$ mm over the piston velocity range $V = 0.01$ to 10 mm $s^{-1}$ and yielded the characterisation parameters given in Table 2. The parameters indicate a strong shear rate dependency, and a very small wall shear yield stress, $\tau_0$, compared to model ceramic pastes. These values were used to assess a number of industrial die designs – including orifice dies and gave reasonable agreement except where the die land diameter was less than 2 mm. Under these conditions there are two potential problems with the analysis: (i) particle size becomes significant compared to capillary dimension and therefore particle deformation phenomena could be expected to modify the rheological behaviour of the dough; (ii) increased shear rates result in bulk deformation of the extruding dough (i.e. non-plug flow). The effects of these phenomena are evident in the second set of characterisation parameters in Table 2, which were obtained for $D = 1$ mm. In this case the parameters no longer have any physical meaning: instead they are effectively reduced to the product a six parameter fit.

**Table 2** *Benbow-Bridgwater parameters (Equation 3) determined for starch dough. Values for a model ceramic paste[2] included for comparison*

| *Parameter* | *Potato Dough (D = 3 mm)* | *Potato Dough (D = 1 mm)* | *Ceramic Paste* |
|---|---|---|---|
| $\sigma$ (MPa) | 0.09 | 0.18 | 0.30 |
| $\alpha$ (MPa $s^m$ $m^{-m}$) | 1.3 | 1.1 | 0.04 |
| $m$ | 0.33 | 0.35 | 0.51 |
| $\tau$ (MPa) | 0.00 | 0.00 | 0.07 |
| $\beta$ (MPa $s^n$ $m^{-n}$) | 0.30 | 0.37 | 0.27 |
| $n$ | 0.36 | 0.24 | 0.72 |

The effect of capillary size on the dough behaviour was quantified by performing a Mooney slip flow analysis[9]. The ram extruder was thus used as a capillary rheometer – albeit using relatively short capillaries – and shear stress/apparent shear rate data collected for dies with $D$ = 1.0, 1.5, 2.0 and 3.0 mm over an apparent shear rate range of 1.8 - 50000 $s^{-1}$. Details of this analysis will be published elsewhere[10]. The Mooney analysis confirmed that the material exhibited wall slip over the range of wall shear stresses involved in this work, with internal deformation occurring at the higher shear rates (smaller duct sizes). The results are presented graphically in Figure 4, which shows the slip velocity and the plug flow velocity recorded for dough flowing through die diameters of 2 mm and 3 mm for the given wall shear stress. The plug flow velocity for 3 mm is within the experimental error estimate of the pure slip velocity, whereas that for 2 mm is significantly larger, indicating that significant internal deformation occurred in these dies.

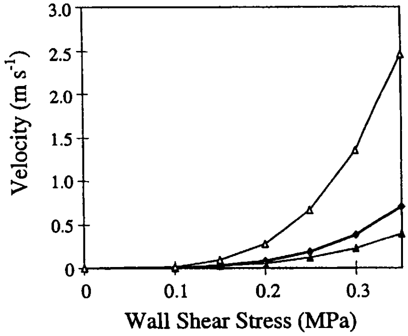

**Figure 4** *Dough slip velocity* ◆ *obtained by capillary flow analysis and expected plug flow velocities for extrusion through 2* △ *and 3* ▲ *mm diameter dies*

The slip velocity data in Figure 4 indicate a strongly non-linear Navier slip condition, i.e.:

$$\tau_w = K_w V_s^q \qquad (5)$$

It was difficult to establish whether these data indicated a wall slip yield stress (i.e. a Herschel-Bulkley type relationship[4]) or not[3]. The modified shear stress-shear rate data were found to fit a power-law relationship (see Equation 1).

The parameters obtained for the dough are given in Table 3, along with those reported in the literature[3,4]. It can be seen that all parameters are of the same order of magnitude, though no two sets of parameters match well. It would not be expected that the parameters reported here should match those in the literature exactly given the differences in material composition and flow conditions. However the differences between the two other works, using nominally the same material, are more surprising, and are indicative of the difficulties frequently experienced by workers using paste-like materials.

**Table 3** *Constitutive model parameters derived for slip flow and bulk shear of starch dough. Data for similar materials from literature*[3,4] *included for comparison*

| *Parameter* | *Potato Dough* | *Potato Granule*[3] *40 %wt water ambient* | *Potato Granule*[4] *50 %wt water ambient* |
|---|---|---|---|
| Bulk Shear | | | |
| $K_b$ (kPa $s^p$) | 154 | 230 | 80 |
| $p$ | 0.256 | 0.14 | 0.16 |
| Wall Slip | | | |
| $K_w$ (kPa $m^{-q}$ $s^q$) | 67 | 8 | 4 |
| $q$ | 0.253 | 0.41 | 0.45 |

### 3.2 Flow Modelling

The validity of the rheological parameters obtained above was tested by using these as input data in the computational fluid dynamics package, *Polyflow*, to simulate extrusion through an axi-symmetric, square-ended die as used in the experiments. The results obtained for an axi-symmetric die with reduction from 25 mm to 3 mm did not give good agreement for either the die entry or the die land section; this may be due to the package not supporting a Herschel-Bulkley wall slip condition. Another factor is that the flow behaviour of this quasi-solid, quasi-fluid dough may not be described adequately by a simple constitutive equation.

An alternative, empirical approach was subsequently adopted to model a set of annular dies. The initial 'plasticity' calculation, using Equation 3, featured a set of characterisation parameters obtained for $D$ = 3 mm, but was noticeably less successful than the lumped approach described below. The $P_{deformation}$ term in Equation (3) was used to model flow into the die, using the same parameters as above. Shear flow components in the die land were determined by replacing the $P_{friction}$ term with data from the curve for slip flow in Figure 4. The experimental data and predictions for a concentric, annular die (o.d. 14.5 mm, i.d. 13 mm, $L$ = 50 mm) are shown in Figure 5. It can be seen that this alteration significantly improves the prediction over the range of velocities.

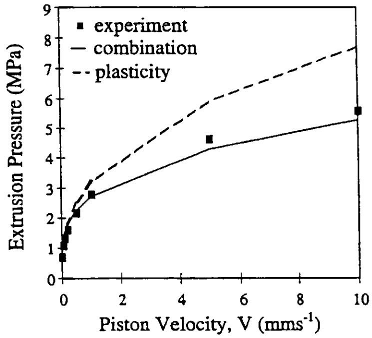

**Figure 5** *Experimental and modelling results for extrusion of dough through a concentric annular die*

### 3.3 Rheology-Microstructural Links

The rheological characterisation suggested that three types of deformation behaviour would be present in the extrudate:

(i) For large diameter ducts ($D \geq 3$ mm), extrudates would contain regions of highly sheared material existing near the wall, corresponding to a slip layer, and a deformation-free region of material transported in plug flow.

(ii) For smaller diameter ducts, ($\leq 2$ mm), sheared material would be present over a noticeable part of the extrudate, consistent with wall slip plus internal deformation due to the larger shear rates involved at similar volumetric flow rates.

(iii) At the smallest diameter ducts, ($\leq 1$ mm), material rheology will be dominated by inter-particle interaction and particle deformation.

Regions of large shear rate, i.e. type (ii), are also generated in the die entry region, the distribution being determined by the nature of the material. The characterisation results suggested that the dough could be described as a plastic in this region, which was also supported by the experimental observation that the material formed zones of static material in the corners of the square-entry dies.

Indications of the likely shear rate distribution in the deformation zone were provided by Horrobin[11], who studied the extrusion behaviour of perfect plastic materials in axi-symmetric dies using the finite element package *ABAQUS*. Figure 6 shows the streamlines and strain rates generated for perfectly smooth and perfectly rough dies. Under the latter condition, the wall shear stress is given by the shear yield stress of the plastic material (here, described by the von Mises' criterion).

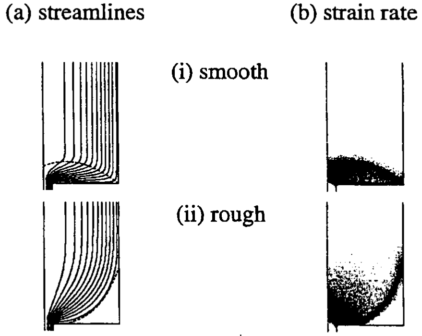

**Figure 6** *(a) Streamlines and (b) strain rates for extrusion with (i) smooth apparatus and (ii) fully rough apparatus (after Horrobin[11])*

The dough is unlikely to manifest the fully rough criterion, but the compaction and die land modelling results indicate that wall friction is important for this material. The noticeable features of the rough condition are the presence of static zones in the die corners, even streamline flow into the die land and a zone of high strain rate on the static zone boundary (where fast moving dough is adjacent to stationary dough in the static zone).

Figure 7(a) shows a cross section of the dough plug remaining in the extruder barrel after an extrusion experiment. The cracks in the material are caused by drying of the sample. The Figure shows very clearly defined static zones and a region of distinctly different material along the static zone boundaries, corresponding to the region of high strain (and shear) rate, which was called 'plastic' starch. It had a rubbery texture and was noticeably denser than the surrounding dough, on drying becoming translucent and brittle. The microstructure of this material is discussed in § 4.1. Its existence demonstrated how the rheology of the quasi-plastic bulk material generated shear rate distributions during extrusion that, in turn, caused irreversible local modification of the material microstructure and rheology.

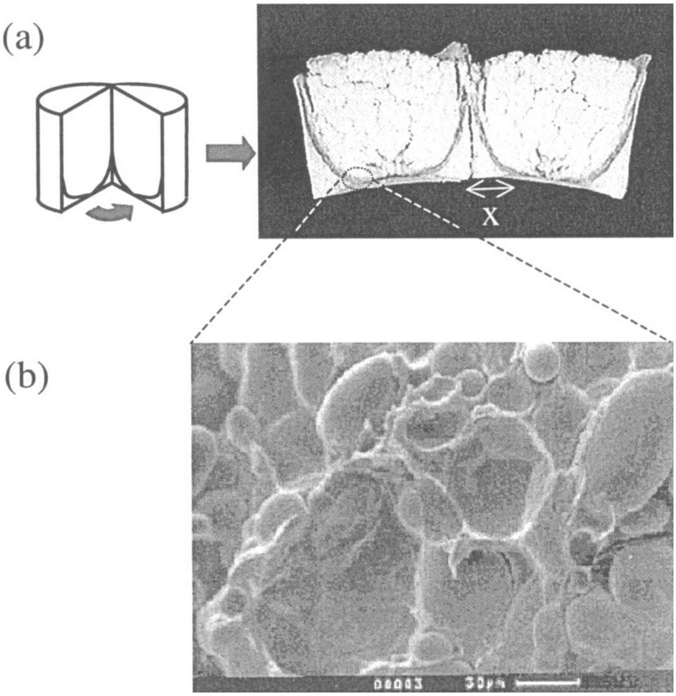

**Figure 7** *'Plastic' material at the static zone boundary: (a) a cross-section through the billet of dough remaining after extrusion; (b) SEM of the microstructure*

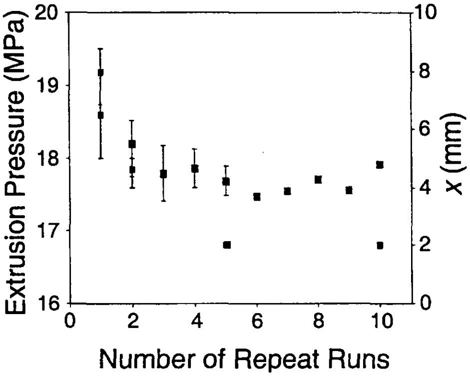

**Figure 8** *The effect on extrusion pressure* ▨ *and static zone size (x)* ■ *of continually re-filling the extruder barrel without cleaning*

The dynamics of the formation of this ’plastic’ starch were investigated by repeated extrusion of dough through the same die, mimicking the operation of a large industrial extruder. It is thought that continual build-up of this material could be responsible for die blockage or even ram and die plate damage. Figure 8 shows that the extrusion pressure did not increase, but settled down to an approximately steady level. The results from parallel experiments, where the dough plug was removed and static zone dimension measured (marked as $x$ in Figure 7(a)), confirmed that the ‘plastic’ starch along the static zone did not grow on continued extrusion. Rather, the static zone was eroded over the repeated experiments suggesting that any 'plastic' starch generated was convected out with the extrudate. The generation of a compacted static zone on the die plate covered by a protective layer of 'plastic' starch raises separate issues for plant sterility and hygiene.

## 4 MICROSTRUCTURAL ANALYSIS

On extrusion the dough demonstrated two surprising features. Firstly, extruded dough, in contrast with the lightly cohesive powder mixture, was highly compacted and had significant strength in tension, supporting its own weight over spans of several meters. Second was the rubbery ‘plastic’ starch developed in areas of high shearing forces, notably along the edge of the static zone boundary.

### 4.1 ‘Plastic Starch’ Generation

SEM examination of the ‘plastic’ starch showed it to be largely composed of an amorphous material (Figure 7(b)). Some particles of native starch and cooked potato were visible in varying quantities within the matrix. The repeated experiments described above indicated that the ‘plastic’ starch was being formed and carried out with the extrudate and hence would affect the structure at least at the extrudate surface (and surface related transport processes such as cooking).

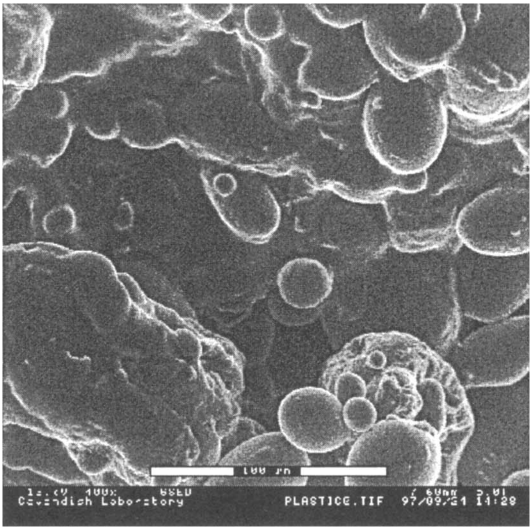

**Figure 9** *ESEM image of the surface of a dough extrudate*

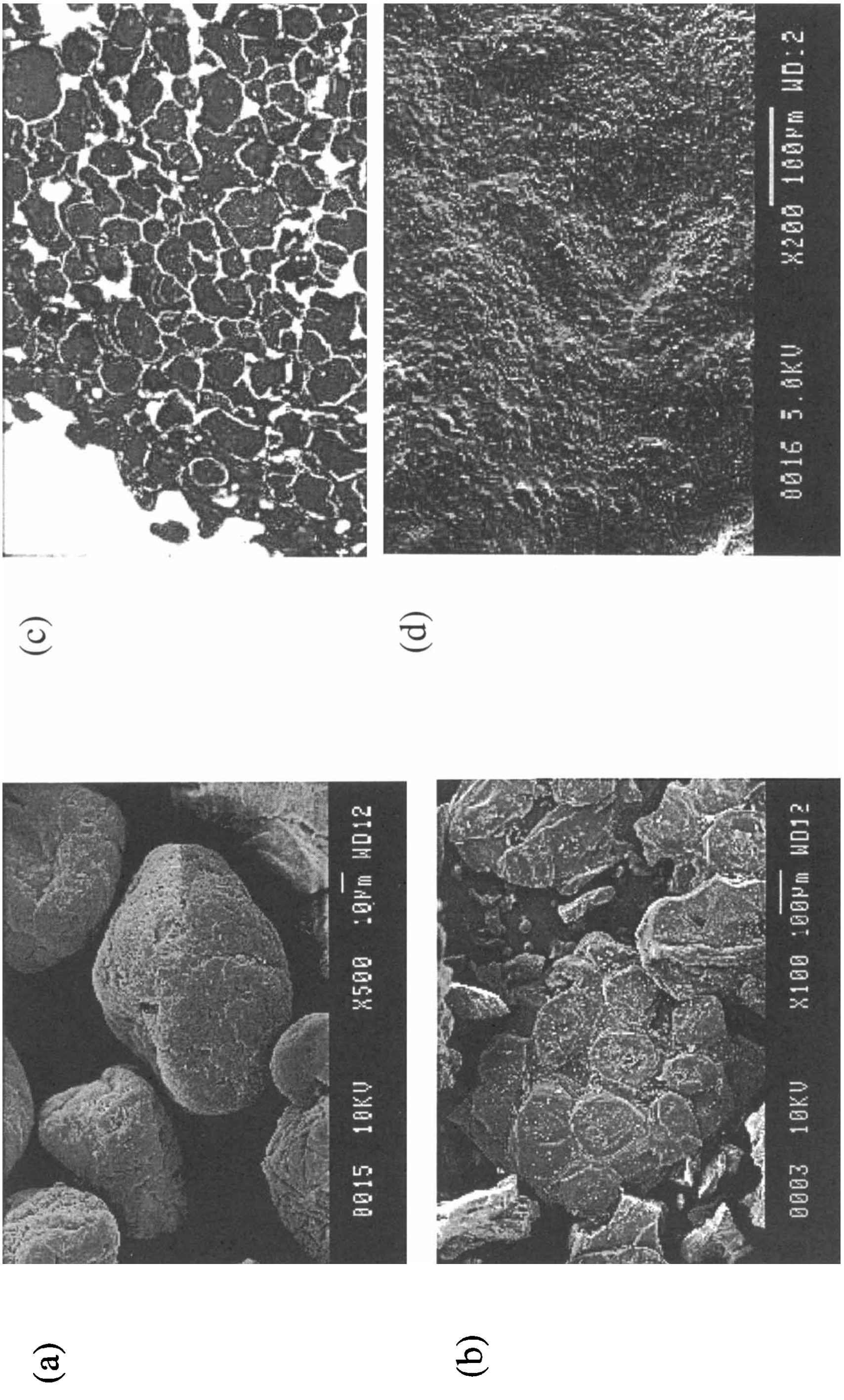

**Figure 10** *Cooked potato powders and extrudate cross-sections: powder composed of (a) material A (intact cells) and (b) material B (ruptured cells); (c) material A (230X) and (d) material B extrudates*

The extrudate was therefore re-examined using electron microscopy. Shear-induced microstructural change similar to that in the die entry region was evident. ESEM analysis of the extrudate surface confirmed that native starch granules and some cooked potato particles were present, as shown by Figure 9, indicating that these shear and stress conditions were not large enough to cause rupture of these particles. The shear stress and strain rate conditions prevalent in Figure 7(*b*) could not be readily quantified, but both Figures 7(b) and 9 show that the undamaged particles were surrounded by a zone of amorphous sheared material. This material was probably derived from the pregelatinised starch in the ruptured component of the cooked potato. This hypothesis was consistent with our hydration results, SEM observation and knowledge of native and gelatinised starches, however a further examination of the effects of shear on each of the components was performed to provide further insight.

## 4.2 Analysis of Mixture Components

Samples of the individual components, at the same water content (40 %wt) were extruded through the longest square ended die ($L/D$ = 48/3 mm/mm) at the largest piston velocity attainable ($V$ = 10 mm $s^{-1}$; $\Gamma$ = 1850 $s^{-1}$). Extrudates were analysed using electron and light microscopy, as well as DSC and WAXS.

Figure 10 shows electron micrographs of cooked potato material. The samples were extrudate cross sections through 3 mm diameter dies. The pictures of the dry powders showed strong differences between material with A - low and B - high quantities of ruptured cells (and hence unconfined gelatinised starch), differences which strongly affected extrudate structure. For material A, the powder consists of discrete particles, i.e. potato cells. The extrudates, produced at similar pressures to those required for the mixture, were fragile and consisted, like its dough, of discrete cells. The strong impaction of the particles upon each other showed the effects of pressure, but there was no sign of damage to the particles through shear.

In contrast, powder B consisted of a mixture of large agglomerated particles containing many cells, and small fragments of apparently structureless material. When extruded, this material broke down to form a homogeneous material with little apparent structure, indicating that shearing forces generated were adequate to fully destroy the pre-damaged potato cells. It was however highly cohesive, and demonstrated visco-elastic properties which were not observed with other components or the mixture. The combination of cooking (during production) and shear (during extrusion) are akin to the condition used to produce thermoplastic starch by screw extrusion, giving a product with similar characteristics[1].

Extrudates of B were stained (with TB) for cell wall material, as shown in Figure 11. Small dark grey flecks indicated that the cell wall had been broken down by the action of shear. These remnants were suspended in a matrix of continuous (pale grey) gelatinised starch. By comparison, extrudates of A showed no continuous material and undamaged cells could be seen. Structural differences were highlighted during sample preparation: the more cohesive extrudate B was held together by the continuous binding material, but the weaker extrudate A began to disintegrate.

**Figure 11** *Toluidine Blue stained extrudate cross-section of material B (460X)*

Despite other differences, there was no evidence of variation in structure over either extrudate cross-section. These observations are consistent with the rheological study, which indicated that flow in the 3 mm dies was dominated by wall slip.

Native starch, however, exhibited very different extrusion behaviour. This component dewatered noticeably during the compaction stage of extrusion so that it was significantly drier when solid extrudate was finally produced, requiring an extrusion pressure c. 100% larger than those involved in the dough mixture and cooked potato tests. Examination of cross sections indicated regions of plug flow in a central core, and particle deformation in the surrounding annulus and at the surface, as shown in Figure 12, even in $D$ = 3 mm extrudates.

The core region (diameter ~ 1 mm) consisted of undamaged native starch granules. The surface layer, approximately 100 μm deep, showed complete loss of birefringence, indicating that the mechanical work input even at this low water content was sufficient to destroy all granular structure. The annular material was visibly tough and transparent, but XP and DIC indicated that the starch granule structure was still intact. The stresses exerted in this region during extrusion were therefore sufficient to compact, but not rupture, the granules.

DSC and WAXS were used to aid determination of the starch state in ambiguous extrudate regions. DSC results are shown in Figure 13, which includes traces from native and gelatinised starch (in excess water at 100°C) for reference.

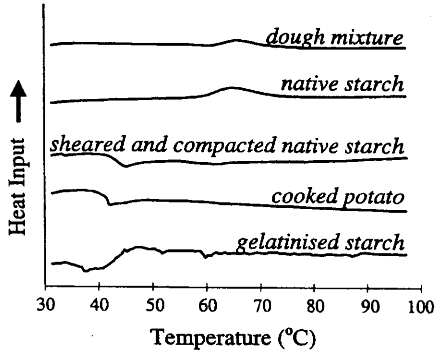

**Figure 13** *DSC of ingredients, dough mixture and extrudates*

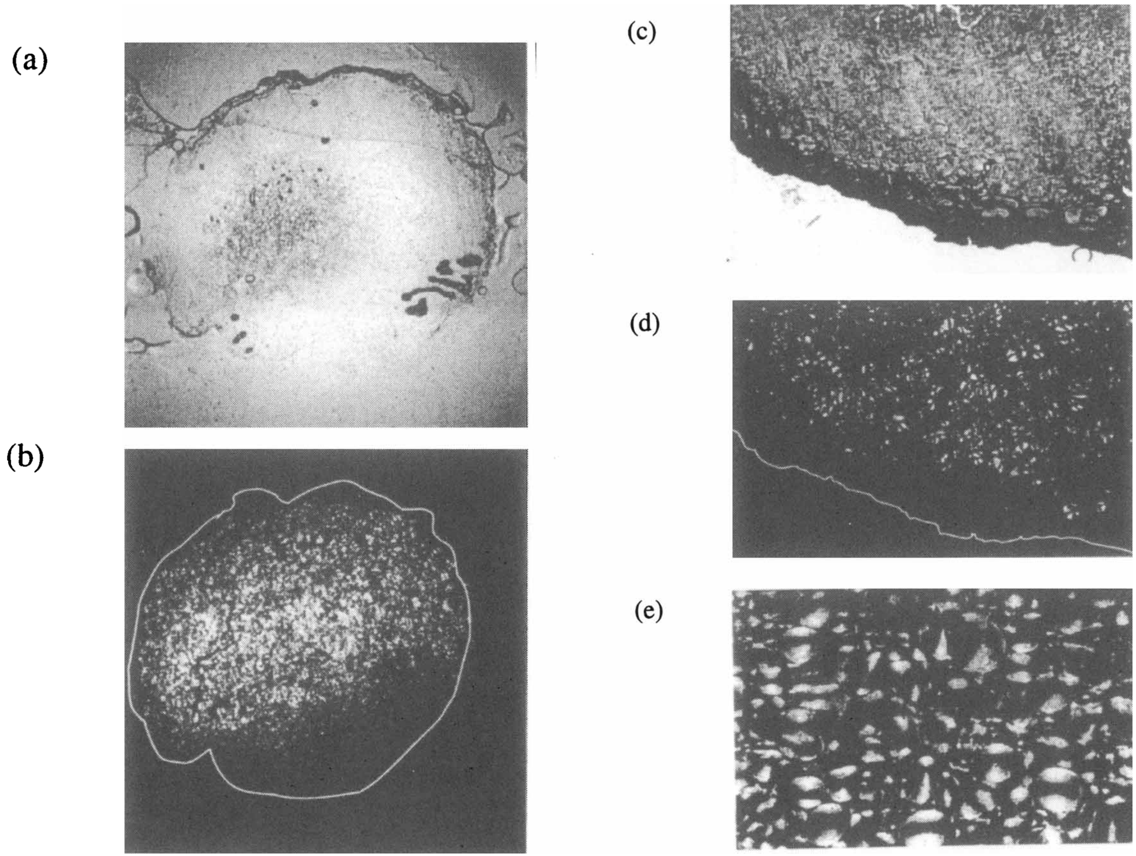

**Figure 12** *Native starch dough extrudates: a cross-section of a extrudate (26X) (a) oil mounted and (b) under XP light; extrudate edge (230X) (c) IV stained and (d) under XP light; (e) DIC of annular region of compacted starch granules (920X)*

Some results were as expected: cooked potato gave a trace similar to the gelatinised starch; undamaged, extruded starch from the core region was unaffected. More surprisingly, the starch from the annular and surface regions, which could not be separated, showed no gelatinisation endotherm, suggesting that it had already undergone, through shear, a transformation similar to gelatinisation. This was in accordance with their visual lack of structure, but surprising given the XP and DIC observations for the annular material.

WAXS results are shown in Figure 14, again with native and gelatinised starch traces for reference. Using this technique it was possible to detect some crystallinity in all samples bar the gelatinised starch. Its presence in cooked potato is presumably due to retrogradation of the gelatinised starch during storage. The result for annular and surface material showed reduced levels of crystallinity compared to native starch, but still significant levels compared to gelatinised starch. This was consistent with the intermediate state of annular material indicated by other tests. It was impossible to test surface material in isolation because it was only produced in small quantities, however it might be expected that this material would have still further reduced levels of crystallinity.

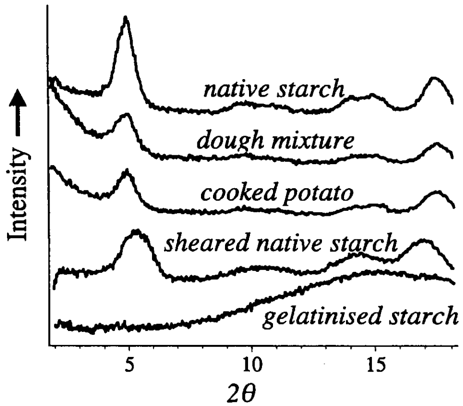

**Figure 14** *WAXS of ingredients, dough mixture and extrudates (2θ is the scattering angle)*

### 4.3 Review

Further elucidation of the dough microstructure was possible using the above results. Native starch granules and intact potato cells were evident over the whole cross-section (Figure 15), with the XP image indicating that starch granules were present in their native form even at the extrudate surface. Both native starch and cooked potato particles were bound together with continuous material similar to that found in the cooked potato extrudates containing high levels of ruptured cells.

DSC results identified a gelatinisation endotherm in the extrudate and therefore the presence of undamaged native starch. WAXS results were ambiguous due to the presence of crystallinity in all ingredients, however it was observed that continuous shear had no effect other than perhaps to densify the material.

These results were consistent once more with the die land plug flow assumption held for low shear flow. It could be argued that there was a thin surface region with a higher concentration of sheared material than the bulk, but this does not necessarily indicate bulk shear flow, as investigation of viscous suspensions suggest that filler particles migrate away from flow surfaces[5].

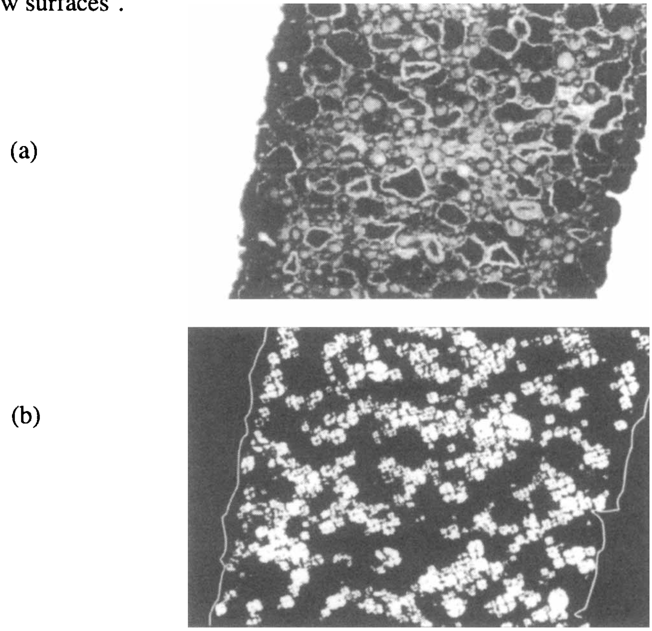

**Figure 15** *Section through an annular extrudate of dough mixture (230X) (a) IV stained and (b) under XP light*

Figure 16 presents a qualitative model for the microstructure-processing relationships occurring in this material. On hydration, most water is absorbed into the cooked potato fraction consisting of ruptured cells. The resultant hydrated, gelatinised starch will be significantly softer than the remaining particulates and acts as a binder for them. As the mixture then travels through the die land it is protected from further deformation by this malleable matrix of gelatinised starch. After extrusion the same material binds the extrudate and prevents loss of shape.

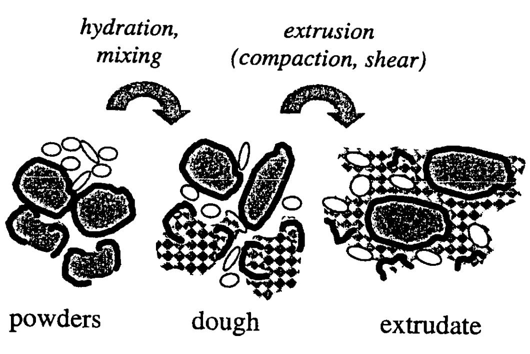

**Figure 16** *Qualitative model of effect of dough mixing and extrusion on the ingredient microstructures*

## 5 CONCLUSIONS

The flow behaviour of the potato starch mixture undergoing extrusion has been investigated using two approaches: a 'plasticity' based model that assumes certain features about the flow pattern, and an approximate capillary flow analysis. The former method described flow into the die land reasonably well, while the latter confirmed that slip flow, or slip flow accompanied by internal deformation, occurred in pure shear in the die land. Particle-particle interactions became more important as the duct size decreased. These materials present challenges for conventional fluid rheological methods: a combination of the two methods gave the best prediction for flow through a set of annular dies.

During extrusion the dough undergoes microstructure-altering shear at die entry. The weakest particles, which are destroyed preferentially, are those with the highest water contents, i.e. the ruptured cooked potato cells. The hydrated, extracellular, gelatinised starch then binds and lubricates the flow of drier, harder remaining particles. The latter were undamaged by extrusion except when subjected to very high shear rates such as those generated at surfaces.

### Nomenclature

| | |
|---|---|
| $A$ | Die land area |
| $A_o$ | Barrel area |
| $D$ | Die land diameter |
| $D_o$ | Barrel diameter |
| $K$ | Walker active coefficient of earth pressure |
| $K_b$ | Bulk shear consistency |
| $K_w$ | Wall slip consistency |
| $L$ | Die land length |
| $\mathrm{m}$ | Bulk yield stress index in equation (3) |
| $n$ | Wall shear stress index in equation (3) |
| $P$ | Extrusion pressure |
| $p$ | Bulk shear strain rate index |
| $q$ | Wall slip index |
| $V$ | Extrudate velocity for pure slip (plug flow) |
| $V_s$ | Wall slip velocity |
| $x$ | Arbitrary static zone dimension (defined in Figure 7) |
| $z$ | Height of dough compact |
| $\alpha$ | Shear rate dependency factor in equation (3) |
| $\beta$ | Wall shear rate dependency factor in equation (3) |
| $\Gamma$ | Apparent shear rate |
| $\dot{\gamma}$ | Shear rate |
| $\mu$ | Coulombic friction coefficient |
| $\sigma_{app}$ | Stress in z direction applied to the dough by the piston |
| $\sigma_o$ | Bulk yield stress at zero flow rate in equation (3) |
| $\sigma_{trans}$ | Stress in z direction transmitted through the dough to the die |
| $\tau$ | Shear stress |
| $\tau_o$ | Wall yield stress at zero flow rate in equation (3) |
| $\tau_w$ | Wall shear stress |
| $\tau_y$ | Yield shear stress |
| $\Psi$ | Stress distribution factor |

## Acknowledgements

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# PROCESSING-STRUCTURE-RHEOLOGY RELATIONSHIPS OF MICROPHASE SEPARATED STARCH / NON-STARCH POLYSACCHARIDE MIXTURES

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## 1 INTRODUCTION

Starch is a mixture of two polysaccharides, amylose and amylopectin, which are packed in the form of partially crystalline granules. The widespread use of starch as thickener is based on the ability of starch granules to swell upon heating in water. Starch is often combined with non-starch hydrocolloids in order to control viscosity, stability and other properties of foods. Several authors have studied the interaction between starch and non-starch hydrocolloids [1-6]. The admixture of non-starch hydrocolloids to starch dispersions results in a strong viscosity increase. This effect is largely due to the accumulation of the non-starch hydrocolloid in the continuous phase[2], that is to phase separation, rather than to a true synergistic interaction between the polymeric components. On the other hand, non-starch hydrocolloids control the rheological properties of starch by influencing the extent of starch granule swelling and amylose leaching[6].

Based on the Flory-Huggins theory of polymers in solution[7,8], blending of two polymers in one solvent will in most cases result in phase separation and the development of two liquid phases, where each polymer is enriched in one of the two phases. Mixing of amylose and amylopectin solutions, for instance, result in phase separation[9]. Similarly, model systems composed of amylopectin and galactomannan were found to phase separate, and phase diagrams have been established[10]. The interaction of starch and non-starch hydrocolloids has been intensively studied in terms of rheology. The present study focuses of the microstructure of starch dispersions in presence of non-starch polysaccharides that dissolve in water and give a thickening effect. Simple aqueous dispersions and more complex systems containing milk have been investigated. In the milk-containing system, which served as model for sauce béchamel, the contribution of additional protein and fat was studied. Besides microscopy, amperometric iodine titration and rheometry have been applied for structural characterisation of the systems.

## 2 EXPERIMENTAL

### 2.1 Materials

Native potato starch was obtained from Blattmann & Co., CH-Wädenswil. Waxy maize maltodextrin (Glucidex 2) was obtained from Roquette Frères, F-Lille. Xanthan gum (Rhodigel) was a product from Meyhall Chemical, CH-Kreuzlingen. Guar gum (Meypro Guar, CSAA M-200) was obtained from Meypro Chemical, CH-Kreuzlingen. Locust bean gum (Portuguese LBG 791) was obtained from Mannesol, UK-Runcorn.

### 2.2 Sample Preparation

*2.2.1 Preparation of Aqueous Starch/Non-starch Hydrocolloid Models.* The starch/non-starch hydrocolloid systems were prepared by either mixing potato starch dispersions with non-starch hydrocolloid solutions (solution-based preparation) or by heating both components together in water (powder-based preparation).

*2.2.1.1 Solution-based Preparations. The stock solutions were prepared as follows:* Waxy maize maltodextrin (15 g/100 g db) was solubilised in deionised water at 85 °C for 15 min. Native potato starch (2.00, 2.25 or 4.00 g/100 g db) was heated to 95 °C in a water bath with an average heating rate of 5°C min$^{-1}$. The samples were kept at 95 °C for 30 min under constant stirring with a magnetic agitator. In one case, the sample was heated to 95 °C without holding time and subsequently cooled to 25 °C in a water bath. Guar and locust bean gum solutions (0.40 or 0.60 g/100 g) were obtained by dispersing the hydrocolloids in deionised water at room temperature for 15 min followed by heating to 95 °C for 30 min. The amount of evaporated water was determined gravimetrically and replaced. Solution-based polysaccharide mixtures were prepared by weighing out the appropriate masses of stock solutions at 95 °C in glass jars. Mixing was accomplished by vigorously shaking the closed jars for 15 s. The mixtures were cooled to 25 °C in a water bath for 2 to 3 h. The maltodextrin/guar gum blend was mixed vigorously for 2 h at 25 °C with a magnetic agitator.

*2.2.1.2 Powder-based Preparations.* Appropriate amounts of potato starch and non-starch hydrocolloid were dry mixed before suspending the mixture in water (total 300 g) and heating at 95 °C for 30 min. During the heat treatment, mixing was carried out with a magnetic agitator and additionally with an impeller-type stirrer. All mixtures were cooled in a water bath at 25 °C for 2 to 3 h.

*2.2.2 Preparation of Starch/Xanthan Models with Milk.* Systems composed of potato starch (2 g/100 g db), xanthan (0.2 g/100 g db), milk and salt (0.33 g/100 g), which were considered as models for sauce béchamel, were prepared by different thermomechanical treatments. The complex model systems were prepared either by a thermal treatment in a beaker at 95 °C for 30 min or by processing the mixture in a scraped-surface heat exchanger (SSHE). The former preparation was similar to the above described preparation of powder-based starch/non-starch hydrocolloid systems with the difference that milk with a fat content of 2.7 % was used instead of water. In one case, calciumstearoyl-lactyl-2-lactylate (CSL) was added in the dispersed state to a potato starch/milk system without salt after the starch had been gelatinised. CSL was stirred into the hot (95 °C) starch/milk system at a concentration of 0.1 g/100 g and the sample was subsequently cooled in a water bath at 25 °C. The treatment of multicomponent starch systems in the SSHE was carried out as follows: Batches of 5 kg were prepared by the addition of skimmed milk powder (0.7 % fat) and pasteurised milk cream (35 % fat) to tap water to give a "milk" with a fat content of 2.7 %. Xanthan powder (0.2 g/100 g) was then slowly added, and the mixture was stored overnight at 4 °C. Salt (0.33 %) and potato starch (2 g/100 g db) were subsequently added, and the mixture was pumped through a SSHE (SMS-Thermalizer TH 10, Luwa: volume 1100 $cm^3$) using a Moineau pump (SB 15, Ecublens: mass flow rate 600 g/min). The SSHE was operated at blade rotation speeds of 110 and 310 rpm and final product temperatures of 95 and 115 °C, respectively. The resulting mass flow ranged from 300 to 600 g/min. The average residence time varied between 110 and 220 s. After the heat treatment, the samples were either directly cooled to 25 °C or homogenised prior to cooling. Small size samples (100 g) were homogenised for 30 s using a laboratory scale homogeniser (Polytron, Kinematica GmbH, CH-Littau). Homogenised samples and the non-homogenised control were stored at 4 °C for up to 6 d. In order to avoid the growth of micro-organisms, the stored samples were stabilised with sodium ethylmercurithio-salicylate (Merthiolate, Fluka No. 71230, CH-Buchs, 0.03 mg/100 g product).

## 2.3 Light Microscopy

Sample preparation for light microscopy was adapted from Langton and Hermansson[11]. Sample droplets of less than 5 mm in diameter were frozen in Isopentan which was cooled by liquid nitrogen. After equilibrium at –20 °C, the samples were frozen on stubs. A Reichert-Jung Cryostat (Leica, A-Vienna) was used at -13 to –15 °C to cut samples into sections of 10 to 20 μm. The sections were transferred onto glass slides and air dried for a minimum of 1 h prior to staining. For staining, a few drops of Lugol's solution (Fluka, CH-Buchs, stock solution: $I_2$ = 14 mM, KI = 44 mM) were applied to the sample on the slide followed by washing with deionised water after 3 min. After staining, one drop of glycerol/water solution (1:1 v/v) was applied onto the slide, and the sample was covered with a cover slip. The samples were examined with a Axioplan photomicroscope (Zeiss, D-Oberkochen) in the bright field mode. Selected samples were observed in the differential interference contrast mode. The maltodextrin/guar gum blend was observed directly after sample preparation in the differential interference contrast mode. A few drops of sample were loaded onto a microscope slide and covered with a cover slip which was placed on top of two cover slips located on each side of the sample. This provided a clearance between the cover slip and the sample to avoid possible artefacts due to sample compression. The sample was sealed with quick-drying clear polish to prevent evaporation of water from the sample. The sample was observed over a period of 90 min.

## 2.4 Rheometry

The rheological properties of aqueous starch systems were determined with a Carri-Med controlled stress rheometer (CS 100, Carri-Med Ltd., UK-Surrey), using a cone-plate geometry with a diameter of 6 cm and an angle of 1 59'. The measurements were carried out after sample preparation at 25 °C. Flow curves were registered in a shear stress range of 0 to 10 $Nm^{-2}$ within 2 min. The viscosity $\eta$ was evaluated at a shear stress of 5 Pa. The viscoelasticity was determined with dynamic measurements in the linear viscoelastic domain. The storage modulus $G'$ and the loss modulus $G''$ were evaluated as a measure of the elastic and viscous components, respectively.

## 2.5 Amperometric Iodine Titration

An estimate of the amount of leached amylose was obtained by amperometric iodine titration[12]. The iodine binding capacity (IBC) of starch dispersions was determined using a Metrohm equipment (Polarizer E585, Potentiograph E576 and Dosimat 655, CH-Herisau). The voltage of polarisation was 140 mV, and the attenuation was set at 5 μA. A 30 g sample containing 100 mg starch, 1 ml 1 M HCl and water was titrated with 0.005 M iodine solution and a titration rate of 1 ml $\text{min}^{-1}$. The sample was constantly stirred during titration. The amount of bound iodine was evaluated graphically[13]. The iodine binding capacity (IBC) was calculated based on dry starch as follows:

$$IBC = \frac{I_b}{S_{tot}} \times 100 \qquad \text{[mg iodine/100 mg dry starch]}$$

where $I_b$ is the amount of bound iodine in mg and $S_{tot}$ the total amount of starch in the titration vessel in mg. The leached amylose was calculated by assuming an amylose content of 24 % for potato starch and an IBC of 19.5 % for pure amylose.

## 3 RESULTS AND DISCUSSION

In a first step, a potato starch dispersion at a concentration level of 2 g/100 g db, which was prepared by heating to 95 °C, was assessed by light microscopy (Figure 1).

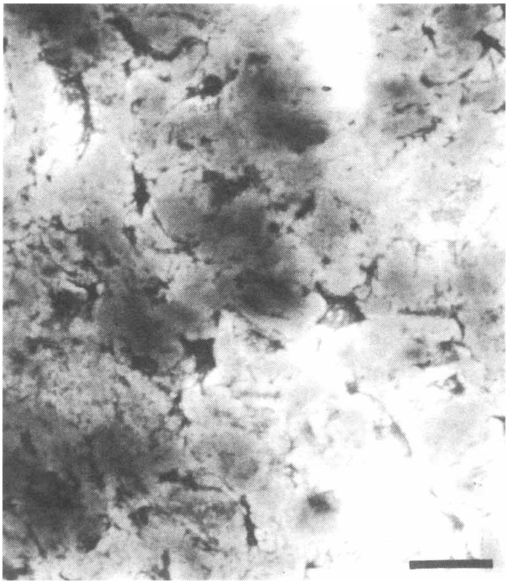

**Figure 1** *Microstructure of potato starch dispersions (2 g/100 g) prepared by heating at 95 °C (scale bar 25 μm)*

Strongly swollen, but not extensively disintegrated starch granules can be observed on the micrograph. The dark areas presented an intense blue coloration on the original micrograph, whereas the lighter areas correspond to light violet staining. The colour differences allow the localisation of amylose and amylopectin as they stain blue and violet, respectively, in presence of iodine. The dark areas on the micrograph correspond to amylose-rich phases.

Gelatinisation of starch can be described as a swelling and solubilisation process with involves partial phase separation of the two starch polymers. The enrichment of amylose in the inter- and intragranular space is due to thermodynamic immiscibility of amylose and amylopectin. Phase separation of starch polymers, which occurs even at moderate concentrations, is promoted by an asymmetry in the affinity of the two polymers to the common solvent water[14]. The polymer fraction with the less favourable interaction with water, i.e. amylose, tends to be driven out of solution[15,16].

In a next step, the interaction between amylopectin and a non-starch hydrocolloid was studied. In order promote the miscibility between the components, maltodextrin was selected as model for amylopectin.

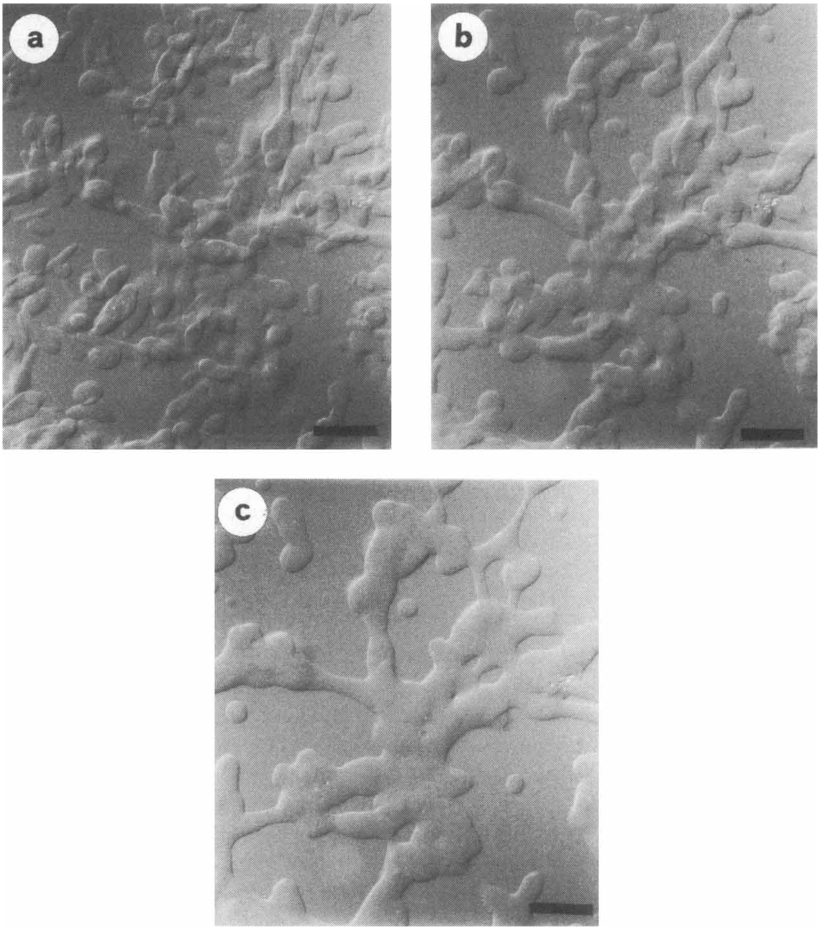

**Figure 2** *Microstructure of maltodextrin (7.5 g/100 g)/guar gum (0.3 g/100 g) mixtures in the differential interference contrast mode, (a) immediately after mixing, (b) 30 min after mixing , and (c) 90 min after mixing (scale bar 100 μm)*

After intensive mixing of a maltodextrin solution with a guar gum solution, the mixture was assessed by light microscopy in the differential interference contrast mode in the hydrated state. Figure 2 a shows a maltodextrin/guar gum mixture immediately after mixing. The micrograph reveals a phase separated system in which droplets of one phase are dispersed in a continuous phase. Based on the concentration of the two polymers and the volume fractions of the phases after bulk phase separation[10], it is reasonable to assume that the guar-rich phase is dispersed in a continuous maltodextrin-rich phase. By holding the system in a quiescent state, the dispersed droplets display coalescence and form an interconnected phase after 30 min (Figure 2b). The structure subsequently undergoes coarsening (Figures 2c). Finally, this mechanism results in the formation of two bulk phases.

Again, demixing of the maltodextrin/guar gum mixture is the result of thermodynamic immiscibility between the two components. Phase diagrams of these systems confirmed that miscibility is restricted to very low polymer concentrations[10]. Demixed biopolymer solutions can be treated as "water-in-water emulsions"[17]. During an emulsion-like phase separation, the droplets gradually coalesce, and two phases are formed with exceedingly small interfacial tensions in the order of $10^{-4}$ to $10^{-1}$ mJ $m^{-2}$ [18]. The miscibility of polymers in solution decrease with increasing molecular weight and with reduced affinity of the polymers for the solvent. Therefore, the miscibility of starch fractions with guar gum is expected to decrease in the order maltodextrin > amylopectin > amylose.

The characterisation of the microstructure of ternary polymer systems composed of amylopectin, amylose and a non-starch polysaccharide is more difficult. The three different phases cannot be identified in the differential interference contrast mode, nor can non-starch polysaccharides be specifically stained to be visualised in presence of starch. Amylose is one of the polymeric fractions which can be reasonably well visualised by iodine staining. The microstructure of mixtures of potato starch with guar and locust bean gum, respectively, was analysed regarding the distribution of amylose. The mixtures were prepared by mixing separately prepared dispersions at a ratio 1:1 or by heating the components together. The micrographs are presented in Figure 3 a-d.

In the control dispersion, which was obtained by dilution of a 4 g/100 g db potato starch dispersion with water at a ratio 1:1, amylose formed a fine continuous phase with some larger amylose structures (Figure 3 a). By mixing the initial starch dispersion (4 g/100 g db) with a guar gum solution (0.4 g/100 g db) instead of water, the amylose fraction became slightly coarser (Figure 3 b). The unstained regions in the micrograph are most probably identical to the guar phase. The mixture of starch and locust bean gum dispersion led to much coarser amylose structures than with guar (Figure 3 c). On the other hand, by heating potato starch together with locust bean gum, a very fine amylose phase can be identified (Figure 3 d).

To summarise, the morphology of the leached amylose phase is largely influenced by the type of admixed hydrocolloid and by the processing conditions. From systematic investigations on synthetic polymer blends, several factors are known to control phase separation, among others miscibility of the phases, mixing conditions, volume fraction of the individual polymers, viscosity of the respective phases and time[19]. Since amylose is the gelling fraction of starch, the dispersion state of amylose is expected to have an influence on the gelling behaviour and the rigidity of the system. Preliminary experiments have shown that the rheological properties of the above described systems are different although the extent of starch solubilisation and amylose leaching were similar (data not shown).

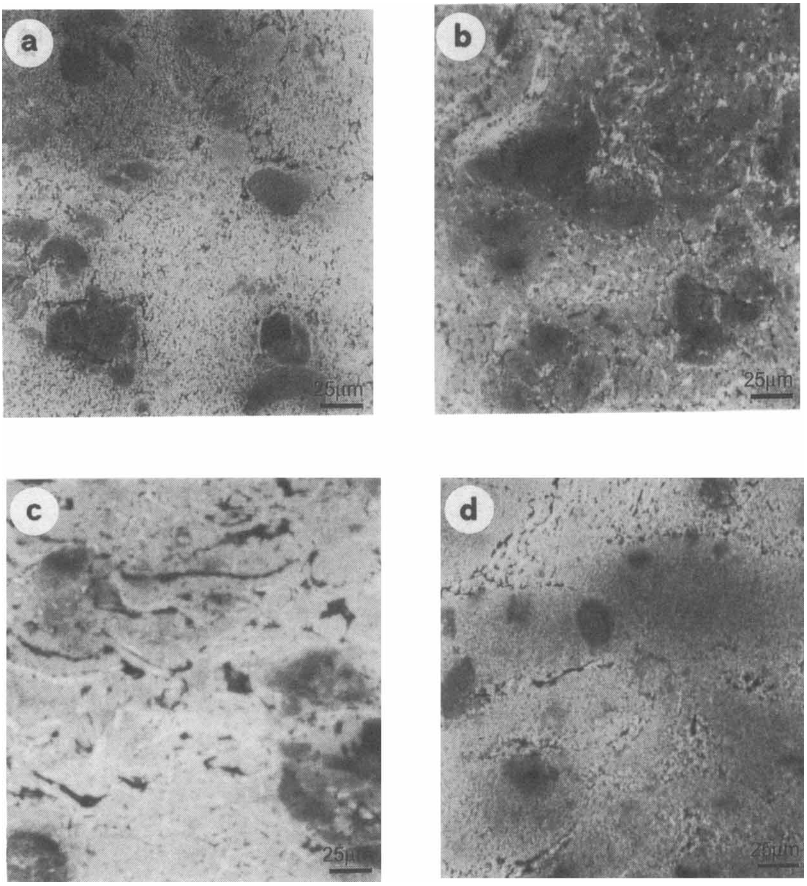

**Figure 3** *Micrographs of potato starch dispersions (2 g/100 g db) combined with guar gum and locust bean gum (0.2 g/100 g db), respectively. (a) control, (b) starch/guar gum, solution-based preparation, (c) starch/locust bean gum, solution-based preparation, (d) starch/locust bean gum, powder-based preparation (scale bar 25 µm)*

Finally, the structure of a more complex system composed of potato starch, xanthan gum and milk was investigated. The components were heated at 95 °C for 30 min at low shear conditions. The systems were characterised by differential interference contrast microscopy as presented in Figure 4. The micrograph shows one largely swollen potato starch granule (diameter ~ 100 µm) with a large dark area in the centre which corresponds to an intragranular enrichment of amylose. The differential interference contrast technique allows to identify the milk fat fraction as small round globules (diameter ~ 3 µm).

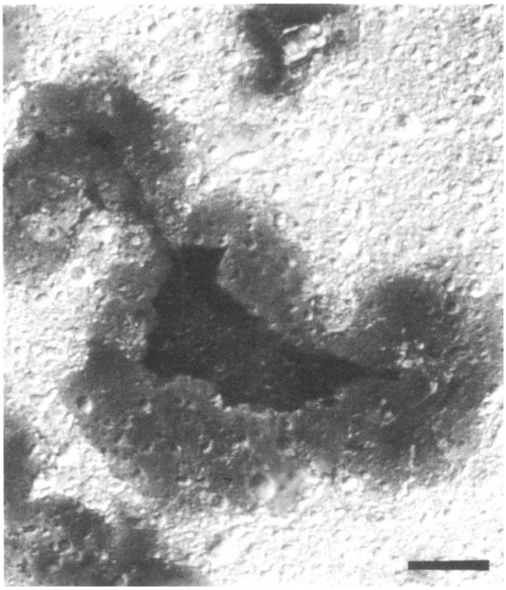

**Figure 4** *Micrograph of a model sauce composed of potato starch (2 g/100 g db) and xanthan (0.2 g/100 g db) heated in milk at 95 °C for 30 min (scale bar 25 μm)*

The influence of milk and xanthan on amylose leaching was quantified by amperometric iodine titration (Table 1). By heating starch in milk instead of water the IBC was reduced from 4.29 to 3.53 mg/100 mg starch db. The addition of xanthan further reduced the IBC to 1.71 mg/100 mg starch which means that only a small portion of amylose (~ 36 %) leached into the intergranular space.

**Table 1** *Influence of milk and xanthan (0.2 g/100 g db) on amylose leaching as detected by iodine binding capacity (IBC) of potato starch dispersions (2 g/100 g db) (mean of two replicates, range in parentheses)*

| *Sample* | *IBC [mg/100 mg starch db]* | *Leached amylose [% w/w total amylose]* |
|---|---|---|
| Potato starch in water (control) | 4.29 (0.04) | ~92 |
| Potato starch in milk | 3.53 (0.08) | ~75 |
| Potato starch-xanthan in milk | 1.71 (0.11) | ~36 |

At the macroscopic level, the multicomponent systems were characterised by dynamic oscillatory measurements. The mechanical spectra in Figure 5 show that the viscoelasticity of starch/xanthan/milk mixtures was enhanced by one order of magnitude as compared to the individual components (starch and xanthan in milk, respectively). It is remarkable that a large increase in thickening power is obtained by a comparatively small increase in total solids (0.2 g/100 g db).

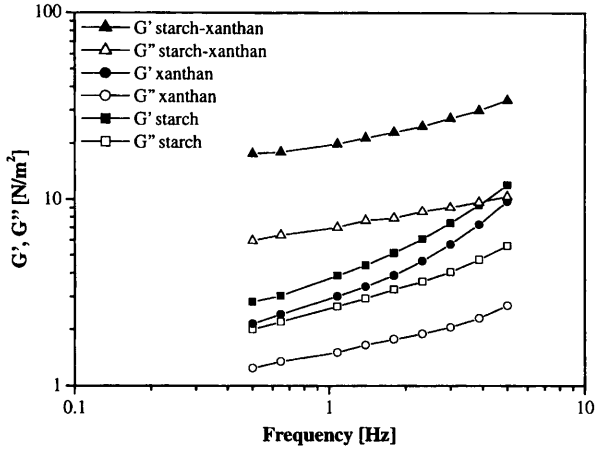

**Figure 5** *Viscoelasticity of potato starch (2 g/100 g)/xanthan (0.2 g/100 g) mixtures and of the individual polymeric components heated in milk at 95 °C for 30 min*

The most outstanding feature of potato starch in the multicomponent system is found in the enrichment of amylose inside the starch granules (Figure 4). This finding is in agreement with the low levels of extragranular amylose as detected by iodine titration (Table 1). Although this phenomenon is also observed in aqueous model systems, phase separation is much more pronounced in complex model systems. Phase separation within the swollen starch granules is most probably favoured by low shear conditions and by the presence of immiscible biopolymers (milk protein and xanthan) and milk lipids. Electrostatic interactions between charged components such as xanthan and milk proteins may have an influence on swelling and phase separation of starch. Electrostatic interactions involving starch are conceivable since potato starch is slightly charged due to the occurrence of phosphate groups. According to Dickinson[20], electrostatic interactions of macromolecules result in the formation of a soluble complex, a coacervate or an amorphous coprecipitate depending on the nature of the polymers and the solvent conditions (pH, ionic strength, etc.). Protein/polysaccharide complexes are considered as food components in their own right offering the possibility to control the texture of multicomponent structured foods.

The influence of the granular starch structure and the release of amylose on the rheological properties of multicomponent starch systems was further investigated by processing complex food models at different time-temperature and mixing conditions. The thermomechanical treatment of food models composed of starch, xanthan, milk and salt was carried out in a scraped-surface heat exchanger (SSHE). The mixing intensity was varied by adjusting blade rotation speed which, in turn, influenced the flow rate.

The microstructure of model systems treated at 95 and 125 °C at 110 and 310 rpm, respectively, are presented in Figure 6 a and b. Mostly intact, although phase separated granules are observed at a preparation temperature of 95 °C and rather low shear conditions (110 rpm). In contrast, extensive destructurisation of starch granules is the result of processing at high temperature and high mechanical energy input (115 °C/310 rpm). The respective IBC and viscosity data are summarised in Table 2. The treatment at 95 °C resulted in high viscosity and lower extent of amylose leaching as compared to the more drastic treatment at 115 °C. Clearly, the viscosity increasing effect of non-starch hydrocolloids is more pronounced in systems with more or less intact granules. On the other hand, the preservation of the granular starch structure is responsible for limited amylose leaching into the intergranular space.

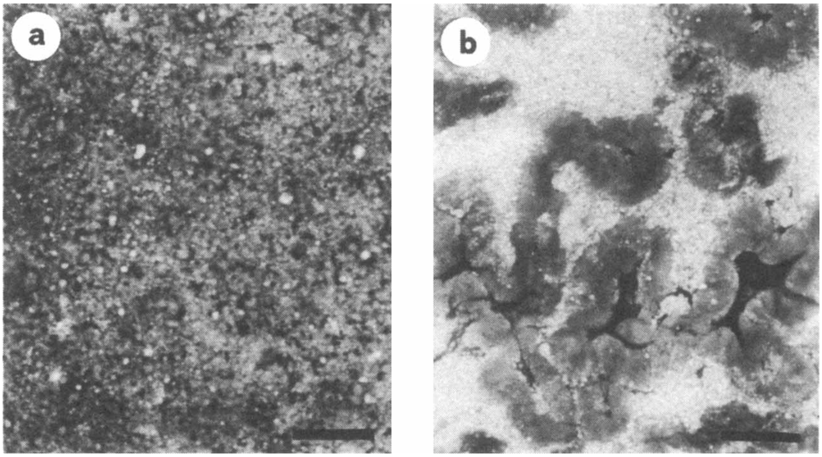

**Figure 6**: *Microstructure of potato starch (2 g/100 g) / xanthan (0.2 g/100g) mixtures heated in milk in a scraped-surface heat exchanger. (a) 115 °C, 310 rpm and (b) ) 95 °C, and 110 rpm (scale bar 50 μm).*

**Table 2** *Influence of processing temperature on shear viscosity at 5 Pa and amylose leaching as detected by iodine binding capacity (IBC) of potato starch (2 g/100 g db) / xanthan (0.2 g/100 g db) mixtures heated in milk in a scraped-surface heat exchanger (IBC: mean of two replicates, range in parentheses)*

| *Temperature [°C]* | *$\eta$ at 5 Pa [Pa s]* | *IBC [mg/100 mg starch db]* | *Leached amylose [% w/w total amylose]* |
|---|---|---|---|
| 95 | 87.000 | 2.03 (0.13) | ~43 |
| 115 | 1.010 | 3.70 (0.05) | ~79 |

Finally, the influence of the extent of amylose solubilisation into the intergranular space on the gelling behaviour of complex model systems was investigated. The storage moduli G' two starch/xanthan mixtures with low and high amount of leached amylose, respectively, were followed as a function of ageing time at 4 °C (Figure 7).

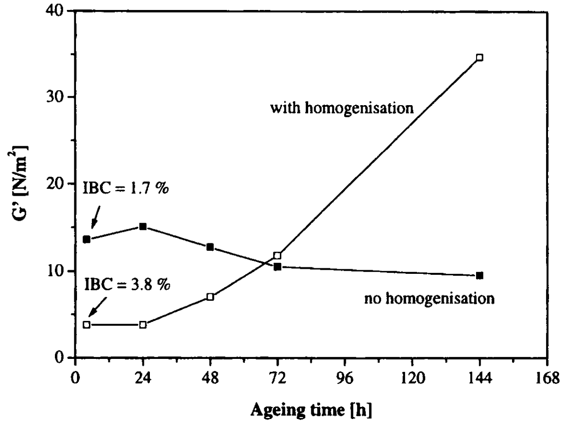

**Figure 7** *Influence of ageing time on the viscoelastic properties at 1 Hz of starch (2 g/100 g) / xanthan (0.2 g/100 g) / milk systems obtained by heating at 95 °C and 110 rpm in the SSHE followed by homogenisation in one case. The iodine binding capacity (IBC) before and after homogenisation is indicated*

Limited amylose solubilisation was obtained by treating a sample at 95 °C and 110 rpm, while extensive amylose leaching was achieved by homogenising the former sample after the thermal treatment. The IBC values were 1.8 and 3.6 mg/100 mg starch db, respectively. In the first case, amylose was mainly contained within the swollen starch granules, while in the second case, the starch granules were almost completely disintegrated (micrographs not shown). As expected, little granule disintegration yielded a system with rather high elasticity which decreased slightly over an ageing period of six days. By degrading the granular structure and liberating amylose, the elasticity was initially reduced but clearly increased during ageing. After six days, the homogenised sample attained a higher elasticity compared to the non-homogenised sample. At the macroscopic level, the non-homogenised sample was liquid-like while the homogenised sample had a gel-like texture.

It is concluded that intragranular amylose does not contribute to the formation of an intergranular network which is the structural basis for the spontaneous formation of a macroscopic gel. On the other hand, release of amylose from the swollen starch granules by degrading the supramolecular structure with a thermomechanical treatment results in a continuous amylose phase enabling the formation of a three-dimensional network which, in turn, induces gelation.

An alternative to the spontaneous gelation of starch is the complexation induced gelation by the addition of complexing emulsifiers[21,22]. Figure 8 shows that the elastic component G' of a starch / xanthan / milk system heated at 95 °C for 30 min is increased by almost two orders of magnitude in presence of calcium stearoyl-lactyl-2-lactylate (CSL). CSL is included in the helical cavity of amylose which results in the aggregation of amylose and formation of a three-dimensional network due to the low solubility of amylose / lipid inclusion complexes in water[21]. In contrast to the spontaneous gelation of amylose, which may require some days, the complexation induced gelation is a rapid process as gelation occurs upon cooling of the system. The controlled gelation of amylose in the continuous phase, by complexing it with emulsifier, allows the possibility to "freeze" a system at a certain stage of phase separation, since polymer diffusion is substantially restricted upon gelation.

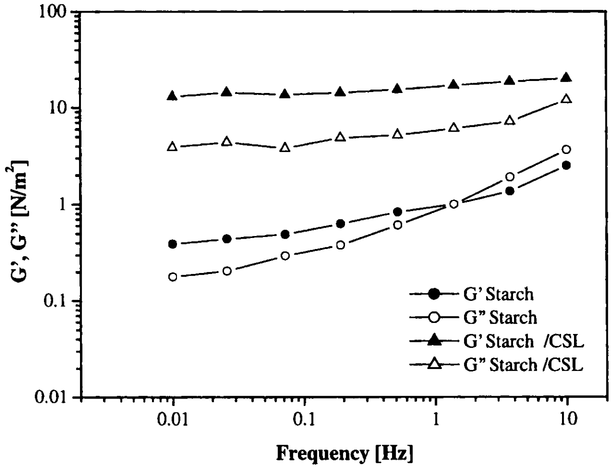

**Figure 8** *Influence of CSL (0.1 g/100 g) on the viscoelastic properties of a potato starch dispersion (2.25 g/100 g) heated in milk at 95 °C for 30 min*

## 4 CONCLUSIONS

The versatility of starch as an aqueous modifier of rheological properties is enhanced by the admixture of non-starch hydrocolloids, such as xanthan and galactomannans. The mixtures are of considerable practical value because of their high thickening power. The effect is primarily due to microphase separation and accumulation of the non-starch hydrocolloids in the continuous phase. The relationships between the microstructure of the amylose-rich phase and rheology deserve further investigation. It is hypothesised that the gelation of an amylose-containing starch can be manipulated by controlling the microstructure of the amylose phases. Starch may be applied as gelling agent, the prerequisites being the selection of an amylose-containing starch, an extensive leaching of amylose from the starch granules, and the existence of a continuous amylose phase. Compared to other gelling agents, starch has the disadvantage that the spontaneous gelation is a slow process which may require several hours or days. To overcome this difficulty, the gelation of starch can be induced by complexation of amylose with monoacyl lipids. If no gelation of starch is desired, polymeric additives and processing conditions should aim at preventing extensive leaching of amylose and at reducing the continuity of the amylose phase close to or beyond the point where phase inversion occurs, i.e. where amylose becomes the dispersed phase.

Foods are multicomponent and multiphase systems. Consequently, interfaces play a critical role in controlling the properties of food. The interaction of starch surface with other macromolecular food components presents a possibility to modify the surface properties. There are indications that the pronounced intragranular accumulation of amylose in the model sauce (Figure 4) is caused by an interfacial phenomenon. One could imagine the modification of the surface material of starch granules in such a way that it acts as a "semi-permeable membrane" allowing the imbibition of water into the starch granule, but restricting the diffusion of solubilised material, i.e. amylose, into the surrounding medium. Finally, it should be noted that in foods with high moisture contents the polymeric ingredients are in a non-equilibrium state. For the food technologist, the challenge in the application of polymer mixtures as thickening and gelling agents with multiphase structures lies in finding a balance between thermodynamics and kinetics.

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# MACROMOLECULAR DEGRADATION OF EXTRUDED STARCHES MEASURED BY HPSEC-MALLS

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## 1 INTRODUCTION

Macromolecular degradation is a well known consequence of the thermomechanical treatments of polymers, as observed in multiple recycling stages of polypropylene[1]. In the case of starch extrusion, fair correlations between intrinsic viscosity $[\eta]$ values, and specific mechanical energy SME have been published: this literature review shows a general agreement on a random chain splitting due to shearing. But, still, this empirical rule is insufficient to understand complete mechanism of breakdown and predict functional properties of extruded starches either for food or non-food applications. The aim of this work is twofold: examine specific susceptibility of linear/branched macromolecules during degradation and determine process variables which govern degradation. For this purpose, high performance size exclusion chromatography coupled on-line with the multiple angle laser light scattering (HPSEC-MALLS) method was used to determine the molecular weight distribution (MWD) and specifically the number-average $\overline{M_n}$ and weight-average $\overline{M_w}$ molar masses of each sample.

## 2 MATERIALS AND METHODS

Native starches were purchased from Roquette Frères (Lestrem, France). High-amylose maize starch contains 70 % of amylose whereas waxy maize starch contains 99 % amylopectin. Two blends are obtained by mixing these two starches, and final amylose levels are 47 and 23 %. Starches are processed on a twin screw extruder (Clextral BC 45) under various conditions of temperature (Tp: 100-180°C), moisture content (MC: 20-33 % on a total weight basis) and specific mechanical energy required during processing (SME: 100-580 kWh/t), details are given by Della Valle *et al.* [2].

The samples were first dissolved in 95% dimethylsulphoxide (DMSO), and then solubilised in water by micro-wave heating as described previously[3], where details on the HPSEC-MALLS procedure are also given. The HPSEC system comprised silica gel columns. Dual detection of solutes is carried out with a MALLS detector (Dawn DSP-F, Wyatt Technology Corporation, US) and a differential refractive index detector (Erma

ERC-7510). This system allows quick fractionation and characterisation of high molecular weight polymers.

## 3 RESULTS AND DISCUSSION

Extruded starches are dispersed in 95% DMSO and solubilised in water with higher yields (86-100%) than native starches (77-91%). Solutions are characterised by HPSEC-MALLS with good sample recoveries (>90%). This method gives the molecular weight distribution (MWD), the number-average $\overline{M_n}$ and weight-average $\overline{M_w}$ molar masses.

### 3.1 $\overline{M_n}$ and $\overline{M_w}$ of native starches

As shown in Table 1, $\overline{M_n}$ and $\overline{M_w}$ of native starches decrease with increasing amylose content.

**Table 1** $\overline{M_n}$ *and* $\overline{M_w}$ *of different amylose content maize starches*

| *Amylose content (%)* | *0* | *23* | *47* | *70* |
|---|---|---|---|---|
| $\overline{M_n}$ (g/mol) | $1.4x10^8$ | $1.1x10^8$ | $2.3x10^7$ | $3.7x10^6$ |
| $\overline{M_w}$ (g/mol) | $1.8x10^8$ | $1.5x10^8$ | $6.1x10^7$ | $1.4x10^7$ |

### 3.2 MWD, $\overline{M_n}$ and $\overline{M_w}$ of extruded starches

Comparing with native starches, extruded starches show a decrease of $\overline{M_n}$ and $\overline{M_w}$, and a shift of MWD to the lower masses, underlining macromolecular degradation. Figure 1 shows a shift of MWD to the lower molar masses for extruded starches, whereas $\overline{M_n}$ and $\overline{M_w}$ decrease: $1.5x10^7$ to $8.4x10^6$ g/mol and $2.4x10^7$ to $1.9x10^7$ g/mol, respectively (Table 2). No evidence of exo-degradation is seen.

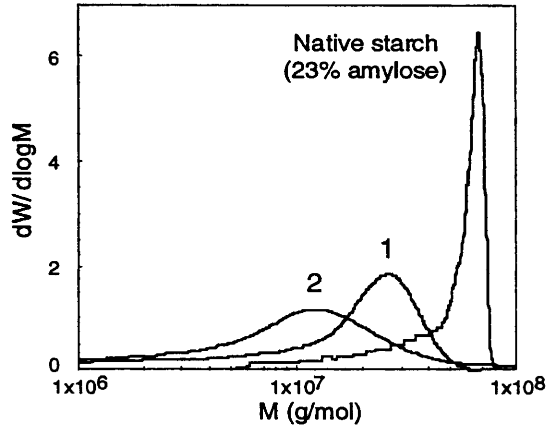

**Figure 1** *Differential logarithmic molecular weight distribution of native and degraded starches with 23% amylose content. (1: SME=120kWh/t, Tp=152°C, MC=27%; 2: SME=370kWh/t, Tp=102°C, MC=33.5%)*

**Table 2** *$\overline{M_n}$ and $\overline{M_w}$ of native and extruded starches with 23% amylose content*

| | $\overline{M_n}$ (g/mol) | $\overline{M_w}$ (g/mol) |
|---|---|---|
| Native | $1.1 \times 10^8$ | $1.5 \times 10^8$ |
| 1 | $1.5 \times 10^7$ | $2.4 \times 10^7$ |
| 2 | $8.4 \times 10^6$ | $1.9 \times 10^7$ |

### 3.3 Temperature influence on macromolecular degradation

This parameter was tested very easily with monodisperse samples of pullulans. Based upon the same monomer and linkages as amylose and amylopectin, pullulans are polymers of α(1, 6) maltotriosyl units. Submitted to high temperatures in a microwave pressure vessel, they presented random depolymerisation. When studying extruded starches, a non clear tendency was evidenced (Figure 2).

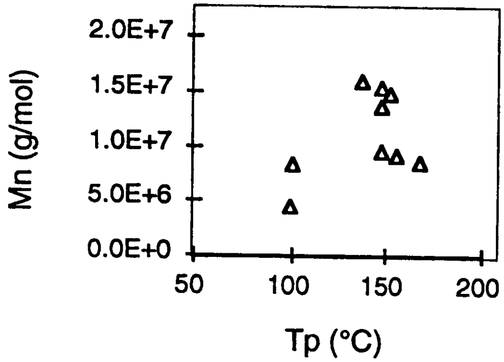

**Figure 2** *Influence of extrusion temperature on $\overline{M_n}$ for 23% amylose content extruded starches*

### 3.4 SME influence on macromolecular degradation

For the same amylose content sample, $\overline{M_n}$ of extruded starches is mainly influenced by SME (Figure 3), and the other variables, [η][4]. They reflect the extent of chain splitting phenomena likely due to the shear stress occurring within the extruder.

Macromolecular degradation of extruded starches varies with amylose content. In fact, $\overline{M_n}$ of different extruded starches versus SME, shows for 0% amylose content samples an important decrease of $\overline{M_n}$ with increasing SME, whereas 70% amylose content samples seem not significantly changed (Figure 5). The progressive decrease of the curves suggests a low susceptibility below $5 \times 10^6$ g/mol. Moisture content does not present a clear influence on macromolecular characteristics (Figure 4).

Amylopectin molecules are more susceptible to macromolecular degradation than amylose ones, as observed by several authors[5]. Amylopectin molecules are 10 to 100 times larger than amylose ones. Submitted to shear they should occupy larger volumes than amylose. This size difference would be responsible of the higher susceptibility of amylopectin molecules to shear degradation. Different susceptibilities to shear degradation between α(1,4) and α(1,6) branching points can not be suspected on the basis of these results.

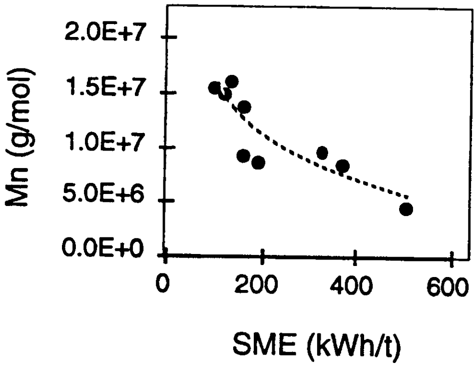

**Figure 3** *Specific mechanical energy (SME) on* $\overline{M_n}$ *for 23% amylose content extruded starches*

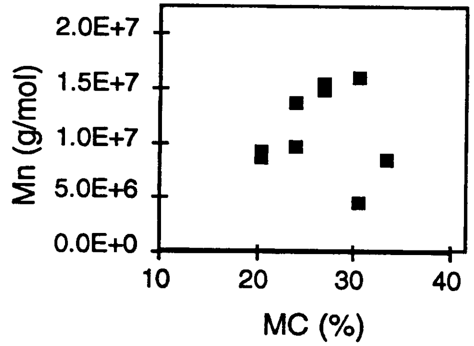

**Figure 4** *Influence of moisture content (MC) on* $\overline{M_n}$ *for 23% amylose content extruded starches*

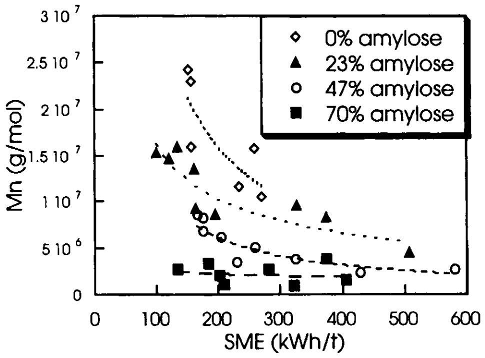

**Figure 5** $\overline{M_n}$ *versus SME, for different amylose content extruded starches*

## 4 CONCLUSION

HPSEC-MALLS method gives additional information on macromolecular characteristics to help understand macromolecular degradation during extrusion processing. This phenomenon is underlined by the disappearance of high molecular weight fractions and the appearance of low molecular weight fractions (mainly higher than $10^5$ g/mol). Shear stress has a strong effect on macromolecular degradation during extrusion-cooking. This effect is enhanced with increasing amylopectin ratio. The larger size of amylopectin could explain its higher susceptibility to shear degradation than amylose.

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# THE IMPACT OF INTERNAL GRANULE STRUCTURE ON PROCESSING AND PROPERTIES

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## 1 INTRODUCTION

Understanding the relationship between the internal starch granule structure and its subsequent break down during processing, has never been more important. For the first time the possibility of deliberately modifying the internal structure via some sort of genetic manipulation has become a realisable goal. Placing the granule structure at centre stage, one must both explore backwards (in time) how alterations in the starch synthetic pathway impact on the internal hierarchical structure, and downstream how these internal changes affect the ease of subsequent processing and the consequences for product stability. This article aims to bring together a discussion of the various structural elements and length scales within the granule, with an analysis of their consequences for both processing and structural stability. As we gain insight into these correlations, we can begin to make recommendations for which changes in structural stability we desire from plant breeders.

## 2 EXPERIMENTAL APPROACHES

### 2.1 Small Angle X-ray Scattering

X-ray scattering is widely used to study structure of semi-crystalline carbohydrate polymers. Wide angle X-ray scattering (WAXS) provides information on the inter-atomic length scale, and hence (in the case of starch) may be used to identify the crystalline polymorph of the amylopectin helices[1]. Small angle X-ray scattering (SAXS) is concerned with probing larger length scale structures from nm distances upwards. In the case of starch granules, the main feature in the SAXS patterns is a peak corresponding to an approximately 9nm spacing [2]. This periodicity arises from stacks of alternating crystalline and amorphous lamellae, in which the amylopectin branch points reside predominantly in the amorphous lamellae. At low scattering angles (q), the scattered intensity increases steeply, indicating the presence of larger length scale structures. This low q data is related to the presence of amorphous growth rings, whose origin is thought to reside in diurnal fluctuations in the way the starch is laid down[3].

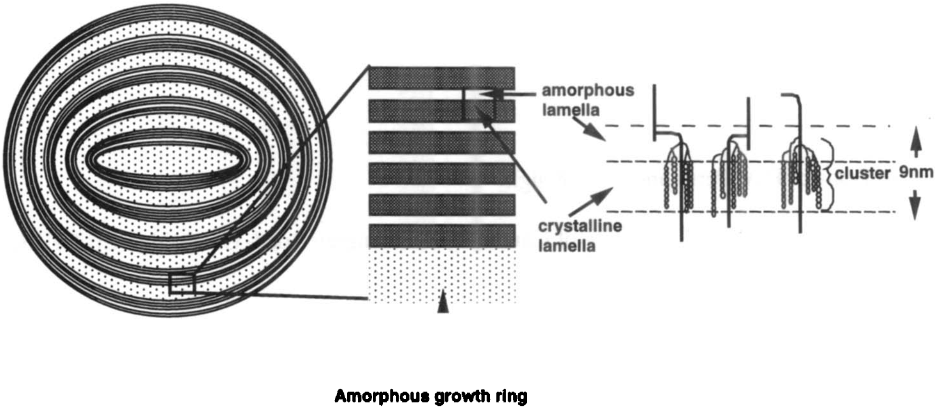

**Figure 1** *Schematic representation of the starch granule showing the 3 different types of regions: amorphous and crystalline lamellae in a repeating stack, and amorphous growth rings*

Modelling of the whole SAXS curves has been carried out, using as a framework the schematic diagram shown in Figure 1 [4]. This model for the internal granule structure lies at the heart of the present paper. The 9nm repeat has been found to be essentially constant across a wide variety of species and cultivars [5]. However, the peak position only provides a measure of the average spacing, and it must be recognised that correlated disorder exists within the lamellae of a granule and variations in its magnitude can potentially occur both within and between granules. SAXS/WAXS experiments were carried out at the SRS (Daresbury Laboratory, UK). Using the high intensity of such a synchrotron source means that real time experiments can be carried out. In this paper we will explore changes in the SAXS during real time heating and cooling experiments to explore the impact of potential processing histories.

## 2.2 Small Angle Neutron Scattering

Small angle neutron scattering (SANS) experiments explore basically the same angular range as SAXS. The difference is that, whereas X-rays scatter off electron density differences, neutrons are sensitive to variations in scattering length density of the nuclei from which they are scattered. Hydrogen and deuterium nuclei have very different scattering length densities, indeed of opposite sign, and thus by replacing some hydrogen atoms by deuterium leads to a ready source of contrast in SANS experiments. Practically this may be implemented by equilibrating starch slurries with $D_2O$ in place of $H_2O$. By changing the relative ratio of $D_20$ to $H_2O$, contrast variation can be achieved, which provides a new fitting parameter not available to SAXS experiments. Such contrast variation experiments were carried out on the LOQ beamline at the RAL neutron spallation source (Didcot UK). The basic model and fitting is therefore the same as for SAXS. It should be noted that SANS provides a much lower flux, and real time experiments are correspondingly harder.

## 3 RESULTS AND DISCUSSION

### 3.1 Effects of Water Content

The absence of the 9nm lamellar peak is well known in dry starch. This has generally been attributed to 'lack of contrast'. Figure 2 shows a comparison of SAXS curves for potato, when wet and dry; the camera length for these experiments has been set up so that the position of the 100 peak (only visible in B type polymorphs and usually observed in the WAXS pattern) can also be observed. It can be seen that both this 100 peak, and the 9nm peak, are absent in the dry system. This observation indicates that the loss of the 9nm peak cannot be attributable to a lack of contrast, but has its origin in some sort of structural rearrangement.

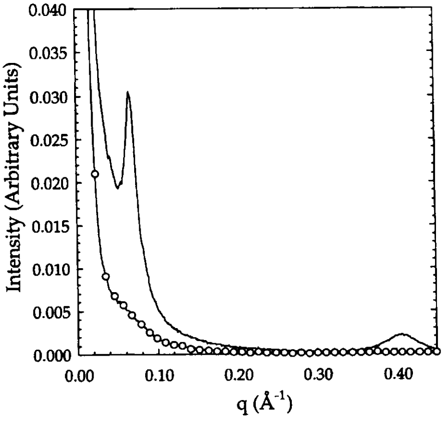

**Figure 2** *SAXS curves for potato starch showing results from hydrated (solid line) and dry (-o-) granules*

To rationalise this observation, the concept of a side-chain liquid-crystalline polymer has been borrowed from the field of (synthetic) polymer physics[6]. The amylopectin molecule, is highly branched, and its side chain branches are known to form double helices, rigid entities with long persistence lengths. Therefore it can be regarded as equivalent to a side-chain liquid-crystalline polymer (SCLCP), as shown in Figure 3. The double helices are equivalent to 'mesogens' which, because they are stiff, wish to align. In the native and hydrated state (Figure 3b), this is readily possible, because the presence of the water molecules can solvate the regions containing the branch points and enable a decoupling between the main backbone of the amylopectin molecule, and the mesogens. This decoupling, allows the competitive tendencies for the ordering of the backbone and side-chains to be reconciled. On the other hand in the dry state, this decoupling does not occur and the lateral packing of the side chains is disturbed (Figure 3a). Both the long range correlations (typified by the 9nm peak) and the local packing of neighbouring double helices (manifested by the 100 peak in potato) are destroyed, although the correlations within the double helices are of course maintained, so that there are still well defined diffraction peaks in the WAXS patterns.

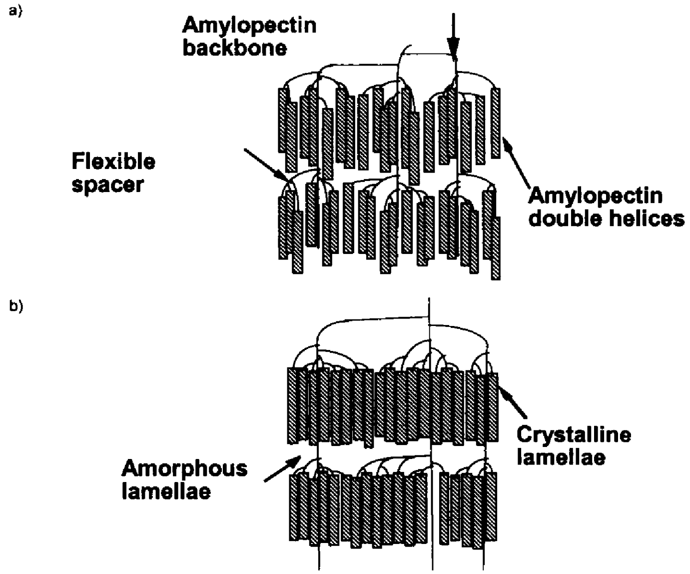

**Figure 3** *Representation of the amylopectin molecule as a liquid crystalline polymer. The double helices, represented by shaded blocks, are the mesogens in the structure. In the dry state (a), the mesogens are disordered. Upon hydration (b) the mesogens line up into smectic-like layers*

Using this framework of a SCLCP highlights the importance of the 'spacer' (again to borrow the language of synthetic SCLCP's); that length of a branch between the part of the side chain involved in double helix formation, and the backbone. The pioneering work of Hizukuri[7] enables some comparisons to be made between the length of this region for different species (Table 1). It can be seen that for potato this spacer length is rather short, but the double helix region itself is comparatively long. For A type starches, such as waxy maize, the converse is true; the decoupling spacer is significantly longer. These findings suggest that one possible explanation for the apparent ubiquity of the average 9nm lamellar spacing is that it is a compromise between the ability of the mesogens to be sufficiently decoupled to permit lateral order to develop in the crystals, and the minimum length of double helix required for stable crystal formation.

**Table 1** *Variation in the number of monomers involved in the double helix (mesogen) and the decoupling spacer, according the data of Hizukuri*[7]

| *Species* | *Modal number of monomers* | |
|---|---|---|
| | i (Mesogen) | ii (Spacer) |
| Tapioca (A type) | 11 | 7 |
| Wheat (A type) | 11 | 7 |
| Waxy rice (A type) | 13 | 6 |
| Potato (B type) | 16 | 3 |

## 3.2 The Response of Organisation in the Granule to Other Solvents

In the absence of water it appears that the lamellar structure in starch is poorly organised. What happens under other conditions? A variety of other solvents, aqueous and non-aqueous, have been investigated. In the case of glycerol and its aqueous mixtures, which are important as processing aids for starch, at room temperature the 9nm peak is not seen for glycerol concentrations above about 85%. Upon carrying out dynamic SAXS experiments during a heating run, it can be seen that this peak appears at elevated temperatures. The temperature dependence of the peak intensity from the 9nm periodicity is shown in Figure 4, where it is correlated with DSC data. DSC traces show that at these glycerol concentrations, a new exotherm is seen at temperatures above room temperature. For each glycerol concentration, the temperature at which this exotherm is seen correlates well with the appearance of the 9nm peak. In other words, the self-assembly of the lamellar structure is an exothermic event. It should be noted that we can check explicitly for glycerol whether self assembly has occurred, and if there is simply a 'lack of contrast' to reveal the peak, by carrying out SANS experiments using deuterated solvents of differing contrasts. By using a selection of different H:D ratio labelled glycerols, at room temperature there is never any sign of a peak, confirming that self assembly has not occurred. In contrast to this behaviour, at 85°C the peak is clearly seen, although its intensity depends on the H:D ratio as one would expect, according to how much solvent has entered the different regions of the granule[8]. The kinetics of the self-assembly process are sigmoidal, indicating a co-operative process of diffusion, of the type known as Case II for polymers[9] as the plasticiser penetrates the granule.

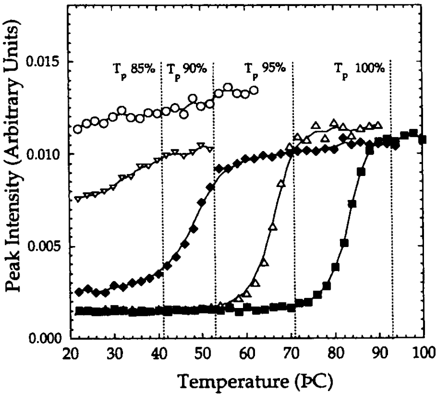

**Figure 4** *Variation in peak intensity with temperature for a variety of concentrations of glycerol solutions in water: -o- 80%; -∇- 85%; -♦- 90%; -Δ- 95%; -■- 100%. The dashed vertical lines represent the peak temperature for the exotherms in the solutions of different glycerol concentrations.*

A similar type of behaviour is seen in non-aqueous solvents, such as ethylene glycol and butane 1,4-diol: an exothermic event is seen in the DSC traces at the temperature at which the SAXS shows the growth of the 9nm peak. The precise temperature at which this event occurs depends on both the solvent which is entering the granule and the particular species, as shown in Figure 5. Since the amorphous lamellae are denser in potato than in waxy maize, as revealed by fitting of SAXS data[10], it is presumed that this controls the ingress of solvent. It is also possible that the presence of amylose may play a role in determining the ease of ingress. The solvent size is another important factor, with ethylene glycol entering the granule more easily than glycerol, due to its lower relative molar mass.

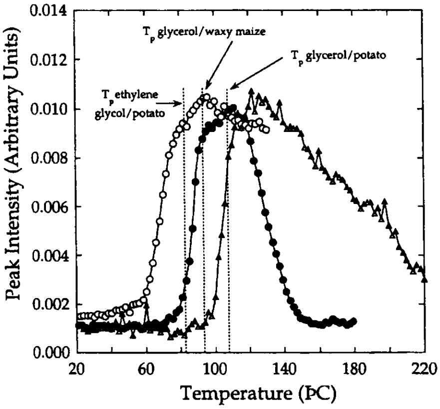

**Figure 5** *A comparison of the behaviour of different starches and different solvents -o- potato in ethylene glycol; -●- waxy maize in glycerol; -Δ- potato in glycerol. The corresponding exotherm peak temperatures are marked by dashed vertical lines.*

It is important to stress that the inclusion of a large number of water molecules in the crystalline structure is not the driving force for self-assembly, since plasticising with diols and glycerol work equally well as crystallising agents. We thus deduce that it is the mobility or entropy of the backbone and spacers which is the key physical process required for the process of self-assembly.

### 3.3 Impact on Gelatinisation Response

In many instances of processing, what is required is the ultimate breakdown of the granule structure via gelatinisation[11]. This may often occur in sugar containing solutions, and it is well known that the requisite temperatures for gelatinisation are pushed up (by comparison with water) in solvents other than pure water. The experiments described here reveal that one explanation for this behaviour is that the solvents are simply not getting into the granule to cause gelatinisation as readily as water does, for which ingress seems to occur instantaneously. Since the solvent plays such a key role in gelatinisation, it is not surprising that conditions which hamper the ingress of solvent and accompanying self assembly of the lamellae lead to a raising of the gelatinisation temperature. Figure 6 shows DSC traces of waxy maize mixed with ethylene glycol taken up past the point of gelatinisation. Ethylene glycol enters the granule fairly easily, therefore the exotherm (above room temperature) associated with the self assembly disappears after storage times (at room temperature) of more than a couple of hours, since assembly has already been completed during the storage period. Whatever the storage conditions, however, the

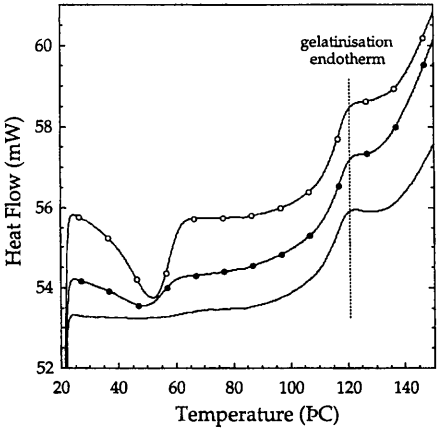

**Figure 6** *DSC traces for waxy maize in ethylene glycol (heating rate of 10°C/min) following room temperature storage for : -o- 45 minutes; ; -●- 90 minutes and – 24 hours.*

gelatinisation response is the same, with the endotherm occurring around 120°C ie. raised by about 60° compared with the case of gelatinisation in water.

Gelatinisation is associated with the loss of double helices together with the loss of lamellar and long range structure. This requires significant chain mobility, which is imparted by a combination of plasticisation and heat. The less effective the plasticisation (which can be inferred from the temperature of the self-assembly exotherm) the higher the temperature needs to be raised to permit sufficient loss of order at all lengths scales. This is shown schematically in Figure 7. Within this framework it becomes clear why the onset of gelatinisation is delayed by the addition of different solvents. The ‘harder’ it is for the

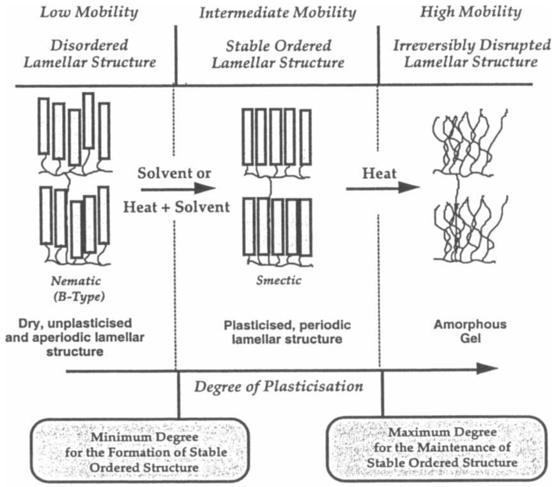

**Figure 7** *Schematic representation of the changes in organisation as plasticisation proceeds*

solvent to get in, which is affected by solvent size as well as type, the higher in temperature and/or time before there is sufficient mobility to permit the granule structure to start to break down. The self assembly shown in the figure of amylopectin helices into well-ordered lamellae is an intermediate step in this process, and monitoring it provides a good indication of the ease with which a particular solvent enters the granule. These ideas apply equally well to a range of different sugar and polyol solutions[10].

## 4 CONCLUSIONS

The use of small angle scattering – both X-ray and neutron – provides useful new insights into the organisation of structure within the granule at length scales characteristic of the lamellar structure. Understanding the conditions under which this structure can self assemble into a regular periodicity with long range correlations as represented by the 9nm peak, helps us to understand the impact of processing conditions. Representing the amylopectin molecule as a side-chain liquid-crystalline polymer is a helpful way of understanding the processes involved in this assembly, and the way the chain branch architecture may impinge on the organisation. It is shown that a necessary requirement for the lamellar organisation to be achieved is sufficient plasticisation, and this can be accomplished by an appropriate combination of time and temperature. The higher the temperature required to accommodate the self assembly, the higher the corresponding gelatinisation endotherm will be. This framework provides a rationale for the well known observation that gelatinisation is delayed to higher temperatures in concentrated sugar solutions.

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# PFG-NMR APPLIED TO MEASURE THE DIFFERENCE IN LOCAL MOISTURE CONTENT BETWEEN GELATINISED AND NON-GELATINISED REGION IN A HEATED STARCH/WATER SYSTEM

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## 1 INTRODUCTION

It is well known that a starch granule has the ability to absorb water with an advancement of starch gelatinisation. However, it is not known how much the gelatinised starch granules absorb water. When some starch granules gelatinise, they enhance their ability to absorb water, and they collect water, not only from their surroundings but also from the neighbourhood of non-gelatinised granules[1]. If starch is not in excess water but in a limited water environment, then this may result in a microscopic inhomogeneity: water-rich and water poor regions (Figure 1), even though the difference in local moisture content has not been detected. In this work, the local moisture content in both water-rich gelatinised region and water poor non-gelatinised region, which change as gelatinisation proceeds, was detected using a Pulsed-Field Gradient (PFG) NMR technique.

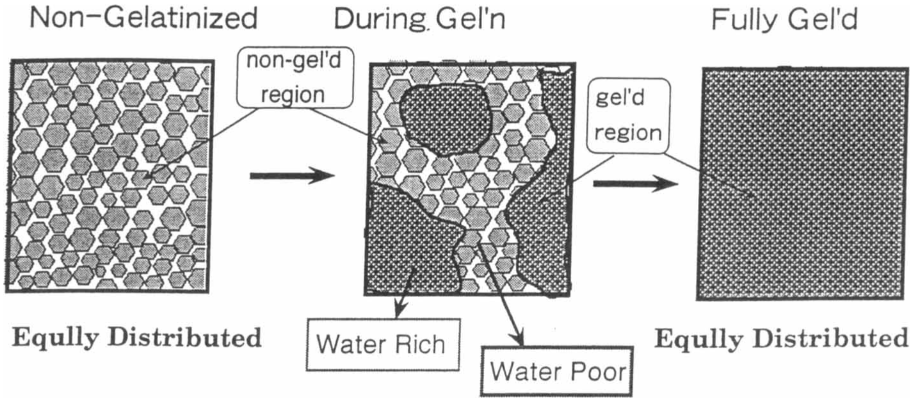

**Figure 1** *Microscopic in-homogeneity in starch/water mixture*

## 2 MATERIALS AND METHODS

### 2.1 Sample Preparation

Rice starch was mixed with water to make about 0.5 g water/ g sample moisture content and put into a Teflon tube. This was wrapped with film and placed at the bottom of an NMR test tube. Then the tube was located at the probehead in the NMR magnet. The temperature of the sample was raised gradually to the target temperature and held there. After some period of settling, a series of diffusivity measurements was made by varying the intensity of the pulse-magnetic field gradient.

### 2.2 NMR Measurement

Water diffusivity in the starch/water mixture was measured by PFG-NMR using the stimulated echo method. The spectrometer used was a Bruker AM200WB with a 4.7 Tesla magnet and a pulse field gradient accessory. A series of PFG-NMR experiments was carried out varying the magnitude of the field gradient ($g$) with a 500 ms diffusion time ($\Delta$). The logarithm of the NMR echo intensity was plotted against $\gamma^2 g^2 \delta^2(\Delta-\delta/3)$; the plot gave a straight line, the slope of which was assigned to the diffusion coefficient of water, where $\gamma$ is the gyromagnetic ratio and $\delta$ is the length of the field gradient pulse. The signal intensity at the echo peak, which is denoted as $S$, is described as:

$$S = S_0 \exp[-kD] \qquad (1)$$

where $D$ is water diffusion coefficient, $k$ is $\gamma^2 g^2 \delta^2(\Delta-\delta/3)$ and $S_0$ is constant.

When the target molecules, which are water in our case, are in two groups, one is in a gelatinised site, G, and the other is in a non-gelatinised site, N (Figure 1). Then the logarithmic plot of the NMR signal intensity may show a multi-exponential curve. The signal intensity, which is the sum of the signals from site G and site N, is expressed as:

$$S = p_G \exp[-kD_G] + p_N \exp[-kD_N] \qquad (2)$$

Where $p$ is an apparent population term.

## 3 RESULTS AND DISCUSSION

The result for non-gelatinised starch/water mixture at 24.5 °C is shown in Figure 2. The logarithm of echo intensity against k gives a straight line. The slope, which gives the diffusivity of water molecules was, $0.7 \times 10^{-5}$ cm$^2$/s. This is about 35% of that of the freely-diffusing water molecules, namely, the diffusion of water molecules which filled the gaps between the starch granules, was detected. This means that free diffusion of water molecule is restricted by the starch granules. However, when the sample was heated to 62.5 °C, the plot did not show a straight line anymore. This may be caused by starch gelatinisation. The gelatinised starch absorbs water into the grain, but this water may still have some freedom in motion, which makes its signal detectable. So the water in the gelatinised granules forms the second phase, which is distributed among non-gelatinised starch/water sites, as illustrated in Figure 1. As the sample temperature increases, the line

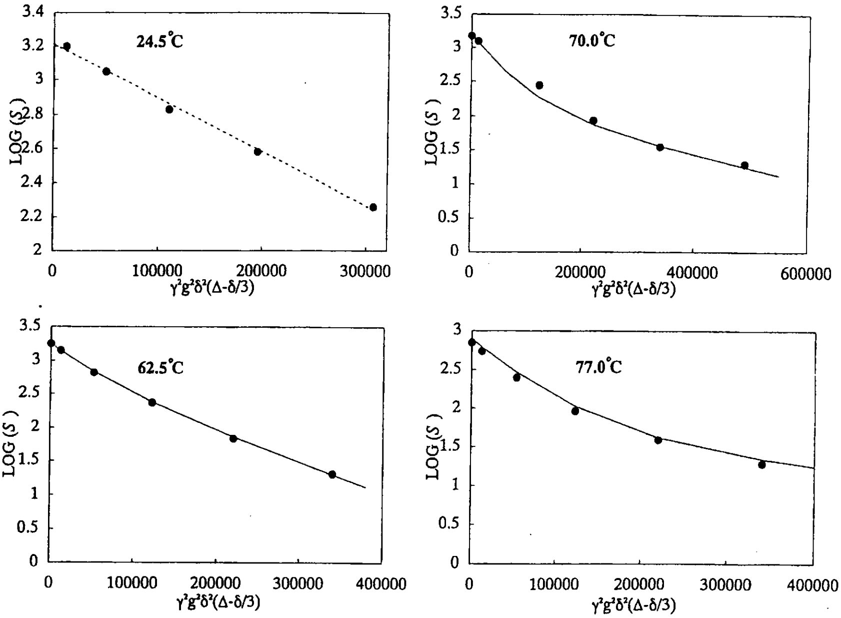

**Figure 2** *The logarithm of signal intensity at the echo peak versus* $\gamma^2 g^2 \delta^2 (\Delta - \delta/3)$. *Parameters other than field gradient intensity g were kept constant. Average moisture content of starch/water mixture was 0.48 wet basis in all experiments*

**Table 1** *Water diffusivity and population obtained by curve fitting*

| *Temp (°C)* | *Component 1* | | *Component 2* | | |
|---|---|---|---|---|---|
| | $D_G$ (cm²/s) | $p_G$ | $D_N$ (cm²/s) | $p_N$ | $p_G/(p_G+p_N)$ |
| 62.5 | $1.7\times10^{-5}$ | 9.03 | $4.5\times10^{-6}$ | 16.8 | 0.35 |
| 70.0 | $1.5\times10^{-5}$ | 16.0 | $2.01\times10^{-6}$ | 9.97 | 0.62 |
| 77.0 | $1.2\times10^{-5}$ | 12.1 | $1.2\times10^{-6}$ | 5.19 | 0.70 |

curved more. These dotted data in Figure 2 were fitted to the two site model using Equation 2. The solid lines in Figure 2 show that the fitting seemingly worked well. The water diffusivity and apparent population obtained by the curve fitting is shown in Table 1. Pairs of large and small diffusivities were obtained, the larger ones being ten times higher. Now these diffusivities need to be converted to moisture content.

Although it is well known that water diffusivity in food is, in general, not constant but strongly depends on moisture content and temperature, the actual dependence is very often unknown. Fortunately, Gomi et al.[2] measured water diffusivity in rice starch/water mixtures at a range of temperatures and moisture contents using a PFG-NMR method. Since they found that two different diffusivities were observed in the partly gelatinised phase, they classified the samples into two categories in order to avoid complexity: one represents non-gelatinised samples in which temperature was kept below 60 °C. The other corresponds to fully heated samples, which were first heated at 95 °C for 60 minutes and cooled. And they proposed the following empirical equation for each category:

For non-gelatinised starch/water mixtures

$$D_{\text{non}} = D_{\text{free}}0.127(m/(1+m)-0.33)\exp[778/(\theta+273)] \qquad (3)$$

where $\theta$ is the temperature and $m$ is moisture content.

For fully heated starch/water mixtures

$$D_{\text{full}} = D_{\text{free}}(1.31m/(1+m)-0.486) \qquad (4)$$

where $D_{\text{free}} = 0.0232\exp[-2070/(\theta+273)]$ [cm$^2$/s] (5)

Figure 3 shows water diffusivity against temperature at selected moisture contents calculated using equations (3) and (4). Under 60 °C, water diffusivity in gelatinised phase is lower than that in non-gelatinised phase. The diffusivity values obtained in this work are marked on the Figure 3. At first, the sample is at room temperature, and the diffusivity starts here, a little below the 0.5 moisture content line. The diffusivity moves along this line, the non-gelatinised starch line, as the sample was heated up to 60 °C, where the starch gelatinisation initiates. At 62.5 °C, the diffusivity splits into $1.7\times10^{-5}$ cm$^2$/s and $4.5\times10^{-6}$ cm$^2$/s. Since there is no reason for the diffusivity in non-gelatinised phase to increase when some granules gelatinise, we presume that the larger diffusivity is for the gelatinised phase where the moisture content increased. Then the moisture content in the gelatinised site corresponds to about 0.65, while the non-gelatinised phase was dehydrated to 0.4. Now the appearance of water-rich and water poor regions can be detected. When the sample temperature is further raised to 70 °C, the water diffusivity decreases, meaning also the decrease of moisture content.

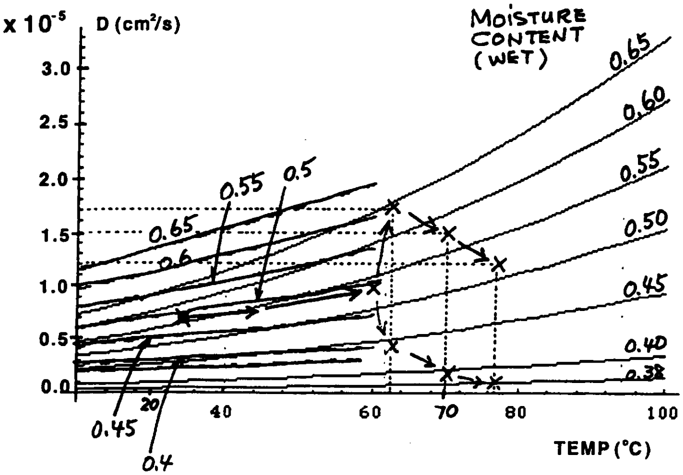

**Figure 3** *Water diffusivity in rice starch/water mixture calculated using empirical equations (Gomi et al.*[2]*)*

This is reasonable, because as the temperature rises, the starch gelatinisation may proceed, and since the newly-gelatinised starch requires more water, and the total amount of water is limited, the demand by the newly-gelatinised starch could be balanced only when all gelatinised granules share the available water in the system. Up to this point, the diffusivity measurement by PFG-NMR seemingly works well. Then how about the apparent population term?

Since the apparent population terms $p_G$ and $p_N$ in Equation 2 is affected by the amount of sample used, comparison between their absolute values are meaningless. But the percentage of $p_G$ and $p_N$ has information. The percentage of component 1(gelatinised phase) is 35% at 62.5 °C, increasing to 62% at 70 °C and 70% at 77 °C. But in fact these percentages do not mean the water percentage existing in the gelatinised phase directly. The NMR signal intensity $S$ is expressed as the sum of signals from gelatinised and non-gelatinised phases. Each signal decays exponentially with the increase of $k$. The coefficient $p_G$ and $p_N$, which we call an apparent population term, is expressed as the following equations:

$$p_G = S_i \eta_G m_G \exp[-\tau/T_{2G}] \tag{6}$$

$$p_N = S_i (1+\eta_G) m_N \exp[-\tau/T_{2N}] \tag{7}$$

where $S_i$ signal intensity just after excitation, $\eta_G$ extent of starch gelatinisation which depends on temperature and moisture content, $m$ moisture content in dry basis, $\tau$ the time of echo kept constant in the experiment, $T_2$ NMR relaxation time. $T_2$ relaxation of water molecule in starch/water mixture is known to depend on moisture content$^3$.

$$T_2=\exp[4.386m/(1+m)+0.6028 \tag{8}$$

It means that the NMR signal from water in high moisture region is sustained longer, while that in the low moisture region decays rapidly. Since the signal detection was made at $\tau$ ms after excitation, the signal from high moisture region may be greatly emphasised. Another point is the amount of water, which is expressed as. In the present experiment, the total moisture content of the sample was kept at 48% wet basis. Figure 4 illustrates to what extent the starch granules gelatinise, as a function of temperature and moisture content. Only a few papers have been published giving information on the extent of gelatinisation. The moisture content in the present experiment was about 50%, which means that water is not in excess but in limiting condition. Figure 4 is the only data available for the extent of gelatinisation, which we need for the discussion on population term. Then we try to use Figure 4. With all the information shown in Table 2, $p$ - term:

$$p = \eta m \exp[-\tau/T_2] \tag{9}$$

was calculated using parameters predicted separately from PFG-NMR experiment. The calculated ratio of $p'_G/(p'_G+p'_N)$ is compared with that of measured $p_G/(p_G+p_N)$. For example, the prediction tells that if about 10% of the total starch is gelatinised at 62.5 °C, then the predicted $p'_G/(p'_G+p'_N)$ value is very near to that of measured value of 0.35. This result shows that this discussion works better than simply an order magnitude estimation.

**Table 2** *Comparison between the measured and the calculated population*

| Temp<br>°C | $\eta_G$ | $M_G$<br>wet | $T_{2G}$<br>ms | $p'_G$ | $1-\eta_G$ | $M_N$<br>wet | $T_{2N}$<br>ms | $p'_N$ | *Calc.*<br>$p'_G/(p'_G+p'_N)$ | *Meas.*<br>$p_G/(p_G+p_N)$ |
|---|---|---|---|---|---|---|---|---|---|---|
| 62.5 | 0.05 | 0.65 | 31.6 | 0.018 | 0.95 | 0.41 | 11 | 0.076 | 0.19 | 0.35 |
| 62.5 | 0.05 | 0.7 | 39.4 | 0.022 | 0.95 | 0.41 | 11 | 0.076 | 0.22 | 0.35 |
| 62.5 | 0.1 | 0.65 | 31.6 | 0.035 | 0.9 | 0.41 | 11 | 0.072 | 0.33 | 0.35 |
| 70.0 | 0.2 | 0.6 | 25.4 | 0.055 | 0.8 | 0.38 | 9.67 | 0.039 | 0.37 | 0.52 |
| 70.0 | 0.2 | 0.7 | 39.3 | 0.089 | 0.8 | 0.38 | 9.67 | 0.047 | 0.65 | 0.62 |
| 77.0 | 0.4 | 0.52 | 25.4 | 0.075 | 0.6 | 0.35 | 8.48 | 0.025 | 0.75 | 0.7 |
| 77.0 | 0.5 | 0.55 | 20.4 | 0.09 | 0.6 | 0.35 | 8.48 | 0.025 | 0.78 | 0.7 |

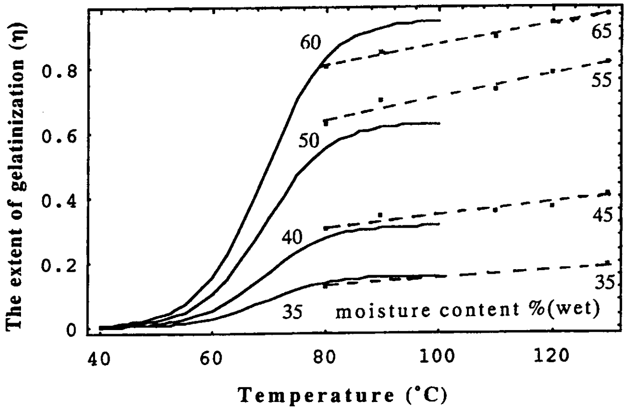

**Figure 4** *The extent of starch gelatinisation (•) versus temperature at various moisture content(wet basis). The solid lines show the • observed by Fukuoka et al.*[4]*. The dashing lines show the •• which are recalculated using published data by Da Silva et al.*[5]

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# RETROGRADATION KINETICS OF MIXTURES OF RICE STARCH WITH OTHER TYPES OF STARCHES

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## 1 INTRODUCTION

The blending of different types of flours and/or starches is an age-old technique used in the manufacture of a wide range of traditional food products[1]. The blending of flours and/or starches serves several purposes. It can be used to obtain desired properties tailored to specific uses which are not inherently obtainable from the individual flours or starches themselves. For example, Stute and Kern[2] have patented blends of unmodified pea and corn starches for use in the preparation of puddings with a greater resistance to syneresis. Obanni and BeMiller[3] have pointed out the possibility of formulating starch blends that behave like chemically modified starches, particularly with regard to pasting behaviour. Another objective is the partial substitution of more expensive or not so easily available materials by cheaper or more freely available indigenous flours or starches while maintaining or even improving product quality.

Interactions between different starches when blended are not well understood. This is particularly so where the phenomenon of starch retrogradation is concerned. The crystallisation process in gelatinised non-waxy starch systems is dominated by amylose in the early gelation stage and by amylopectin over long-term storage[4,5]. This ability of starch molecules to aggregate and crystallise is of considerable commercial interest since it is a major factor contributing to the textural properties of starch-based food products. For example, staling or increased stiffness of such products during storage has been attributed primarily to amylopectin retrogradation[6-8]. However, there are instances where starch recrystallisation is promoted to enhance the structural, mechanical, or organoleptic properties of certain starch-based products[9].

While the retrogradation tendencies of individual starches have been studied extensively, those of mixtures of starches have received scant attention. Manipulation of the rate or extent of starch retrogradation by the simple expedience of blending different flours or starches is of technological importance to the food industry. Staling of starch-based products can, for example, be effectively retarded by incorporation of waxy starches into the formulation[10, 11]. Conversely, waxy corn starch gels have been observed to retrograde to a greater extent when commercial starch hydrolysates are incorporated[12]. del Rosario and Pontiveros[13], who conducted a limited study of the retrogradation behaviour of dilute 1% solutions of 50:50 (w/w) mixtures of various starches at pH 7 during storage at 10° and 30°C, found extensive interactions between the starches. More recent DSC studies, albeit very limited, have indicated that certain starch blends may have a reduced tendency to retrograde as compared with the individual starches at the same starch concentration[3]. Clearly, interactions do occur and starch molecules do not necessarily behave independently when they are combined. The nature of such interactions is, however, not well-defined and further investigation is warranted.

The present study, involving the use of a novel pulsed low-resolution NMR technique[14, 15] to monitor starch (amylopectin) retrogradation during storage, restricts itself to mixtures of rice starch with other starches in more concentrated gel, rather than sol, systems. Such systems are particularly relevant to Asian countries where many indigenous food products are made from combinations of rice flour/starch with other types of flours/starches. For example, in the production of rice vermicelli (known in Malaysia as *beehoon*), at least two other starches, usually corn and sago, are used together with rice flour.

## 2 MATERIALS AND METHODS

Potato and corn starches were obtained from BDH Ltd (Poole, UK) and rice starch from Sigma Chemical Company (St. Louis, MO, USA). Mungbean and sago starches were local commercial products which were purified using the method described by Juliano[16] involving repeated steeping of the materials in NaOH solution.

Moisture content was determined from the loss in weight on drying triplicate 2-g samples in an air-oven at 105°C to constant weight. Amylose content was determined in duplicate using the colourimetric method of Williams et al.[17] involving the formation of blue iodine-amylose complex.

Mixtures of rice starch with different proportions of corn, sago, potato, and mungbean starches were prepared by dry mixing. Starch gels, at a fixed dry starch/water ratio of 1:2, were then prepared in NMR tubes as described by Teo and Seow[14]. Gelatinisation involved heating at 98°C for 90 min. Distilled demonised water was used to prepare the gels. The retrogradation tendencies of the gels derived from mixtures of starches were measured in triplicate, as a function of storage temperature (5°, 15° and 25°C) and time, using the pulsed NMR method of Teo and Seow[14]. The classical Avrami equation was used to determine the kinetics of starch retrogradation[14].

## 3 RESULTS AND DISCUSSION

The pulsed NMR method used in the present study for the study of starch retrogradation is based on the differentiation in NMR signals from protons in the solid and liquid components of any particular system following a 90° radiofrequency (r.f.) pulse[14]. Molecular aggregation of starch should result in a decrease in the amount of hydrogen nuclei able to contribute to the liquid phase signal as relatively mobile polymer chains become increasingly less mobile. It has been demonstrated that starch retrogradation proceeds biphasically[4], the rapid early stage and the slow later stage being dominated by recrystallisation of amylose and amylopectin, respectively. Teo and Seow[14] have clearly shown that increases in magnitude of the NMR signal from the "solid-like" component of concentrated starch gels (such as those used in the present study) during storage mainly reflect the retrogradation of amylopectin. Measurements by the pulsed NMR method were found to give very highly significant correlation ($P<0.001$) with firmness (maximum force on compression) measurements using the Instron Universal Testing Instrument for corn starch gel, bread and rice cup-cake[15].

As previously described by Teo and Seow[14], retrogradation rate constants (*k*) of the various samples were calculated from Avrami plots which yielded straight lines. Figures 1–4 show the retrogradation behaviour of mixtures of rice starch with mungbean, potato, sago, and common corn starches, respectively. For any particular mixture, as expected, starch retrogradation proceeded faster as storage temperature was lowered from 25° to 5°C, confirming once again the already well-known negative temperature coefficient associated with the overall kinetics of retrogradation over this range of temperature[14].

At the same starch/water ratio and temperature, all starches used for blending were found to exhibit a higher retrogradation tendency than rice starch which has the lowest amylose content among the different types of starches employed in the present study (Table 1). It has previously been shown that the rate of retrogradation of starch gels on ageing generally increases with increasing amylose content of the starch[14]. The level of amylose in the potato starch sample studied appeared to be unusually high, but may be accounted for by biological variability.

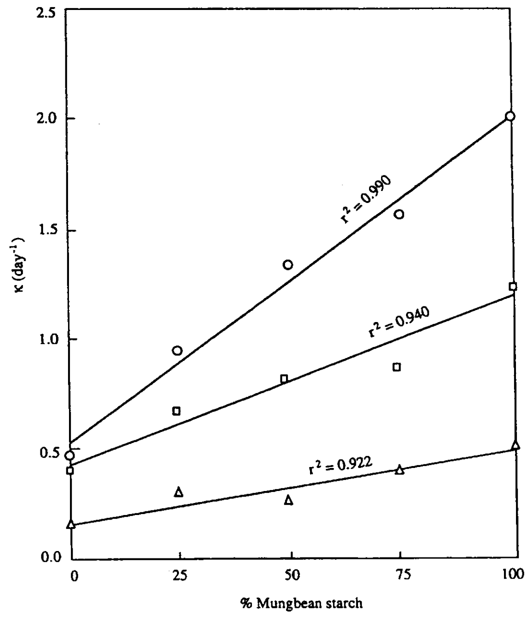

**Figure 1** *Rates of retrogradation of rice/mungbean starch gels at 5° (o), 15° (□) and 25°C (Δ)*

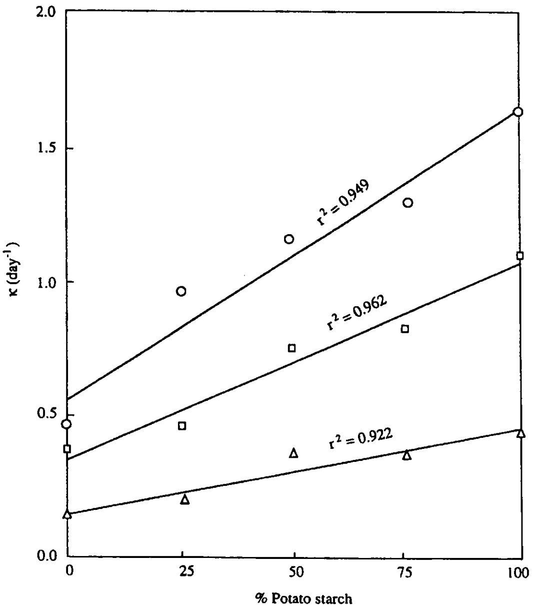

**Figure 2** *Rates of retrogradation of rice/potato starch gels at 5° (o), 15° (□) and 25°C (Δ)*

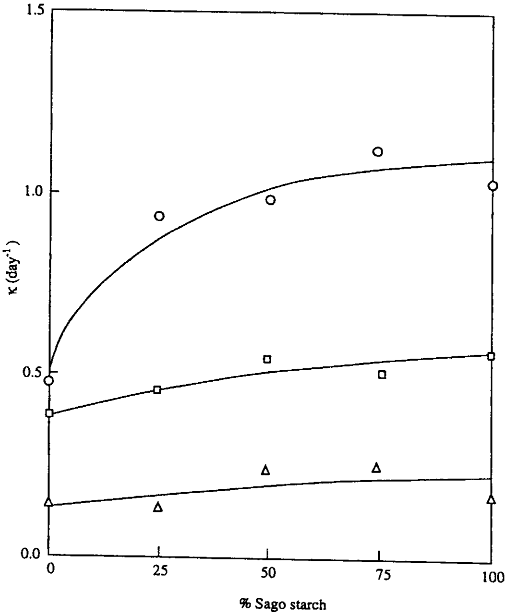

**Figure 3** *Rates of retrogradation of rice/sago starch gels 5° (o), 15° (□) and 25°C (Δ)*

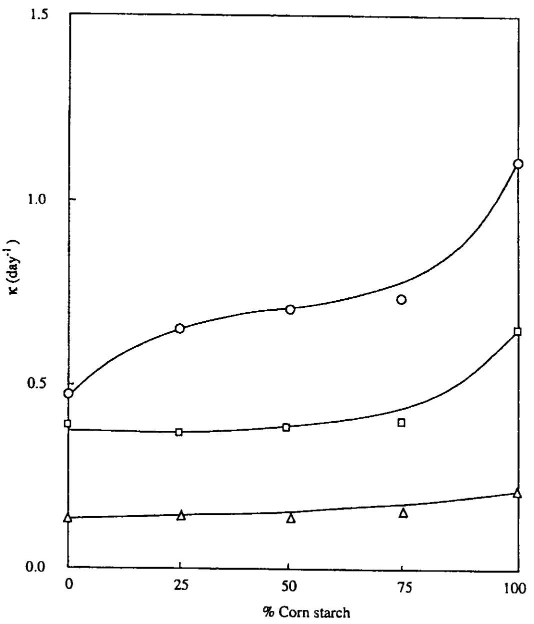

**Figure 4** *Rates of retrogradation of rice/corn starch gels at 5° (o), 15° (□) and 25°C (Δ)*

In any particular mixture, the rate of retrogradation fell between those of the individual component starches. In other words, rice starch, which has a low tendency to retrograde and the lowest among the starches used in the present study, retrograded faster when blended with the other starches. A similar observation was earlier made by del Rosario and Pontiveros[13] who studied the retrogradation behaviour of very dilute (1%) starch solutions.

**Table 1** *Amylose contents of various starches used in the present study*

| *Type of starch* | *Amylose content (%, dry basis)* |
|---|---|
| Rice | 22.5 |
| Corn | 28.5 |
| Sago | 28.5 |
| Potato | 36.0 |
| Mung bean | 36.8 |

However, the present results contradict those obtained by Obann*i* and BeMiller[3]. Using DSC, those workers observed that a normal rice and potato starch blend (50:50) exhibited lower retrogradation than the individual starches at the same concentration of 33% solids. The heating regimes used for starch gelatinisation in both studies were obviously different and this may be a factor that can account for the contradictory results. Under the gelatinisation conditions applied in the studies by Obanni and Bemiller[3], the starch granules were mostly disintegrated. However, in the present study, light microscopy (the results of which are not shown here) revealed that starch granule integrity was generally maintained although extensive swelling and deformation had apparently taken place. It has, in fact, been shown that thermal history has a profound effect on the retrogradation behaviour of maize starch[18] and presumably, therefore, also on starch blends. Thus, a pertinent factor to consider is the varying extents of residual molecular order in gelatinised starch samples subjected to different heating regimes. Disintegration of granules would probably result in a continuous phase comprising a mixture of co-solubilised amylose and amylopectin whereas maintenance of granular integrity would lead to an amylose-rich continuous network with the swollen starch granules forming the dispersed microphases[19].

In the absence of any significant interaction between the different starches, the rate of retrogradation would be linearly related to the level of substitution of rice starch for any other type of starch. This appears to be true in the case of mungbean and potato starches (Figures 1 and 2). On the other hand, as shown in Figure 3, replacement of rice starch with increasing levels of sago starch influenced retrogradation in different ways which appear to be dependent on both the storage temperature and the proportion of sago starch in the mixture. Little interaction was observed at 15° and 25°C, the effects of combining both starches on retrogradation rate being substantially additive. Synergistic effects were, however, very much in evidence when the samples were stored at 5°C, the rate of retrogradation of any mixture being much higher than predicted by a simple additive effect. It is clear that low temperature storage not only accelerates starch retrogradation, but also accentuates whatever interactions between the different starch polymers that lead to higher than expected retrogradation rates. Sago starch apparently dominated the retrogradation kinetics of the mixture at 5°C. A 25% replacement of rice starch with sago starch was sufficient to increase the rate of retrogradation to a level close to that of a 100% sago starch system.

The starch retrogradation profile was entirely different from that described above when common corn starch was present. It is apparent from Figure 4 that the rate of retrogradation of mixtures up to a level of 75% common corn starch was not drastically different from that of a 100% rice starch system. In other words, rice starch predominantly controlled the retrogradation behaviour of such starch mixtures systems. The observed rates of retrogradation for such systems were lower than those predicted in the absence of any interaction, thereby indicating an antagonism between rice and corn starch molecules.

From the foregoing results and discussion, the retrogradation behaviour of mixtures of non-waxy starches (one of which is rice starch) may be schematically simplified as shown in Figure 5. Rice starch is designated as starch X in this diagram. Three main types of interactions may occur when rice starch is combined with any one of the other starches. Type I represents the condition where the starch (amylopectin) molecules from the different sources behave independently in the mixture. The rate of retrogradation of the mixtures can, therefore, be predicted by a linear relationship.

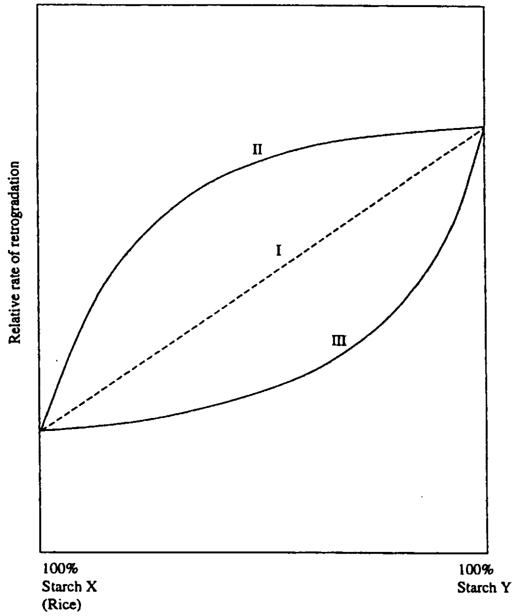

**Figure 5** *Schematic representations of the retrogradation tendency of mixtures of starches showing (I) additive, (II) synergistic, and (III) antagonistic effects*

Types II and III display non-linear but opposite retrogradation behaviour, the former showing synergistic interactions and the latter exhibiting antagonistic interactions between the starch molecules from different botanical origins.

The actual mechanisms responsible for starch-starch polymer interactions are as yet unknown and warrant further investigation. Present knowledge appears inadequate to formulate a scientific basis for predicting the effects on retrogradation resulting from the blending of different types of native starches. There are apparently many factors (such as amylose/amylopectin ratio, chain length, degree of branching of the starch molecules, degree of gelatinisation, and extent of starch granule swelling or disruption) that can influence the course of such interactions, but in ways yet to be fully understood. Nevertheless, appropriate blending of different native starches may provide a simple practical avenue for manipulating, at least to some extent, the retrogradation behaviour of a starch-based end-product.

## Acknowledgement

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# EFFECTS OF SUGARS ON GELATINISATION AND RETROGRADATION OF CORN STARCH

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## 1 INTRODUCTION

Starch is widely used as a thickening, stabilising, or gelling agent in food industries due to its inexpensive and abundant supplies. In a native state, starch is present in the form of granules, composed of an amylopectin skeleton in an interpenetrating amylose matrix.[1] Amylose is an essentially linear $\alpha$-1,4-linked D-glucan chain and amylopectin is a highly branched molecule containing short chains of $\alpha$-1,4-linked D-glucan chains with $\alpha$-1,6-linked branches. When dry native starch granules are suspended in water and then heated, starch granules absorb water and swell to some extent. A loss of X-ray crystallinity and birefringence are observed, suggesting melting of ordered structures in granules. Amylose partly separates from amylopectin and leaches out of granules. These phenomena are generally called gelatinisation.[2] Then, on cooling and during a subsequent storage, the initially ordered structures in starch dispersions are partially recovered, which is called retrogradation.[2] Retrogradation of starch proceeds in two stages. In the first stage, the gelation and crystallinity in the dispersion develop quickly mainly by amylose. The second stage is slow development of the crystallinity of amylopectin, which may continue for weeks. A starch dispersion can turn into a gel through thermal-treatments while the resultant gel continues to be firmer and releases water during following storage due to slowly progressing retrogradation. If retrogradation of starch takes place in food products, it leads to deterioration in food qualities (*e.g.*, bread staling or firming). Sugars are known to retard this process.[2-4] Sugars are not only inherently found in starch ingredients but also used in various food products sometimes at very high concentrations as in some confectionery systems. In this study, we investigated effects of sugars on gelatinisation and retrogradation of corn starch by uniaxial compressional and dynamic viscoelastic tests of thermally-processed starch gels and also by differential scanning calorimetry (DSC). We selected corn starch because of its predominant production in the nation over other starches from different origins. Effects of a relatively novel sugar, trehalose, as well as those of glucose and sucrose were examined. Trehalose,[5] $\alpha$-D-glucopyranosyl-$\alpha$-D-glucopyranoside, has been known for its unique physiological functions as a water substitute in many plants or as a blood sugar of insects so that its utilisation in foods, cosmetics, pharmaceutical, or medical industries are anticipated to be enhanced nowadays owing to its commercial availability.

# 2 MATERIALS AND METHODS

## 2.1 Materials

Corn starch and trehalose were supplied by Sanwa Cornstarch Co. Ltd. (Nara, Japan) and Hayashibara Co. (Okayama, Japan), respectively. Glucose and sucrose were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemicals were of reagent grade quality.

## 2.2 Gel Preparation

Weighed powders of corn starch with/without sugar were dispersed in distilled water in a separating flask by a motorised stirrer (HEIDON BL600, Shintoh Science Ltd., Tokyo, Japan) with a Teflon mixing blade at 200 rpm for 60 min at room temperature. Sodium azide was added to avoid microbial growth. The dispersions were then heated in an oil bath: the temperature reached 95°C in 20 min. Mixing was continued for another 30 min at 400 rpm at 95-100°C. Boiled distilled water was then added to adjust the concentration. The compositions of gels with sugar were starch:water:sugar = 2:7:1 whereas those without sugar were starch:water = 2:7 or 2:8. The hot mixture was poured into cylindrical Teflon moulds (20 mm diameter and 20 mm height) and held at 25°C for 60 min and then stored at 5°C up to 3 weeks.

## 2.3 Measurements

*2.3.1 Uniaxial Compressional Tests.* Gel properties under large strains were investigated by uniaxial compressional tests. Sample gels equilibrated at 25°C for one hour were placed on Rheoner RE-3305 (Yamaden Co. Ltd., Tokyo, Japan) with a 20-kgf load cell and compressed with a plunger (40 mm diameter) at a crosshead speed of 0.05 mm/s to break gels.

*2.3.2 Dynamic Viscoelasticity.* The storage *E*' and loss *E*" Young's modulus of gels were determined by applying small strain longitudinal vibrations. After equilibration at 25°C for an hour, gels were placed on the stage and glued to the parallel plate test fixture of the measuring apparatus (Rheolograph gel, Toyo Seiki Seisakusho Ltd., Tokyo, Japan) that subjects longitudinal vibrations at a frequency of 3 Hz and amplitude of 100 $\mu$m.[6] The temperature dependence of *E*' and *E*" were determined by raising the temperature in steps of 5°C up to 60°C. Measurements were done after a 20 minutes equilibration at each temperature.

*2.3.3 DSC.* Corn starch powder and distilled water with/without sugar were directly weighed into a silver pan to make the same concentrations as those of the gels for rheological measurements mentioned above. Total weight of the samples was adjusted to be 50 mg. After keeping at 5°C overnight, the pans were set in a DSC apparatus (DSC-8240A, Rigaku Co. Ltd., Tokyo, Japan) and then the temperature was raised from 25°C to 130°C at 1.0°C/min. After the first heating run, the pan was quenched to 5°C and stored for up to 3 weeks at 5°C. In the second heating run measurements, the temperature was raised from 5 to 130°C at 1.0°C/min.

# 3 RESULTS AND DISCUSSION

Figure 1 shows changes in breaking properties of corn starch gels with/without sugars determined by uniaxial compressional tests. All the gels showed a similar tendency: the stress at fracture (fracture stress) continued to increase during storage at 5°C for up to three weeks while the strain at fracture (fracture strain) decreased during the first stage of storage of up to one week and remained almost constant thereafter. Increasing fracture stress and decreasing fracture strain during storage have been observed for corn,[7] potato,[8] and wheat starch gels.[8] Adding sugars decreased the fracture stress in comparison with gels without sugars (starch:water = 2:7, open circles). However, such decreases in the fracture stress were less than those when water was added to the same extent (starch:water = 2:8, closed circles). Therefore, adding sugars appeared to prevent gel-firming during storage by retarding retrogradation although adding sugars was not so effective as adding water. Gels with glucose showed the smallest fracture strain except for the day 3, suggesting brittle gels were formed. Gels with sucrose appeared to be relatively strong, having relatively large fracture stress and fracture strain. Trehalose formed soft but tough gels with relatively small fracture stress and large fracture strain.

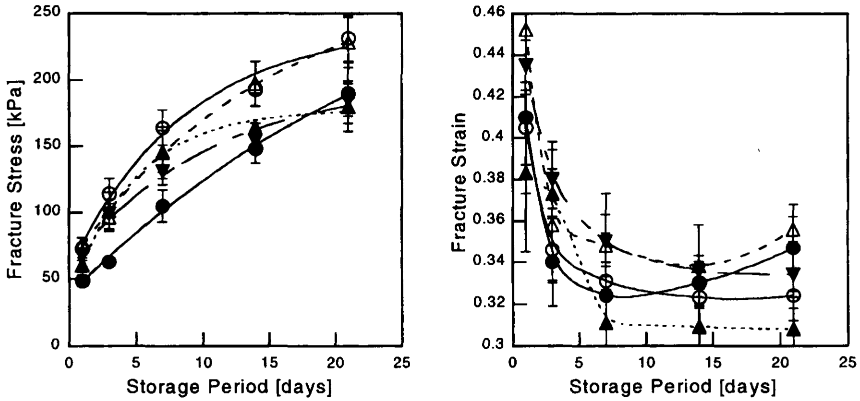


**Figure 1** *Changes in fracture stress and fracture strain of corn starch gels with / without added sugars during storage at 5°C. Starch : Water = 2:7* (○), *2:8* (●); *Starch : Water : Sugar = 2:7:1 (glucose* ▲*), (sucrose* Δ*), (trehalose* ▼*)*

The initial Young's modulus was determined based on the initial slope of the force-deformation curve obtained by uniaxial compressional tests. Figure 2 shows the initial Young's modulus of corn starch gels with/without sugars plotted against the storage period. Clear trends can be seen throughout the entire storage period, that is, the initial Young's modulus decreased by adding sugars or water in the order of water > trehalose > sucrose > glucose, suggesting that the retrogradation of starch during storage was retarded by adding sugars or water in the same order. It is known that the retrogradation of starch proceeds faster with increasing starch concentration[2] and that sugars interact with starch chains in the amorphous regions of starch granules to stabilise those regions.[3] Therefore, sugars seem to retard retrogradation by diluting the effective starch concentration and/or by stabilising gelatinised structures in starch gels. Trehalose, which is known for its water substituting function in biological systems,[5] showed similar effects to water on preventing retrogradation in terms of developments of the initial Young's modulus during storage.

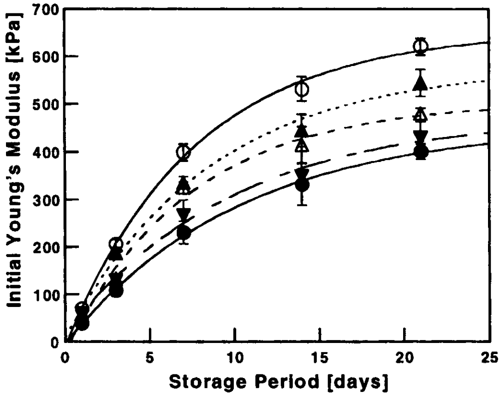


**Figure 2** *Effects of sugars on developments of initial Young's modulus of corn starch gels during storage at 5°C. Starch : Water = 2:7 (○), 2:8 (●); Starch : Water : Sugar = 2:7:1 (glucose ▲), (sucrose △), (trehalose ▼)*

A starch gel can be considered as a system of tightly packed swollen granules glued together with a thin amylose gel layer.[8] For such a system, a linear relationship between the fracture stress and the modulus should be observed since the Young's modulus is mainly determined by the stiffness of the swollen granules while the fracture stress would depend mainly on the stiffness of the granules and on the fracture stress of the thin amylose layer between the granules to a lesser extent.[8] In this study, linear relationships between the fracture stress and the initial Young's modulus were observed for both gels with and without added sugars as shown in Figure 3, suggesting that an increase in fracture stress during storage is ascribed to an increase in the stiffness of swollen granules. Smaller increases in the fracture stress than the initial Young's modulus would be because of the contribution of the fracture stress of amylose matrix as pointed out by Keetels et al.[8]

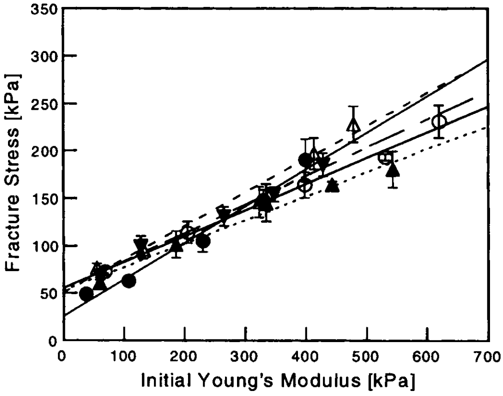


**Figure 3** *Linear relationships between fracture stress and initial Young's modulus of corn starch gels with / without sugars stored at 5°C for 1–21 days. Starch : Water = 2:7 (○), 2:8 (●); Starch : Water : Sugar = 2:7:1 (glucose ▲), (sucrose △), (trehalose ▼)*

Effects of such amylose layer contribution would be constant since reordering of amylose during storage is usually complete within one day or so.[2] Therefore, increasing fracture stress during storage after one day appears to be due to increasing stiffness of the swollen granules consisting of amylopectin and unleached-out amylose. On the other hand, the fracture stress of the thin amylose layer around the swollen granules would determine the fracture stress at zero modulus (intercept).[8] As shown in Figure 3, adding water decreased the intercept while adding sugars did not change it. While adding water plasticises a starch dispersion, sugars are known to reduce the amount of amylose leaching out of starch granules during gelatinisation.[9] This may be one of the reasons why adding sugar did not affect the fracture stress at zero modulus.

Figure 4 shows the storage $E'$ and loss $E''$ Young's modulus and loss tangent $\tan\delta$ ($= E''/E'$) of corn starch gels with/without sugars plotted against the storage period. For all gels, $E'$ increased and $\tan\delta$ decreased during storage, suggesting that retrogradation led to firmer and more solid-like gels. Glucose and sucrose added gels showed even higher values of $E'$ than gels without sugars although the initial Young's moduli (Figure 2) showed opposite trends. However, the storage modulus of gels with sugars decreased in the order of trehalose > sucrose > glucose, consistent with the results of uniaxial compressional tests (Figure 2). Trehalose appeared to contribute to a formation of relatively soft gels with low $E'$ and high $\tan\delta$.

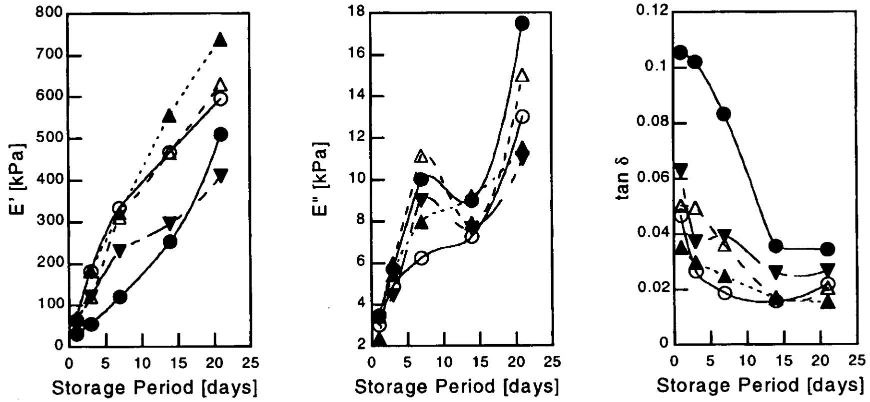


**Figure 4** *Effects of sugars on developments of storage modulus and loss tangent of corn starch gels during storage at 5°C. Starch : Water = 2:7 (○), 2:8 (●); Starch : Water : Sugar = 2:7:1 (glucose ▲), (sucrose △), (trehalose ▼)*

The temperature dependence of the absolute values of the complex Young's modulus $E^*$ ($= |E' + iE''|$) is shown in Figure 5. For all gels with/without sugars, $E^*$ decreased with increasing temperature, suggesting that starch gel networks were formed mainly by secondary physical bonds such as hydrogen bonds.[10] $E^*$ became small at 60°C for all examined gels. In other words, decreases in the moduli when the temperature is raised from 25 to 60°C were enhanced by storage.

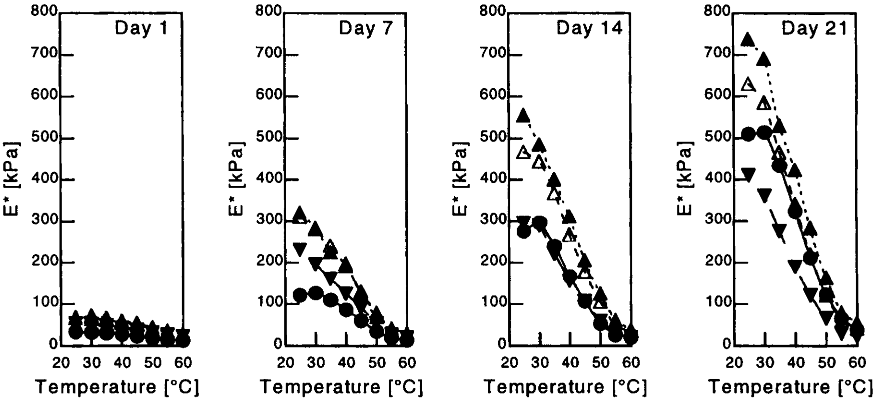


**Figure 5** *Effects of storage on temperature dependence of complex Young's modulus of corn starch gels. Starch : Water = 2:7 (○), 2:8 (●); Starch : Water : Sugar = 2:7:1 (glucose ▲), (sucrose △), (trehalose ▼)*

The first-run heating DSC curves of starch dispersions are shown in Figure 6. All samples showed the first and the second gelatinisation endothermic peaks at around 65-70°C and around 98-100°C, respectively. Yoshimura et al.[7] have shown two endothermic peaks in DSC heating curves for corn starch: the peaks at around 70°C are due to gelatinisation, *i.e.*, changes in ordered structures of amylopectin which lead to swelling of starch granules, while the peaks at around 100°C correspond to disintegration of amylose-lipid complexes. The gelatinisation peak temperature $T_p$ and the endothermic enthalpy $\Delta H$ are summarised in Table 1. Adding sugar shifted $T_p$ to a higher temperature and slightly increased $\Delta H$ while adding water lowered $T_p$ and decreased $\Delta H$.

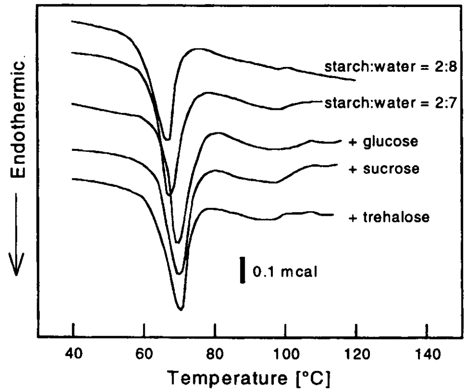


Figure 6 *First-run heating DSC curves for corn starch gels with/without added sugars.*

**Table 1** *Gelatinisation Temperature and Enthalpy*[a]

| *Sample (starch/water/sugar)* | $T_o$ *[°C]* | $T_p$ *[°C]* | $T_c$ *[°C]* | $\Delta H$ *[mJ/mg-starch]* |
|---|---|---|---|---|
| 2/7/0 | 59.3 | 66.3 | 76.4 | 11.6 |
| 2/8/0 | 59.5 | 65.9 | 75.5 | 10.4 |
| 2/7/1 (glucose) | 62.0 | 68.5 | 76.8 | 11.6 |
| 2/7/1 (sucrose) | 62.8 | 69.8 | 78.5 | 12.0 |
| 2/7/1 (trehalose) | 62.1 | 68.5 | 76.8 | 12.1 |

[a] *$T_o$, onset temperature; $T_p$, peak temperature; $T_c$, conclusion temperature; $\Delta H$, gelatinisation enthalpy.*

These results are consistent with those in the literature[4,9,11,12] in which disaccharides usually increase the gelatinisation temperature more than monosaccharides. Adding water is considered to plasticise starch dispersions to reduce the thermal stability of the system while sugars appear to have anti-platicising effects compared to water.[9] The presence of sugars in starch gels have been found to reduce water activity[3] and decrease considerably the translational mobility of water.[13] Therefore, effects of sugars may be attributed to reduction of available water for gelatinisation. However, the magnitudes of changes in the values of $T_p$ and $\Delta H$ have been found to be much larger than those predicted by considering the reduction of the amount of water due to hydration by sugar.[4] Additionally, different solutes changed gelatinisation temperatures of starch dispersions to different extents even at the same water activity.[3] Binding between starch chains and sugars seems to contribute to increasing heat stabilities of starch dispersions as well. There exists evidence showing direct interactions between sugar and starch during heating based on changes in the chemical shifts of sugars determined by $^{13}$C NMR.[14]

The second-run heating DSC curves are shown in Figure 7. While the second gelatinisation peaks remained similar at positions and in shapes, the first gelatinisation peaks shifted ca. 25°C toward lower temperature than those in the first-run heating for all the samples. During storage at 5°C, $T_p$ decreased and $\Delta H$ increased for all samples (Figures 8 and 9).

These results suggest that retrogradation proceeded during storage since decreasing $T_p$ is considered due to decreasing heat stabilities and increasing $\Delta H$ is considered due to increasing the amount of the retrograded fraction of starch. Adding sugars appears to raise $T_0$ but lower $\Delta H$ suggesting that sugars suppressed retrogradation.

The degree of retrogradation was evaluated as the ratio of the re-gelatinisation enthalpy in the second run DSC heating curve to the gelatinisation enthalpy in the first run DSC heating curve[4] and was shown in Figure 10. Dilution by water retarded retrogradation, consistent with results in the literature.[2] The sugars slightly promoted retrogradation that occurred within 5 days storage at 5°C while inhibited retrogradation during a longer storage period of up to three weeks (Figure 10). Additionally, results of rheological tests (e.g., Figure 2) suggested that diluting starch dispersions with water was more effective for preventing gel firming during storage than adding sugars. Therefore, sugars seem to prevent retrogradation not only by lowering the starch fraction in the system but also by interacting directly with starch granules. The higher stiffness of the gels with sugars than

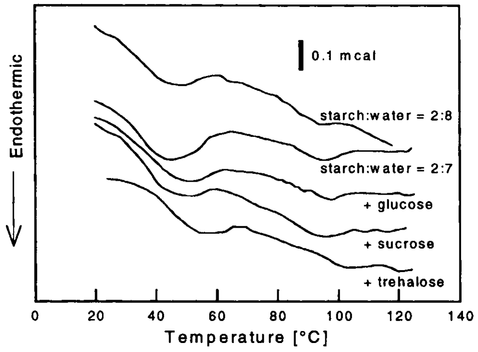
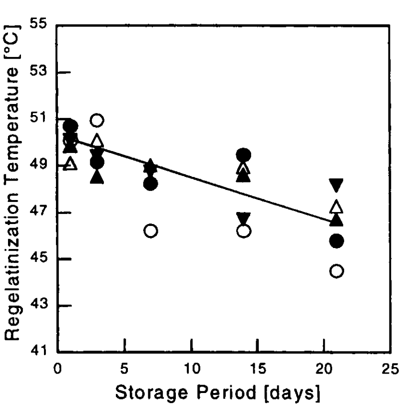


**Figure 7** *Second-run heating DSC curves for corn starch gels with/without added sugars stored at 5°C for 21 days.*



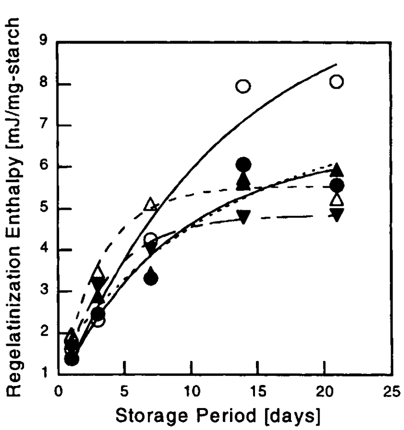


**Figure 8** *Changes in re-gelatinisation peak temperature $T_p$ in the second-run heating of corn starch gels with/without added sugars during storage at 5°C.*

**Figure 9** *Changes in re-gelatinisation enthalpy $\Delta H$ in the second-run heating of corn starch gels with/without added sugars during storage at 5°C*

*Starch : Water = 2:7 (○), 2:8 (●); Starch : Water : Sugar = 2:7:1 (glucose ▲), (sucrose △), (trehalose ▼)*

that without sugars (even at a lower degree of retrogradation) would be because of an apparent reduction of the amount of water available for starch chains due to hydration of sugars. Among sugars the inhibition of retrogradation is more effective in the order trehalose > sucrose > glucose, which were in line with the results of rheological experiments (e.g., Figure 2). Stronger inhibiting effects of sucrose on retrogradation than those of glucose are also found in many literatures based on various experimental results using creep tests,[15] DSC,[4] or X-ray diffraction.[1] The origin of an even higher effectiveness of trehalose on suppressing retrogradation is not yet clear. Uedaira et al. have pointed out that a saccharide molecule with a larger mean number of equatorial OH groups had a stronger stabilising effect on the water structure because an equatorial OH group fits well into the tridymite structure of water.[16,17] They have also proposed the concept of dynamic hydration number as an essential quantity characterising hydration abilities of solutes. The dynamic hydration number is determined based on the spin-lattice relaxation times of $H_2{}^{17}O$ in a solution and a good linear relationship with the number of equatorial OH has been found. Additionally, good correlations between the dynamic hydration numbers and stabilising effects of saccharides against retrogradation of starch have been revealed.[12,15] Both the order of the dynamic hydration number (trehalose 25.4, sucrose 25.2, and glucose, 18.6)[16,17] and the number of equatorial OH per molecule (trehalose 7.2, sucrose 6.3, and glucose 4.6)[16,17] are also in good correlations with anti-retrogradation effects of sugars (trehalose > sucrose > glucose) shown in this study.

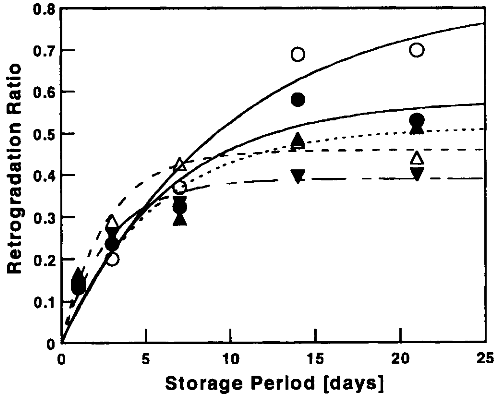


**Figure 10** *Effects of sugars on progressing retrogradation of corn starch gels during storage at 5°C. Starch : Water = 2:7 (○), 2:8 (●); Starch : Water : Sugar = 2:7:1 (glucose ▲), (sucrose △), (trehalose ▼)*

## 4 CONCLUSIONS

Adding sugars to a starch dispersion could cause specific sugar-starch interactions, competition between starch and sugar for available water, and influences on water structure. Therefore, sugars would affect gelatinisation and retrogradation of starch at various stages in different ways. However, we observed some trends in effects of sugars on corn starch gels formed through heat-treatments: (i) sugars promoted solid-like characters of gels and increased firmness under small strain, (ii) anti-plasticising effects of sugars compared to water were observed in terms of increasing gelatinisation temperature, and (iii) sugars slightly promoted retrogradation during the first 5 days of storage at 5°C while inhibited retrogradation during a longer storage period up to three weeks. Comparisons of fracture properties of gels stored at 5°C for 21 days suggested that adding glucose formed more brittle gels while softer yet tougher gels were formed by adding trehalose. Trehalose was the most effective in the examined sugars for suppressing retrogradation of corn starch, suggesting efficiencies of trehalose for maintaining initial qualities of starch products for a relatively long period.

## Acknowledgements

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# IMPLICATIONS OF GENETIC CHANGES IN STARCH GRANULAR STRUCTURE TO GELATINISATION BEHAVIOUR

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## 1 INTRODUCTION

Starch granules are composed of alternate ordered and disordered, or amorphous, regions. The ordered areas consist of amylopectin chains formed as double helices, some of which are organised into small crystals, or crystallites.[1] The double helices may be densely packed, as in cereal starches, and termed A-type crystals, or less densely packed, as in tuber starches such as potato, and termed B-type crystals. In accord with these crystal types, cereal and potato starches, are termed A- and B-type starches, respectively. Legume starches, such as those from pea, contain both A- and B-type crystals and are termed C-type. A series of mutants have been developed in pea, each of which differ in the granular structure of the starch within their seeds.[2]

When starch granules are heated in water, the ordered structures disrupt at a specific temperature, and this disruption is accompanied by irreversible swelling, the whole process being termed gelatinisation. We have determined the effects of structural changes in starch granules occurring in different pea mutants and related these to gelatinisation behaviour.

## 2 GRANULAR STRUCTURE IN PEA MUTANTS

The proportions of ordered to disordered structures and of A- to B-type crystallites, were measured. The proportion of amorphous material in the starches was determined using $^{13}$C CP/MAS NMR. The relative area of the characteristic peak at 84.2 ppm was used as a measure of the disordered material content, as described previously.[3] Based on this measurement, starches from the mutants can be ranked as follows:-

$$r > rug5 > rug3 > \mathrm{WT} > rug4 > rb \geq lam.$$

The ≥ symbol signifying that the different values attributed to *lam* and *rb* starch were similar to the level of error associated with the measurements.

The polymorph composition (crystalline type) in the starches was determined using an x-ray powder diffractometer with a fixed divergence slit. This type of slit allows a corresponding increase in the irradiated area of the sample as the angle of the goniometer is decreased. This results in the peaks at small angles appearing to be relatively large, which is important for identifying small peaks at small angles. On the other hand, the proportion of A- and B-type crystallites was determined using an x-ray powder diffractometer with an automatic divergence slit. With this type of slit the irradiated area of the sample remains constant irrespective of goniometer angle, which is required for this type of measurement. The proportion of A- and B-crystal types was calculated for starch from each of the mutant lines using the peak areas that are characteristic for each crystal type,[4] and the results are illustrated in Table1.

**Table 1** *Structural characteristics of starches from a range of pea seed mutants*

| *Genotype* | *% A-polymorphs** | *% B-polymorphs** | *Peak temperature ( °C*)* |
|---|---|---|---|
| *rug3* | 63 | 37 | 70.0 |
| *rug4* | 59 | 41 | 65.4 |
| *rb* | 58 | 42 | 66.1 |
| Wild type | 57 | 43 | 61.8 |
| *rug5* | 46 | 54 | 49.0-57.0 |
| *lam* | 30 | 70 | 58.6 |
| *r* | 0 | 100 | 52.5-60.0 |

* - error = +/- 2%
** - error = +/- 0.1°C

Starches from mutants containing mutations affecting the supply of substrate during starch synthesis (*rb*, *rug3*, *rug4*) had an increased A-type crystal content, although this increase was very small and difficult to measure for starches from the *rug4* and *rb* mutants. Conversely, starches from mutants containing mutations that directly affect the synthesis of the starch polymers (*r*, *rug5*, *lam*) had an increased B-type crystal content, the largest increase being for starch from the *r* mutant, which had only B-type crystals and by definition, therefore, was B-type starch.

## 3 GELATINISATION

It has been shown, that when relatively low starch concentrations and low heating rates are used, the gelatinisation process can be considered to consist of a large number of equilibrium states, *i.e.* it may be considered to be a quasi-equilibrium process.[2] Under these conditions the thermodynamic parameters are not influenced by the experimental conditions and these parameters can be used, therefore, to characterise the starch. All of the DSC experiments on starches from the pea mutants were carried out in conditions that satisfy the requirements of a quasi-equilibrium process.[5]

We discovered that starches from the range of mutants fell into two groups with regard to their gelatinisation behaviour. The first group consisted of starches from the wild-type and from those mutants where either the substrate supply (*rb*, *rug3* and *rug4*), or amylose synthesis (*lam*), was affected. The second group contains mutants that directly affect the synthesis of amylopectin (*r* and *rug5*).

Starches from the first group of mutants showed a sharp peak of heat capacity change during gelatinisation, accompanying the disruption of crystallinity (Figure1). The change in heat capacity for this group of starches is typical for a first order phase transition and can be characterised by the peak temperature ($T^P$). $T^P$ is regarded as a characteristic of granule thermal stability. We have found differences for $T^P$ between the starches produced by the first group of mutants (see Table 2).

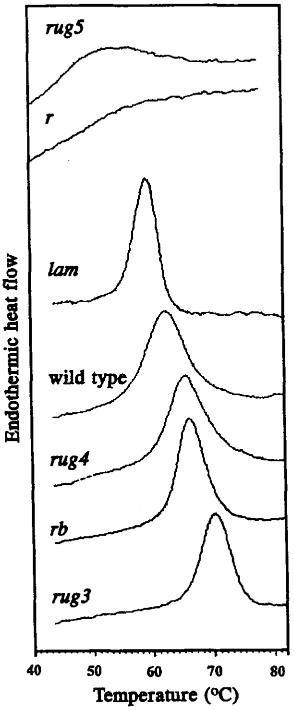

**Figure 1** *Differential scanning calorimetry of starches from wild type and mutant peas. The rate of heating was 1°C/min and the concentration of starch in suspension was 2-4%*

**Table 2** *Relationships between starches from a range of pea mutants for starch granule characteristics*

| *Measured parameter* | *Relationship* |
|---|---|
| % Disordered material | *rug3* > *WT* > *rug4* > *rb* ≥ *lam** |
| % A-polymorphs | *rug3* > *rug4* ≥ *rb* ≥ WT > *lam** |
| Peak temperature of gelatinisation | *rug3* > *rb* > *rug4* >WT > *lam** |

* Use of the ≥ symbol indicates that the difference between the values is smaller than the error of the measurements.

It can be assumed that the thermal stability of starches is dependent on their crystalline structure. The relationships between the starches from this range of mutants, with regard to the proportions of double helices, A-polymorphs and $T^P$ are illustrated in Table 2. The similarity in ranking order demonstrated that there was a positive correlation between the value for $T^P$ and the proportion of A-polymorphs. The difference in the proportion of A-polymorphs between starches from the wild-type, *rb* and *rug3*, however, was very small and probably could not influence the $T^P$ values. In the case of starches from these three mutants, a negative correlation was found between the proportion of amorphous material and $T^p$.

It follows from Flory's equilibrium theory of melting point depression,[6] that the more dense A-polymorphs should disrupt at a higher $T^p$ than the less dense B-polymorphs when other parameters remain the same. This hypothesis has been supported experimentally by observations that the $T^p$ for B-type starches and spherulites is lower than for A-type.[3,7,8] From a general point of view, it also can be stated that an increase in the content of amorphous material may result in a decrease in $T^p$. It can be suggested, therefore, that the proportion of ordered to disordered structures and the proportion of A- to B-type crystals can account for the particular $T^p$ values for the starches from this group of mutants.

Using polarised light microscopy, we have observed that the disruption of crystallinity during gelatinisation for the starches from this group of pea mutants begins from the central area of the granule and is propagated along the granule, accompanied by granular swelling. Similar observations have been made using starches from potato and maize.[3] The suggestion has been made that swelling of the disrupted parts of the granule decreases the melting temperature of neighbouring crystals, resulting in a rapid progressive disruption of the crystalline areas.[3] It is apparent, therefore, that the swelling of granules plays the role of a driving force for the melting of crystallites during gelatinisation.

We have shown that the disruption of crystallinity for starches from the second group of mutants (*r* and *rug5*) occurs over a wide range of temperatures. In these starches the crystallinity was found to be slowly reduced across the whole granule and that this process was accompanied by very little swelling. The DSC gelatinisation patterns for these two starches were very wide (Figure 1) and it was not possible to determine a $T^p$, it was only possible to determine the peak temperature range (Table 1). This type of heat capacity change is more typical for starch melting,[9] when there is no available water for swelling during the disruption process. The difference in swelling power for the starches from the *r* and *rug5* mutants, compared with the wild-type, are shown in Figure 2.

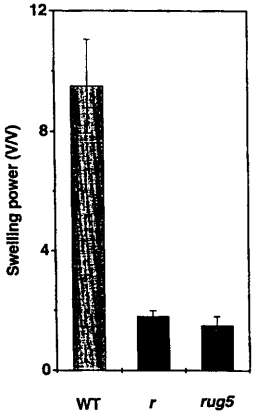

**Figure 2** *Swelling power (volume/volume) of starches from WT and r and rug5 mutant peas. Determined by comparing the volume of individual granules in water at 25°C and after heating in water to 80° C, using a microscope hot stage. Granule areas were calculated using an image analyser. Granule volume was approximated from the volume of a sphere with a diameter equal to the diameter of a circle with the same area as the starch granule image*

The very low swelling power for starches from this second group of mutants indicates that swelling does not play a very important role in their gelatinisation. In this case, we can suggest that the crystallites in different parts of the granules disrupt independently during gelatinisation. It is likely that differences in the thermal stability of crystallites within the granules of these starches result in the disruption process occurring over a very wide range of temperatures.

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# STARCH ETHERS OBTAINED BY MICROWAVE RADIATION - STRUCTURE AND FUNCTIONALITY

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## 1 INTRODUCTION

Starch ethers i.e. cationic starches, carboxymethylated starches, hydroxyethylated and hydroxypropylated starches are a wide range of industrial products of different degree of substitution, excellent physicochemical properties, useful in many branches of industry, especially in papermaking, building, and even in food manufacture. A scale of modified starch applications depends on their competitiveness in relation to synthetic products, with regard not only to the physicochemical properties but also to the prices. Due to the fact that microwaves are known to be capable of generating heat deep inside the penetrated medium, the suggestion of applying microwave ovens to starch processing seems very promising. However successful design of microwave processing must include not only thermal properties of the product to be processed, which are relatively insensitive to temperature differences, but also a number of interrelated electric properties which vary extensively with the processing frequency and time-temperature profiles of the product. Dry unmodified starches, are thought to be electrically inert[1] due to the statement that at microwave frequencies, the basic electric characteristics - the dielectric constant and loss factor - are largely determined by salt and moisture contents of the product[2]. In our previous work it was found that time - temperature profiles of microwaved starch layers depend on their moisture content[3], and the samples of low moisture content showed a rapid rise in temperature, while in those of higher moisture the rise was less pronounced. It was also found that microwave radiation of moistened starch samples, of moisture content over 20% causes structural changes similar to those induced by heat-moisture treatment[3,4]. The problem of the influence of microwave radiation on the reactivity of different substances still remains unsolved. It is believed that microwave radiation (2450 MHz) does not activate specific bonds in molecules and consequently this forms of treatment will not lead to any kinetic differences compared to other form of heating[5]. In spite of this, there has been a growing interest in the use of microwave heating synthesis, so called 'MORE chemistry' (Microwave Oven induced Reaction Enhancement), and in some processes catalytic effect is claimed[5-11]. Consequently, solid state reactions are considered as the most promising area of applying microwave ovens instead of traditional heating methods[11]. This type of processing is conventionally applied in starch industry to produce derivatives especially of ester type, but the process is expensive due to the high energy consumption .

Our attempts of application of microwave ovens in order to obtain inorganic starch esters proved that microwaves shorten reaction times, but do not affect molecular and submolecular structure of the derivatives as compared to conventional technology[12]. Promising results of previous investigation on esterification encouraged us to undertake attempts to apply microwave radiation to starch etherification. The aim of this work was to examine the influence of microwave processing on structure and functionality of cationic starches.

## 2 MATERIALS AND METHODS

### 2.1 Cationisation of starch

*2.1.1 Microwave assisted reactions.* Microwave assisted cationisation process was carried out according to the Polish patent description[13]. To this aim commercial potato, wheat, corn, waxy corn, and pea starches were sprayed with the solution of N,N,N-trimethyl-2-epoxypropylammonium chloride to incorporate 0.34% of nitrogen (basis of starch dry mass), alkalised with 1% calcium hydroxide and 1% sodium metasilicate (basis of starch dry mass) and carefully mixed to unification. The moisture contents of the prepared blends were adjusted to 35%. Then, the starch samples were dried in a Panasonic microwave oven emitting radiation of 2450 MHz frequency and 0.5 W/g energy, to reduce moisture content level below 20%. In order to obtain products of high degree of substitution a several stage process was applied[13,14].

*2.1.2 Suspension reactions.* Commercial potato, wheat, corn, waxy corn and pea starches were slurried in the water solution of 3-chloro-2-hydroxy-N,N,N-trimethylpropylammonium chloride (0.34% of nitrogen basis of starch dry mass). The suspensions were alkalised with the solution of sodium hydroxide (2 moles per 1 mole of cationisation agent) and the reaction were carried out at the temperature of 35°C during 6 hours. Then the reaction mixtures were neutralised with diluted hydrochloric acid, filtered, washed twice with water and air dried.

### 2.2 Analytical methods

*2.2.1 Rheological properties.* The course of gelatinisation was monitored with a Brabender viscograph under the following conditions: measuring cartridge 0.07 Nm; heating/cooling rate 1.5°C/min; thermostating 30 min.

*2.2.2 Nitrogen content.* Cationic starches obtained in the microwave process were first purified with hydrochloric acid. To this aim, starch samples were suspended in 5% solution of hydrochloric acid, filtered, and washed with water to remove chloride ions and air dried. The nitrogen contents was determined according to EN ISO 3188 standard.

*2.2.3 GPC investigations.* Starch samples were dispersed in a DMSO / water mixture (9:1) at room temperature, then heated while shaking at 90°C for 30 min. The solution was subsequently filtered through a 5 mm Durapor filter and injected into an aqueous system consisting of four Shodex Sugar (Showa Denko K.K.) columns. The Shodex columns (KS-806, 804, 803 and 802) were linked in a series and maintained at 50°C. The model 410 Waters refractive index detector with the cell temperature maintained at 50°C was used to detect carbohydrate peaks. Data were collected and analysed using Millennium Chromatography Manager PDA software. Molecular mass (up to 788000) was calibrated with pullulan Shodex STANDARD P-82 (Showa Denko K.K.).

*2.2.4 Microscopic examinations.* The starch samples to be examined by light microscopy were prepared by the smear method. To this end starch suspensions were heated at the initial gelatinisation temperature (as measured according to Brabender), and at 95°C. A drop of the resulting paste was applied to a microscope slide and, on cooling, the smear was stained with iodine according to Kaczyñska et al.[15], and observed with an Olympus BX60 light microscope. The starch samples to be examined by scanning electron microscopy were prepared according to Fornal[16] and observed with a Jeol JSM 5200 microscope.

*2.2.5 X-ray diffractometry.* X-ray diffractometry was carried out with a TUR 62 Carl Zeiss X-ray diffractometer under the following conditions: X-ray tube CuKα (Ni filter); voltage 30 kV; current 15 mA; scanning from $\Theta=2°$ to 18°.

*2.2.6 Flocculation of cellulose suspensions.* The effectiveness of cationic starches as flocculating agents for cellulose suspensions was evaluated by adding different amounts of cationic starch solution to the standard suspension of microcrystalline cellulose, and determination of a volume of settleable solids according to Standard Method for the Examination of Water and Wastewater No. 2540 F (prepared and published jointly by American Public Health Association, American Water Works Association, Water Environment Federation). Standard cellulose suspension was prepared according to modified European Standard EN 872, where 3.0 g microcrystalline cellulose for thin-layer chromatography was added to 1000 ml distilled water and mixed until it was completely uniform.

## 3 RESULTS AND DISCUSSION

One of the most important parameters influencing usability of cationic starches in the paper industry is degree of substitution. Due to their excellent functional properties - cationic starches of DS = 0.02-0.04 make a substantial part of modified starches for the paper industry market. Their prices mostly depend on cationisation agent consumption, so it is important to apply technological processes of high efficiency. As it is shown in Table 1 microwave processing is a convenient way to obtain cationic starches of DS ≈ 0.04. The suspension reaction which is believed to provide the cationic starches of the best quality, needs a very long time to reach a satisfactory degree of substitution (in the case of normal corn, wheat, and pea starches only about 50% of cationisation agent reacted after six hours). In most cases of microwave assisted reactions at least 97% of cationisation agent was consumed. The only exception was pea starch which reacted only with 85% of cationisation agent. In the case of the high substituted cationic starches (DS > 0.1) it is necessary to apply a several stage process[13,14], which makes the microwave assisted reaction less advantageous.

Cationic starches of DS = 0.02 - 0.04 (Table 1) revealed quite good effectiveness as flocculating agents for cellulose suspensions. Starch macromolecules containing cationic charges of quaternary ammonium groups attracted slightly anionic cellulose particles. The flocculation activity was related to the presence of cationic charges since the attempts at application of cationic starches as flocculation agents for bentonite suspensions completely failed. In this case, addition of the cationic starches did not cause flocculation of bentonite particles and even prevented sedimentation of the suspension. However the correlation between the degree of substitution and flocculation activity of investigated starches was not simple. Thus, increasing degree of starch substitution resulted in decreasing flocculation activity.

**Table 1** *Effectiveness of cationic starches as flocculating agents for cellulose suspensions in relation to degree of substitution and reaction conditions*

| *Starch origin* | *Reaction type* | *Nitrogen content* [%] | *Degree of substitution* | *Solubility in cold water* | *Volume of settleable solids after addition of different amount of cationic starch* [ml] | | |
|---|---|---|---|---|---|---|---|
| | | | | | *2 ppm* | *6 ppm* | *10 ppm* |
| Potato | Microwave assisted | 0.33 | 0.04 | – | 20 | 26 | 28 |
| Potato | Suspension | 0.30 | 0.03 | – | 21 | 22 | 27 |
| Potato | Three stage microwave assisted | 1.11 | 0.13 | + | 20 | 22 | 20 |
| Potato | Five stage acc. to EP 0874000 | 2.30 | 0.27 | + | 14 | 15 | 17 |
| Wheat | Microwave assisted | 0.34 | 0.04 | – | 17 | 19 | 20 |
| Wheat | Suspension | 0.17 | 0.02 | – | 25 | 25 | 27 |
| Corn | Microwave assisted | 0.24 | 0.03 | – | 20 | 24 | 25 |
| Corn | Suspension | 0.19 | 0.02 | – | 21 | 23 | 25 |
| Waxy corn | Microwave assisted | 0.33 | 0.04 | – | 23 | 28 | 30 |
| Waxy corn | Suspension | 0.26 | 0.03 | – | 25 | 28 | 31 |
| Pea | Microwave assisted | 0.29 | 0.03 | – | 18 | 20 | 28 |
| Pea | Suspension | 0.19 | 0.02 | – | 16 | 20 | 22 |
| Blank determination | | | | | 14 | | |

The incorporation of cationic substituents into the starch molecules significantly affected physicochemical properties related to the starch-water interactions. The substitution of cationic groups (DS = 0.02 - 0.04) into the starch molecules resulted in a decrease of pasting temperature accompanied by a rapid increase in viscosity within a narrow temperature range and the occurrence of a sharp viscosity peak (Figures 1-5). The extent of these changes in microwave assisted ethers was more significant than that in products of the suspension reaction, which could be the result of a higher degree of substitution[17]. The increase of the amount of substituted quaternary ammonium groups up to DS = 0.13 resulted in solubilisation of modified starch in cold water (Table 1).

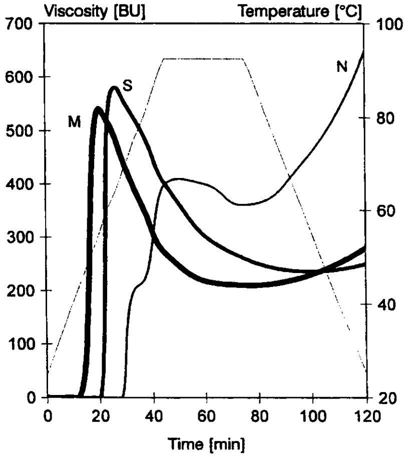

**Figure 1** *Brabender viscosity curves (c=3,3%) of potato starch cationic ethers as compared to native starch: N - native potato starch; S - suspension reaction product; M - microwave assisted reaction product*

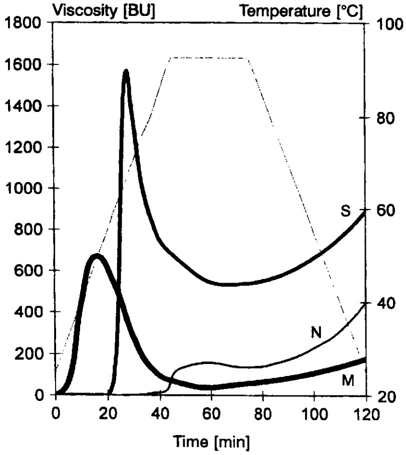

**Figure 2** *Brabender viscosity curves (c=8%) of wheat starch cationic ethers as compared to native starch: N - native wheat starch; S - suspension reaction product; M - microwave assisted reaction product*

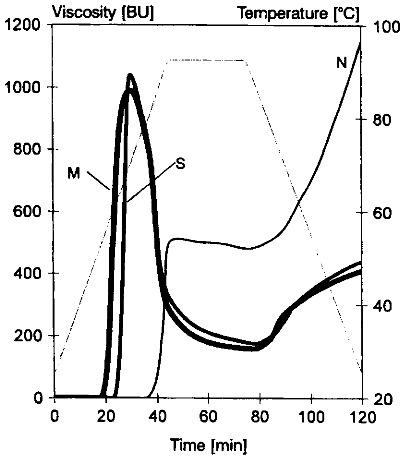

**Figure 3** *Brabender viscosity curves (c=8%) of corn starch cationic ethers as compared to native starch: N - native corn starch; S - suspension reaction product; M - microwave assisted reaction product*

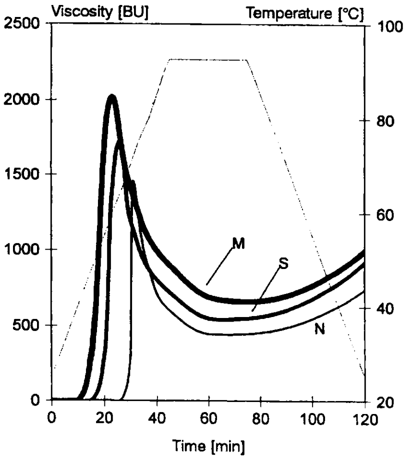

**Figure 4** *Brabender viscosity curves (c=8%) of waxy corn starch cationic ethers as compared to native starch: N - native waxy corn starch; S - suspension reaction product; M - microwave assisted reaction product*

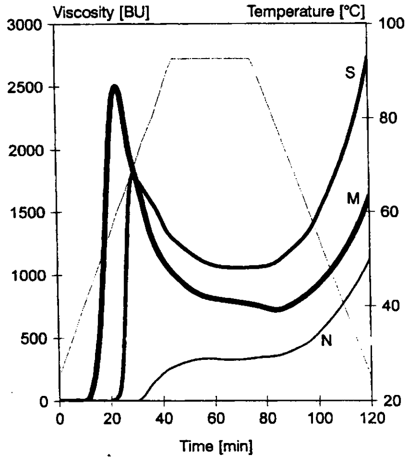

**Figure 5** *Brabender viscosity curves (c=8%) of pea starch cationic ethers as compared to native starch: N - native pea starch; S - suspension reaction product; M - microwave assisted reaction product*

The above observations were confirmed by light microscopy. Native starches heated at pasting temperature give a characteristic behaviour - amylose leakage out of the starch granules[3,4]. Cationic starches of low degree of substitution (DS = 0,02 - 0,04) revealed similar solubilisation mechanism (Figure 6A, 6B) i.e. amylose leakage out of the starch granules. At the temperature of 90°C the solubilisation of native starches is advanced, amylose leaks completely out of granules, but amylopectin still forms aggregates which are the remnants of the granules[3,4]. Cationic starches at the temperature of 90°C (pictures not shown) formed uniform mixture of completely soluble amylose and amylopectin. The colour of amylose and amylopectin - iodine complexes changed only a little as compared to native starches. Amyloses of different starch origin formed deep blue complexes, whereas amylopectins formed coloured complexes varied from orange in case of waxy corn starch, to brown, in case of normal corn starch (pictures not shown). The increase of degree of the substitution resulted not only in cold water solubility but also of complete change in complexing phenomena. Potato cationic starch of DS = 0.13 solubilised in cold water formed a yellow smear, and only small remnants of starch granules could be observed (Figure 6C) . The sample of DS = 0.27 was completely soluble in applied conditions and formed an orange complex with iodine (Figure 6D).

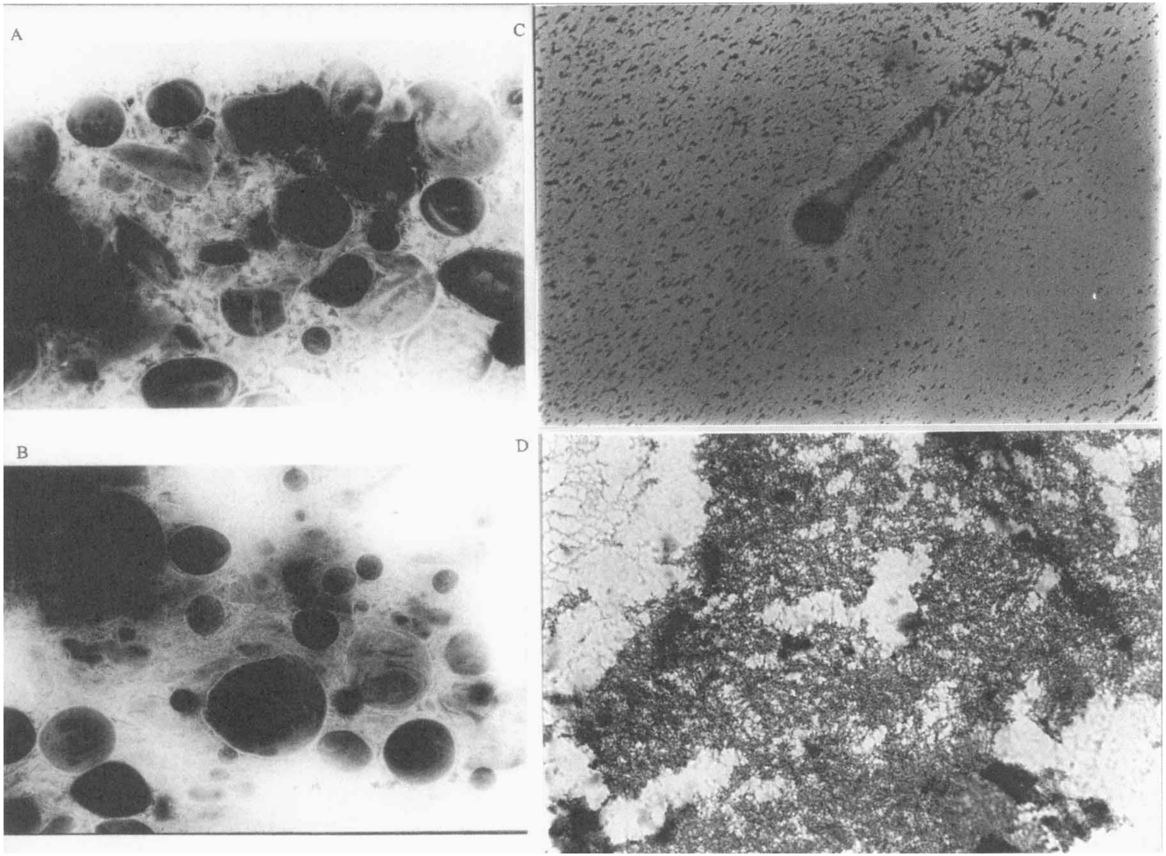

**Figure 6** *LM microphotographs of the potato cationic starch ethers. Microwave assisted reaction product of DS = 0.04 heated at initial gelatinisation temperature (A); suspension reaction product of DS = 0.04 heated at initial gelatinisation temperature (B), microwave assisted reaction product of DS = 0.13 solubilised in cold water (C), five stages acc. to EP 0874000 reaction product of DS = 0.27 solubilised in cold water (D)*

Changes in physicochemical properties of cationic derivatives related to starch-water interactions were confirmed by GPC analysis (Figures 7-9). GPC curves of native potato and pea starches (Figures 7, 8) comprised two peaks - of amylopectin and amylose. After the modification process, the two peaks could not be observed any longer. GPC curves of all cationic starches comprised one broad peak shifted to a lower retention time as compared to native amylopectin (Figures 7-9). A decrease of retention time and a broadening of GPC peak as a result of cationisation indicated a change in hydrodynamic behaviour of starch molecules rather than an increase in average molecular mass. It was previously shown that the relationship between linear dimension and molecular mass in a freely jointed polymeric chain (random coil) depends on macromolecule-solvent interaction[18,19]. The retention time of GPC peak of suspension cationic starches was always lower as compared to microwave products. These differences pointed to some degradation phenomena occurring during microwave assisted cationisation.

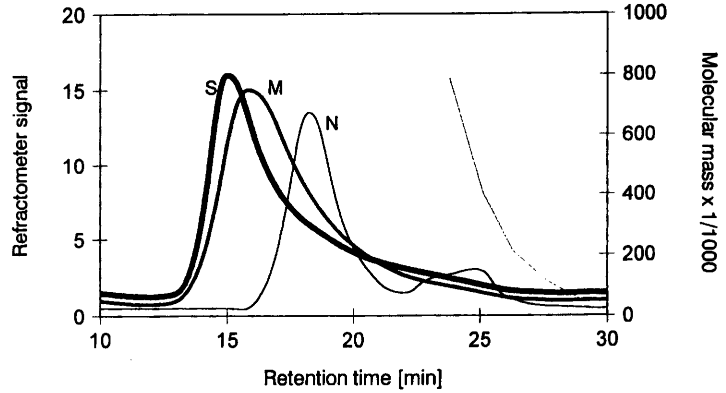

**Figure 7** *The GPC curves of potato cationic starch ethers as compare to native starch. Native starch (N); Microwave assisted reaction product of DS = 0.04 (M); Suspension reaction product of DS = 0.04 (S)*

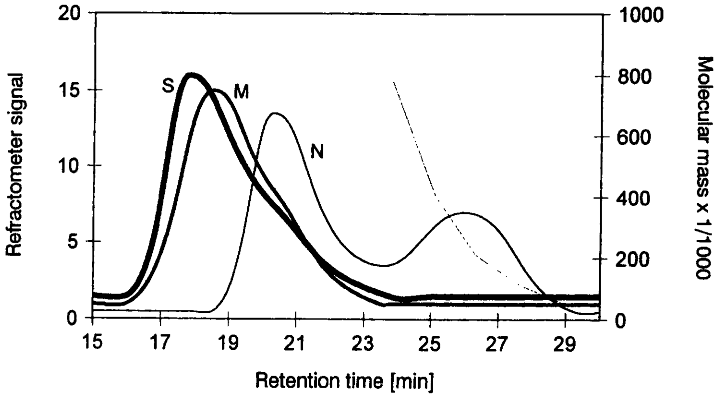

**Figure 8** *The GPC curves of pea cationic starch ethers as compare to native starch. Native starch (N); Microwave assisted reaction product (M); Suspension reaction product (S)*

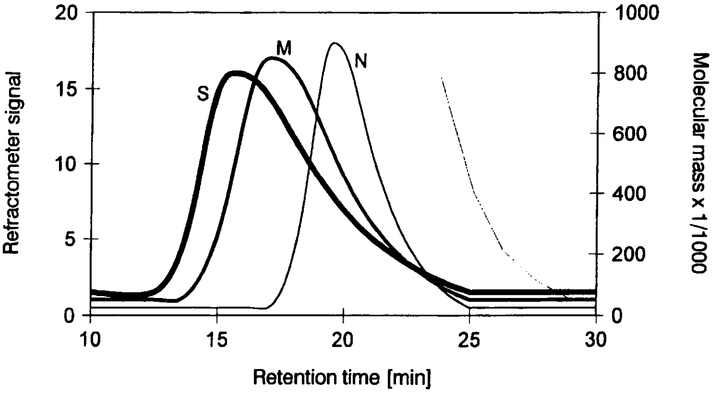

**Figure 9** *The GPC curves of waxy corn cationic starch ethers as compared to native starch. Native starch (N); Microwave assisted reaction product (M); Suspension reaction product (S)*

This hypothesis was confirmed by SEM investigations (Figures 10,11). Scanning electron microphotographs of cationic starches proved that modification process induced some deterioration of starch granular structure. The strong alkaline conditions applied both in suspension and microwave assisted cationisation processes caused some gelatinisation phenomena resulting in a leakage of starch material out of the granules. The extent of this change was more significant in the products of microwave assisted reactions than in suspension reactions products, but in all cases was very limited. The increase of the degree of substitution to DS > 0.1 caused more massive damages of starch granules indicating more advanced gelatinisation phenomena, but the granular structure of highly substituted potato cationic starches was still observed. The extent of changes caused by cationisation depended not only on type of processing but also on starch origin. Tuber and cereal starches revealed similar, rather small amylose leakage, whereas pea starch was extensively damaged, even more than potato cationic starch of high degree of substitution.

Significant damage of granular starch structure suggested similar changes in crystal structure. However X-ray diffraction investigation proved that microwave assisted cationisation process at low degree of substitution (DS ≈ 0.04) did not change the type of X-ray diffraction pattern, and only slightly influenced the degree of crystallinity (Figures 12-14). The increase of degree of substitution over DS = 0.1 significantly decreased crystallinity of potato cationic starch (Figure 12). Highly substituted potato cationic starch of DS = 0.27 was almost completely amorphous. These observations suggest that material leaking out of low substituted cationic starch granules, observed on SEM pictures, was mainly an amorphous fraction. At low degree of substitution the crystal part of starch material forming granule framework remained almost unaffected. Extensive substitution with cationic groups, where over one per four anhydroglucose units contained ionic group caused X-ray diffraction annihilation, in spite of maintenance of the shapes of starch granules.

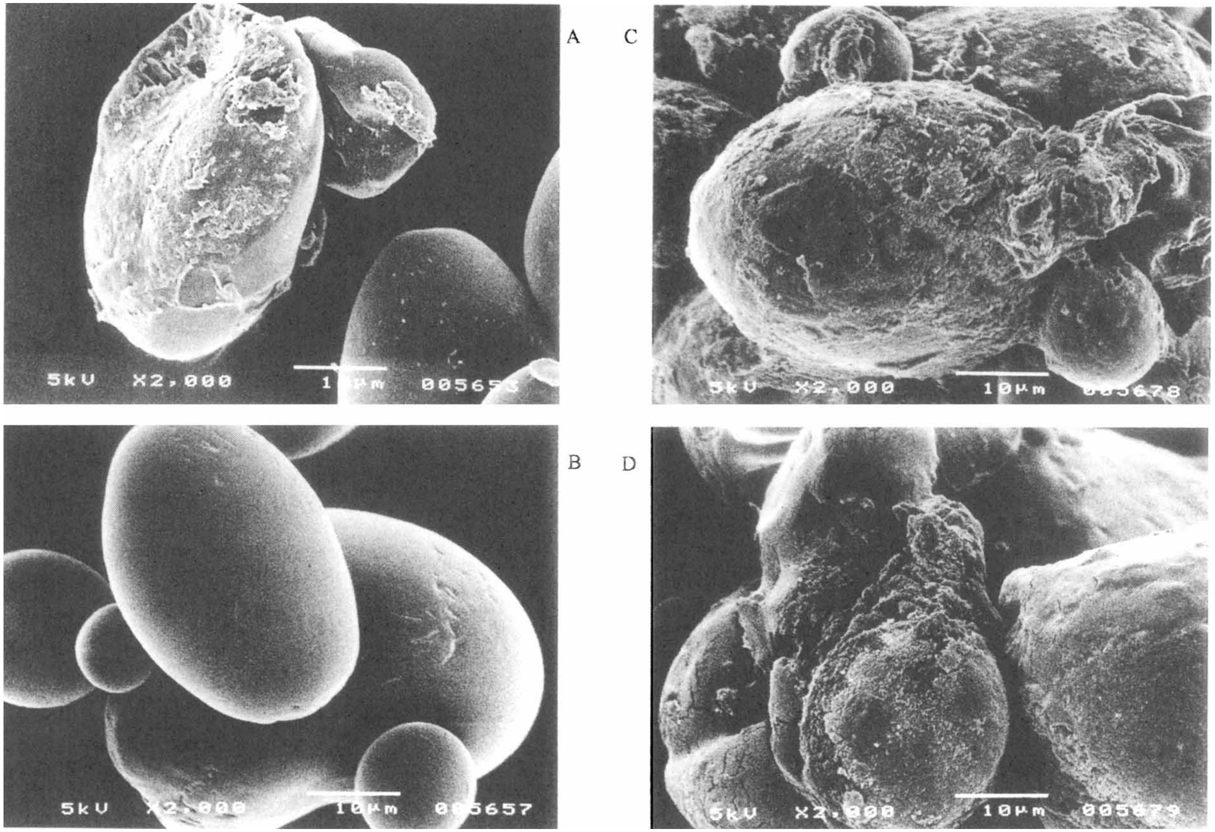

**Figure 10** *SEM microphotographs of potato cationic starch ethers. Microwave assisted reaction product of DS = 0.04 (A); suspension reaction product of DS = 0.04 (B), microwave assisted reaction product of DS = 0.13 (C), five stages acc. to EP 0874000 reaction product of DS = 0.27 (D)*

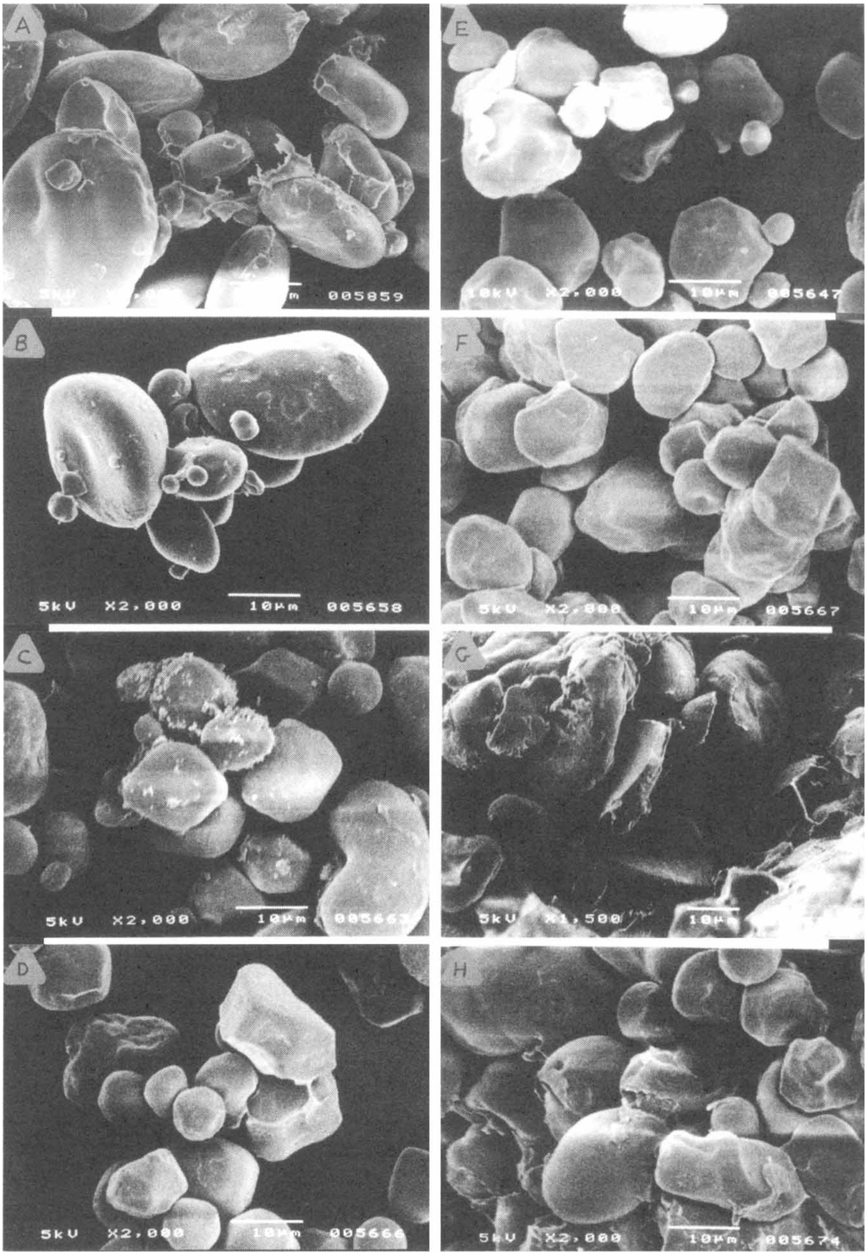

**Figure 11** *SEM microphotographs of cationic starch ethers of DS = 0.02 - 0.04. Microwave assisted reaction product of wheat starch (A); suspension reaction product of wheat starch(B), microwave assisted reaction product of corn starch (C), suspension reaction product of corn starch (D), microwave assisted reaction product of waxy corn starch (E), suspension reaction product of waxy corn starch (F) microwave assisted reaction product of pea starch (G), suspension reaction product of pea starch (H)*

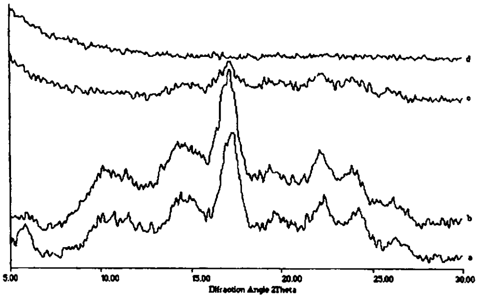

**Figure 12** *X-ray diffraction patterns of the potato cationic starch ethers. Microwave assisted reaction product of DS = 0.04 (a); suspension reaction product of DS = 0.04 (b), microwave assisted reaction product of DS = 0.13 (c), five stages acc. to EP 0874000 reaction product of DS = 0.27 (d)*

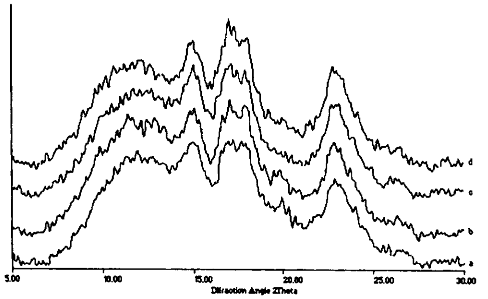

**Figure 13** *X-ray diffraction patterns of cationic starch ethers of DS = 0.02 - 0.04. Microwave assisted reaction product of corn starch (a); suspension reaction product of corn starch (b); microwave assisted reaction product of waxy corn starch(c); suspension reaction product of waxy corn starch (d)*

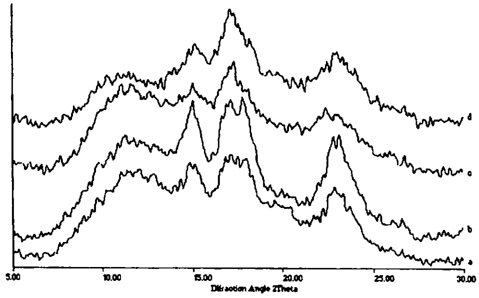

**Figure 14** *X-ray diffraction patterns of cationic starch ethers of DS = 0.02 - 0.04. Microwave assisted reaction product of wheat starch (a); suspension reaction product of wheat starch (b); microwave assisted reaction product of pea starch (c); suspension reaction product of pea starch (d)*

## 4 CONCLUSIONS

- Microwave processing is a convenient way to obtain cationic starches of DS ≈ 0.04.
- The incorporation of cationic substituents into the starch molecules significantly affects their physicochemical properties related to starch-water interactions. This results in the decrease of the gelatinisation temperature, changes of the swelling characteristics, and affects molecular mass distribution, solubilisation and complexation with iodine.
- Degradation phenomena accompanying cationisation, observed as change in molecular mass distribution and damage of granular structure, is more pronounced in microwave reaction products than in suspension reaction products.
- The extent of above changes depends not only on type of processing but also on the starch origin and is the most pronounced in the case of pea starch.
- Changes in physicochemical properties of cationic starches up to DS ≈ 0.04 are not reflected in their crystal structure.
- High degree substitution with cationic groups causes gradual decrease in starch crystallinity and at DS = 0.27 the starch is completely amorphous.

## Acknowledgement

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# AMYLOPECTIN CRYSTALLISATION IN STARCH

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## 1 STARCH CRYSTALLISATION

The biosynthesis of starch and the associated physico-chemical properties have been the subject of extensive research for many decades. Whilst the major enzymatic steps and responsible enzymes associated with the progressive deposition of starch have been largely characterised, the exact spatial location and regulatory control of these enzymes within plant tissues are still unclear. Similarly, the exact mechanisms involved in the crystallisation of starch α-glucans and the consequence of these processes on physical properties are also unclear. This paper provides an overview of recent work in our laboratory to understand starch (especially amylopectin) crystallisation and how this affects properties and potential commercial applications.

### 1.1 Background

The biochemistry associated with starch biosynthesis has been well reviewed elsewhere[1-5]. There is enzymatic partition between the cytosol and the amyloplast within which starch granules are synthesised. Three major amyloplast-based enzyme systems are responsible for the dedicated biosynthetic processes. The first, ADP-glucose pyrophosphorylase generates ADP-glucose from glucose-1-phosphate and ATP (liberating pyrophosphate). The second, starch synthase joins glucose residues provided from ADP-glucose to pre-existing α-glucan chain non-reducing ends (which liberates ADP). Starch synthase is subdivided into two major forms. Soluble starch synthase (SSS) is responsible for amylopectin biosynthesis whilst granule bound starch synthase (GBSS) is responsible for amylose biosynthesis. Finally, starch branching enzyme inserts α-(1-6) bonds into α-(1-4) glucan chains and is responsible for the highly branched nature of amylopectin (which contains about 5% α-(1-6) bonds and 95% α-(1-4) bonds). Amylose molecules contain proportionally more (>99%) α-(1-4) bonds and are only lightly branched. The relationship between the biochemistry of starch synthesis and the creation of starch granule structure is rarely addressed.

The composition and structural aspects of starch granules and the constituent α-glucans have been reviewed elsewhere[6]. Basically, amylopectin molecules ($M_w$ typically[7] cited as $10^7$-$10^9$) radiate from the hilum (centre) of starch granules to the periphery. The average chain length of the constituent α-(1-4) chains is of the order of 20-25 glucose units long although there is a hierarchical order of chain lengths as proposed by Hizukuri[8-9]. According to this scheme, A-chains are α-(1-6) bonded to B-chains. The B-chains are/may themselves be α-(1-6) bonded to other B-chains which, depending on their length, (and consequently number of amylopectin clusters within crystalline laminates they traverse radially within the molecule) are defined as $B_1$-$B_4$. Depending on this hierarchy, the A- and B-chains are ultimately linked (α-(1-6) bonds) to the long single C-chain of the amylopectin molecules (and contain the only free reducing group). Amylose is a lightly branched α-glucan with $M_w$ typically[7] cited as $10^5$-$10^6$, and about 9-20 branch points per molecule[10] which α-(1-6) bond α-(1-4) glucan chains of variable length. Overall, the amylose molecules contain 2-11 chains with between 200-700 glucose residues per chain[6]. In native starch granules the amylose may be free or, as in cereal starches where lipid (free fatty acids and/or lysophospholipids) occurs, lipid-complexed (V-type helix)[11-12]

The exterior chains of amylopectin (which comprise A- and $B_1$ chains) form double helices. These are on average typically 18-20 glucose residues long[13] (or perhaps 14-18 as reported elsewhere[7]). Usually cereal starches are reported to have shorter chains than tuber starches[8-9]. The single helix repeat (pitch) within a double helix represents six glucose residues. Hence, for eighteen glucose residues, three complete single helix repeats (three full pitch lengths) will be present. We assume that this is the optimal length of the amylopectin unit chains and consequently amylopectin double helices because these spontaneously-formed double helices become too large and rigid to permit much further elongation by the starch SSS (see below). This concept is, it is recognised probably at odds with the 'trimming' process described by others[14] in relation to starch biosynthesis.

Wu and Sarko[15] showed with amylose double helices (used to model double helices of amylopectin exterior chains for both A- and B-type polymorphs) that there was a repeat distance of 1.05nm along the helix axis. For this model, each strand was a 6(-3.5) helix. The 6 representing 6 glucose units per pitch with 3.5Å (0.35nm) advance per monomer, giving a pitch length of 21Å (2.1nm). The helix in starch granules is probably left-handed (and hence the minus sign) where 18 residues would be equivalent to 6.3nm and 20 residues 7.0nm. Cameron and Donald[16] have measured the internal dimensions of starch granules using small angle X-ray scattering (SAXS). Using this approach they have shown that growth rings contain semi-crystalline radial (shell) regions of about 140nm thick interspersed with broad amorphous radial regions of at least the same thickness. These semi-crystalline shells contain 16 radiating clusters of amylopectin exterior chains, comprising 6.64nm laminates of amylopectin double helices interspersed with 2.21nm laminates of relatively thin amorphous regions. Gallant *et al*[17] have proposed that a larger scale of order exists within starch granules and contain 5 to 50 amylopectin clusters. These blocklets range in size from 20 to 500nm depending on the number of clusters they contain.

## 1.2 Crystallisation

*1.2.1. Biosynthesis.* The crystallisation of starch during biosynthesis is a difficult process to manipulate as biochemical events impinge upon physical events and *vice versa*. Three major approaches have been adopted to moderate the crystallisation and have generated a great deal of information about starch structure.

The first approach has focused around characterising the physico-chemical properties of starches at various days after anthesis (DAA)[18]. However, not a great deal of effect on crystallinity has been identified using this approach although the composition of starch does change (increasing amylose and lipid content) during development[19].

The second approach has been to generate specific mutant or transgenic forms of plants and characterise the consequence in terms of the biochemical flux and physico-chemical properties. This area has and is receiving a great deal of attention with a view to generating novel starches using biotechnology. The major problem with this approach is that it is extremely difficult to target specific modifications to starch on the basis of modifying the key biosynthetic enzyme expression. The transgenic approach is particularly difficult to modify with a view to moderating amylopectin crystallinity although some recent work on *rug* mutants of peas[20] has proved very interesting in this respect.

The third approach concerns modifying growth temperatures to which plants are subjected and identifying what the consequence is with respect to key biosynthetic enzyme activity (SSS is especially sensitive to increasing temperatures) and amylopectin crystallisation. Elevated growth temperatures cause little significant effect on amylopectin structure. Similarly the number of double helices measured directly using $^{13}$Carbon Cross-polarisation/Magic Angle Spinning-Nuclear Magnetic Resonance ($^{13}$C CP/MAS-NMR), amount of hydrogen bonding by differential scanning calorimetry (DSC) or amount of crystallinity using wide angle X-ray scattering (WAXS) remains roughly constant. However, elevated growth temperatures cause enhanced registration of amylopectin crystallites with perhaps enhanced rigidity of amorphous regions[21-24]. These molecular events restrict granule hydration and consequently elevate gelatinisation temperatures. We often describe the direct effects of growth temperature on starch crystallisation as '*in vivo*' annealing[23].

It is evident from the preceding text that starch crystallisation is a complex process. Our research on the environmental regulation of starch biosynthesis shows clearly that whilst key biosynthetic enzymes have reduced activity as growth temperature increases, α-glucan structures remain essentially identical. This slowing down of starch deposition favours double helix registration and rigidity of amorphous regions and hence elevates the gelatinisation temperatures. The formation of double helices and their growth to an optimal length probably (at this point) prevents starch synthase activity and hence controls the length of the exterior chains of amylopectin molecules.

*1.2.2. Annealing.* The annealing process involves heating starches at or below their onset gelatinisation temperatures ($T_0$ by DSC) in enough water to facilitate molecular mobility. The molecular underpinning of this technique has been discussed in detail by these authors (Tester and Debon) in recent publications[13,25,26] and the following text is largely derived from this work.

The composition of starch granules remains essentially unchanged (except for a small amount of leached α-glucan) when granules are annealed. We have shown that although annealing causes a significant increase in the gelatinisation temperature of waxy and normal starches, this is not due to a significant increase in the amount of double helix hydrogen bonding (DSC), number of amylopectin double helices ($^{13}$C CP/MAS-NMR) or amount of crystallinity (WAXS).

For native wheat starch, the amount of crystallinity by WAXS is about 36% which corresponds to a double helix content of about 46%[27]. This is interesting for two reasons. Firstly, it shows that not all double helices are in crystalline regions. Secondly, that there is great potential for double helices located outside crystalline domains to become part of these domains if such a mechanism could exist (by for example annealing). However, as described above, the DSC, NMR and WAXS data indicate that annealing causes enhanced registration of pre-existing crystalline regions rather than creation of more crystalline material. In other words, the double helices excluded from crystalline domains do not have enough mobility during annealing to become part of the crystalline domains.

We have proposed[13,25,26] that the increase in gelatinisation temperature as a consequence of annealing is caused by enhanced registration of amylopectin double helices with perhaps a little enhancement of helix length. The amorphous regions probably become more rigid as a consequence of the annealing process also. Amylose lipid complexes (which we believe are located in specific domains) are not annealed at the temperatures used to anneal the amylopectin crystallites as their dissociation temperature is much higher (typically 95 to 115°C). Overall, the more ordered nature of starch granules post annealing restricts water penetration and consequently gelatinisation temperatures are elevated. Small improvements to helical length (removing imperfections at the ends) may also contribute to the increase in gelatinisation temperatures. These molecular events make it much harder for granules to swell (a consequence of gelatinisation).

In high amylose starches the crystallisation pattern is different from waxy and normal starches[13]. In the waxy and normal starches lipid free amylose does not form any significant amylose-amylose or amylose-amylopectin double helices. This is probably because of the high amylopectin content, short amylopectin compared to long amylose chain lengths and favourability of amylopectin exterior chains to spontaneously form double helices during biosynthesis. Hence amylose and amylopectin are segregated. However, in high amylose starches, crystalline regions comprise amylopectin-amylopectin, amylopectin-amylose and amylose-amylose double helices (with single V-type amylose-lipid single helices in common with normal and, to a much lesser extent, some waxy starches). Annealing of these starches facilitates compartmentalisation of intermixed amylose and amylopectin chains into amylose-amylose and amylopectin-amylopectin.

## 1.3 Starch Polymorphic Form

The crystallisation of α-glucan chains within starch granules is a complex process as discussed above. The composition of starch granules affects this crystallisation process, as does the molecular structure of the α-glucans. The A-type polymorph is more stable than the B-type, and both forms can be formed in the laboratory using linear α-glucans to model the crystallisation properties of the exterior chains of amylopectin. Heat moisture treated B- and C-type starches can readily be converted to the A-type form but not *vice versa*. According to some authors[28,29], the A-type polymorph formation is favoured when chain lengths are shorter, crystallisation temperatures are higher, polysaccharide concentrations are greater and the crystallisation process is slower. Hence, whilst A- (cereal) and B-type (tuber) polymorphic forms of amylopectin are different, these crystalline forms need not necessarily reflect the slightly longer exterior chains within the B-type starches[30] but the actual crystallisation process itself. We are currently working on the relationship between the polymorphic crystalline form of starch (using WAXS) and functionality. This aspect of starch chemistry is of great industrial significance but very poorly understood.

## 1.4 The Future

It is important that the work on transgenically modified starchy crops continues so that the consequence of gene expression (and hence enzyme activity) on starch structure and properties is understood. However, many questions related to structure-function relationships can be understood with starches currently available. Because of the major importance of starch in food and other industrial products and processes, it is critical that all attempts are made to unravel the complexities of starch crystallinity and how this impinges upon physical properties. Whilst this is not easy it is critical to release the full potential of starches.

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# AN APPROACH TO STRUCTURAL ANALYSIS OF GRANULES USING GENETICALLY MODIFIED STARCHES

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## 1 INTRODUCTION

Starch granules are mainly composed of macromolecular polymers of α,D-glucosyl units, amylose and amylopectin[1]. Amylose is a linear glucan with α,1-4 glycosidic linkages and limited branching that produces few non-reducing end-groups. Its average molecular weights range is between $10^5$ and $10^6$g.mol$^{-1}$ [2]. Conversely, amylopectin is a highly branched polymer composed of short linear chains [degree of polymerisation (dp) =15] branched on longer chains (dp=45) by α,1-6 linkages. Its molecular weight is greater than $10^8$g.mol$^{-1}$ [3]. Depending on botanical origin, the structural features of amylose and amylopectin differ. The proportion of amylose in starch ranges from 0 in *waxy* (*wx*) maize starch up to 70-80% in *rugosus* (*r*) pea starch (*i.e.* wrinkled pea). Amylose content is known to influence both nutritional and technological properties such as susceptibility to enzymatic hydrolysis, gelling and pasting behavior[4]. It is accepted that the cluster organisation of amylopectin chains allows the short chains, and part of the long chains to form double helices which can pack together in crystalline zones[5]. However the respective contributions of amylose and amylopectin to crystallinity are still unknown.

Depending on their botanical origin, native starches display different diffraction patterns, *i.e.* A-type and B-type. When crystallising linear chains in solution, the A-type is favoured thermodynamically, and the B-type kinetically[6]. The structure of the A-type is obtained preferentially under conditions of high crystallisation temperature, high polymer concentration, and short chain length[7]. For native starches, amylopectin molecules from A-type starches have shorter constitutive chains and larger numbers of short-chain fractions than those from B-type starches[8]. Many studies have been based on the average structural features of whole amylopectin[6,7,9] without considering the possible influence of the internal structure of clusters on crystallisation.

The main targets of the present study were to:

1. Assess the binary composition of starch.
2. Investigate the fine structure of amylopectin in order to determine how cluster features may determine the crystalline type obtained.
3. Estimate the contribution of amylose to crystallinity.

The originality of this work is the availability of a wide range of starch granule structures, therefore a wide range of mutations. Maize starches, for which numerous genotypes have been obtained by classical breading, have been used. These maize mutants have been provided by Limagrain company.

The main enzymatic activities in starch biosynthesis are due to granule bound starch synthases (GBSS), soluble starch synthases (SSS) and branching enzymes (BE)[10]. In the present work, starch in which granule ultrastructure is mainly due to one specific enzyme activity was obtained by selection of double maize starch mutants for which two of these biosynthetic enzyme activities were shut down. The double mutants of maize chosen are: *wx ae* (waxy-amylose extender), *wx du* (waxy-dull), *ae du* (amylose extender-dull) and *du su2* (dull-sugary2) whose main biosynthetic activities are due to SSS, BE, GBSS, GBSS and BE respectively. Corresponding single mutants are studied as well. Starches from these double mutants allow work to be done on a wide range of amylopectin content (from 35 to 99%), and a ratio of short to long chains varying from 1:9 to 4:3. They cover a wide range of crystalline structures, with a crystallinity level varying from 19 to 48%, and an A to B type polymorphs ratio from 0 to 100%.

## 2 IS STARCH A BINARY MIXTURE OF TWO POLYMERS?

Starch macromolecular composition was analysed by size exclusion chromatography (SEC), iodine-binding capacity (IBC) measurements, differential scanning calorimetry (DSC) and complexation with concanavalin A. These methods are based on structural or functional differences between the two theoretical macromolecules present in starch. SEC (with a 2.6 x 200 cm column) on HW75 S gel was used as the reference method for analysing the macromolecular composition of starches.

In addition to the variation of amylose/amylopectin ratios, the mutants used reveal a third population of α-glucans, shown by size exclusion chromatography. In between amylopectin and amylose, a third peak is observed (Figure 1). This peak corresponds to a population called intermediate material because its hydrodynamic radius is between that of amylose and amylopectin, and the lambda max obtained from iodine complexation is also in between that of amylose and amylopectin (Table 1).

**Table 1** *Amylopectin $\lambda_{max}$ (nm) and amylose content in maize mutant starches. (SEC: size exclusion chromatography, Con A: concanavalin A, DSC: differential scanning calorimetry, IBC: iodine-binding capacity, nd: not detected; standard deviation in parentheses).*

| | *Amylopectin* | *% Amylose* | | | |
|---|---|---|---|---|---|
| | *λmax (nm)* | *SEC* | *ConA* | *DSC* | *IBC* |
| *wx* | 535 | nd | nd | nd | nd |
| *wxdu* | 520 | nd | nd | nd | nd |
| *aewx* | 545-580 | nd | 7 (0.5) | nd | 14 (2) |
| *du* | 575-585 | 27 | 45 (0.5) | 55 (1) | 45 (4) |
| *su2* | 580-590 | 24 | 50 (2) | 58 (0.5) | 45 (1) |
| *dusu2* | 580-605 | 34 | 58 (0.5) | 66 (4) | 60 (2) |
| *aedu* | 560-570 | 30 | 56 (0.5) | 64 (1) | 56 (2) |
| *ae* | 565 | 54 | 63 (1) | 60 (2) | 63 (3) |

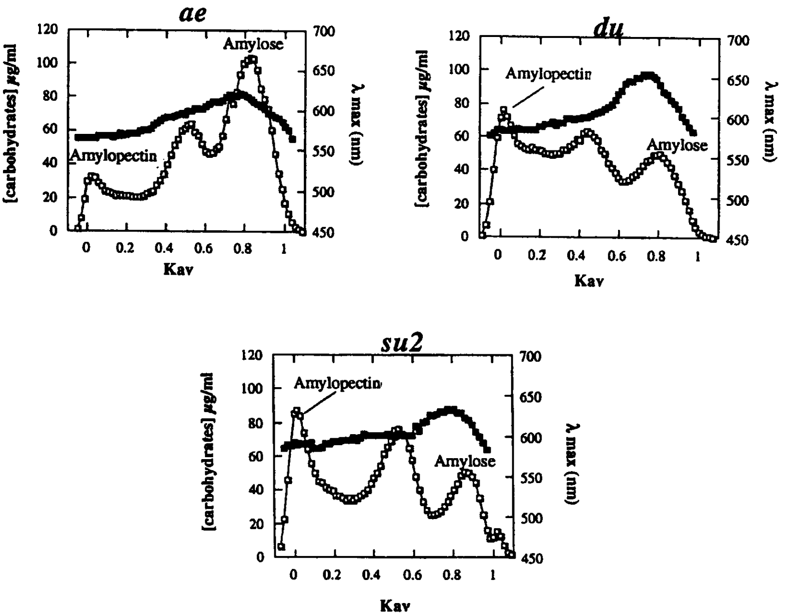

**Figure 1** *SEC profiles of maize mutant starches (a) ae, (b) du, (c) su2. -■- total carbohydrates (µg/ml). -□- λmax (nm)*

The different methods used to determine amylose content such as precipitation with concanavalin A, DSC or iodine binding capacity have been compared with results obtained from size exclusion chromatography (Table 1)[11].

All these tested methods lead to an overestimation of the amylose content. The intermediate population reacts with iodine, lipids and conA. This reactivity which cannot be predicted leads to a very difficult interpretation of the amylose content determination by usual methods, for instance simple test based upon iodine staining. This intermediate population is composed by chains long enough to complex with iodine and lipid, and has enough non reducing ends to complex with concanavalinA. This population synthesised for all the starches studied, except for waxy mutants, corresponds to five to fifty percent of the all macromolecules.

## 3 WHAT IS THE DETERMINANT OF EACH CRYSTALLINE TYPE?

Fine structures of amylopectin from an A-type and a B-type double-mutant maize starches have been investigated in order to determine how the distance between branching points in a cluster is related to the crystallisation pattern of the chain arrangement.

Studied samples needed to have different crystalline type, the same level of crystallinity in order to get rid of the influence of the crystallinity level, and to be composed only of amylopectin to avoid any influence of amylose. These three conditions have been satisfied by studying starches from double mutants: *wxdu* (A-type) and *aewx* (B-type) whose crystallinity level are similar (45%). Cluster and the upper part of the clusters where all the branch points are located (BZC : branching zone of the clusters) were isolated using

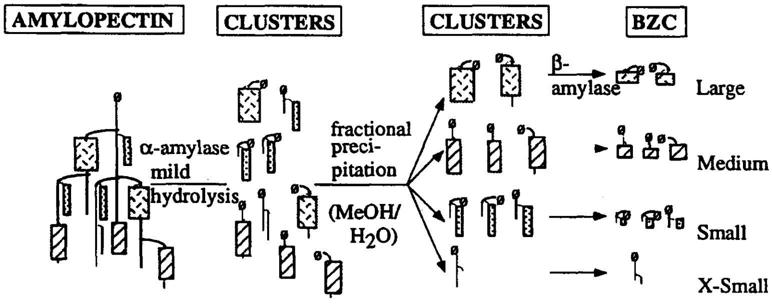

**Figure 2** *Methods used to isolate the clusters and the branching zone of the clusters (BZC) to get a complete characterisation of the structure of amylopectin*

successive hydrolysis involving alpha and beta amylases (Figure 2)[12]. First, in order to cut long internal chains and to isolate the clusters, a mild hydrolysis with α-amylase was performed. Then, the clusters were precipitated according to their sizes using different methanol / water ratios. Different fractions of large, medium, small and very small clusters were obtained. On each fraction, an extensive hydrolysis with β-amylase was performed in order to reduce the chain length to a minimum and to isolate the BZC.

Clusters of amylopectin from *wxdu* starch, which shows A-type crystallinity are big and are composed of numerous chains (Figure 3). Constitutive chains are short and branch points are close. This organisation leads to a high branching density: 0.18 per BZC. Conversely, for amylopectin from *aewx* starch, which shows a typical B-type crystallinity, clusters are composed of few chains, longer than those of *wxdu* amylopectin. BZC of *aewx* amylopectin are characterised by a long distance between branch points and a low branching density: 0.13 (Figure 4).

A parallel might be drawn between the characterisation of clusters and BZC with the corresponding crystalline type. Previous crystallographic studies[13,14] coupled with molecular modelling have lead to proposed models for the organisation of A and B-type crystallites. The differences between the two allomorphs relate to the packing of double

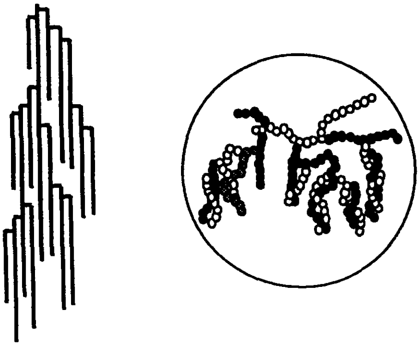

**Figure 3** *Proposed structure of a wxdu cluster : (left) a cluster in symbolised form, and (right) a cluster as an organised structure*

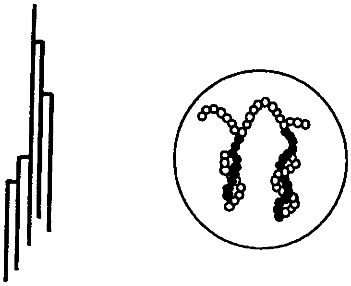

**Figure 4** *Proposed structure of a aewx cluster: (left) a cluster in symbolised form, and (right) a cluster as an organised structure*

helices in the crystal unit cell and the quantity of water molecules stabilising these double helices. In the B-type structure, double helices are packed in a hexagonal unit cell in which each has three neighbors[13]. In the A-structure, double helices are packed in a monoclinic unit cell in which each has 6 neighbors[14]. In the case of type A model, the packing of the double helices is dense, and distances between doubles helices in the unit cell are short. For B-type polymorph, the packing of the double helices is less dense due to the higher distance between double helices in the unit cell. These data are in agreement with the models of clusters we proposed. Crystalline type would be due to the number of chains within a cluster and how they layout. Moreover, these models are in agreement with data on *waxy* rice and potato amylopectins fine structures[15,16,17].

## 4 INVOLVMENT OF AMYLOSE AND INTERMEDIATE MATERIAL IN CRYSTALLITES

Amylopectin clusters involved in crystallites of native starch are composed of a few chains (4 or 5) for B-type starch (*aewx*) (Figure 4), whereas those from A-type amylopectin (*wxdu*) are composed of more chains (~10 to 20) (Figure 3)[12]. In the case of an A-type cluster, removing one or two chains from such a structure does not influence its branching density. The new cluster created is still able to form double helices, so able to participate in the crystalline organisation (Figure 5 b). An added apparent amylose segment would not have any strong influence on A-type crystallisation, in that the role of amylopectin conceals any apparent amylose contribution (Figure 5 c). Conversely, when considering a cluster from B-type starch amylopectin with two chains removed (Figure 5 e), the obtained cluster is unable to form double helices, so it can no longer participate in crystalline organisation. One or two apparent amylose segments added to such a cluster (Figure 5 f) would induce the formation of a novel cluster able to participate in crystallinity for B-type starch, and the role of apparent amylose in such a cluster is significant. Consequently, the involvement of apparent amylose in crystallites even if it occurs in both crystalline type, would be more detectable in B-type than in A-type, crystallites.

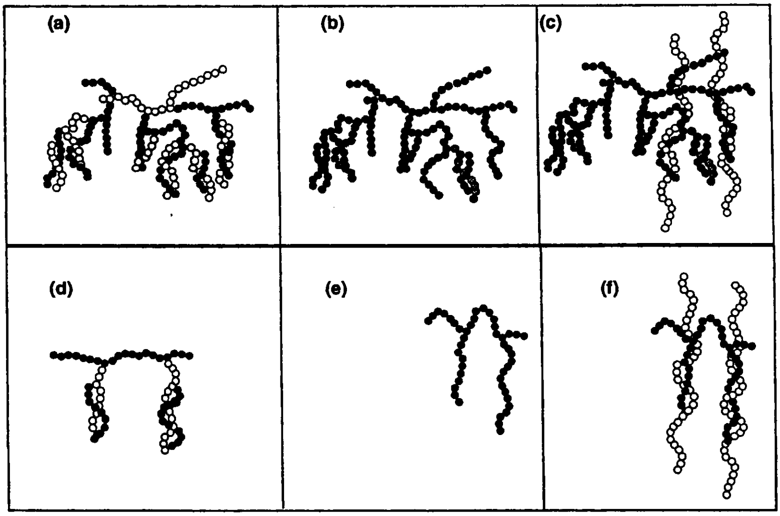

**Figure 5** *Involvement of amylose in crystallites: (a) an A-type cluster an organised structure, double helices are composed of short chains from amylopectin, (b) an A-type cluster without two short chains, (c) the presence of two apparent amylose chains (white circles) do not have a strong influence on A-type cluster crystallisation, (d) a B-type cluster an organised structure, double helices are composed of short chains from amylopectin, (e) a B-type cluster without two short chains, (f) the presence of two apparent amylose chains (white circles) allow the B-type cluster to be involved in the crystalline organisation*

## 5 CONCLUSION

Starch appears to be different from a simple binary mixture of two polymers. From a genetic point of view, this study showed that starch biosynthesis without some of the enzymes involved in granule construction produces a polysaccharide different from amylopectin and amylose. This polysaccharide shows an intermediate structure between the two well-known starch macromolecules, which creates difficulties for the amylose assay. Moreover, our experiments clearly indicate the structural heterogeneity of macromolecules between genotypes and within a specific genotype. Results from statistical averaging approaches to determine amylose content regularly used should be considered carefully.

In maize mutant starches, the crystalline type obtained is not correlated to the amylose content. The fine structure of amylopectin is determinant for the obtained polymorph. The paradigm is that the chain length of the short chains of amylopectin is the parameter most important. However, it appears from the collected data that the distance between branch points inside a cluster, the branching density of the cluster and the size of the cluster are more important. Moreover, the structural models proposed for clusters from A and B type crystallites are in agreement with crystallographic models and are able to explain the hypothetical involvement of amylose or of the intermediate material inside the B-type crystallites.

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# MECHANISMS OF THE ACTION OF PORCINE PANCREATIC α–AMYLASE ON NATIVE AND HEAT TREATED STARCHES FROM VARIOUS BOTANICAL SOURCES

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## 1 INTRODUCTION

In a typical British diet, starch contributes about 60-70% of the 'available' or 'glycaemic' carbohydrate, but different starch containing foods containing isoglucidic amounts of available glucose, result, after ingestion, in different postprandial rises in blood glucose and insulin levels[1]. Indeed a variable fraction of ingested starch can escape digestion altogether[2]. The molecular/structural basis for the observed differences in the glycaemic index of foods is unclear but factors that are likely to influence the activity of pancreatic α-amylase include (a) the botanical source of the starch, (b) food texture affecting the ability of the starch granules to swell and (c) the amylopectin/amylose ratio[3-7]. The presence of non-starch polysaccharides in the diet also inhibits digestion of carbohydrate in a number of ways, depending critically on the type of polysaccharide consumed[8-10].

Full 3-D structures for human and porcine pancreatic amylases have been determined by X-ray crystallography which indicate that the active site region contains 7-11 subsites for sugar residues to ensure maximal binding. In addition, the enzyme seems to possess a starch binding domain remote from the active site which is probably important for tethering the enzyme onto its insoluble substrate[11,12]. Such domains appear to be a common feature of polysaccharidases[13-15].

Standard enzyme kinetic work is based on a model in which interaction occurs between enzyme and substrate molecules in solution, but it was pointed out many years ago[16], that the reaction between α-amylase and starch is a two-phase system and if the reaction mechanism involves a kinetically-significant absorption step, the relationship between reaction rate and enzyme concentration is not exactly 1:1, but will take the form of a Freundlich equation:

$$\log v = n\log E_0 + \log(k_{cat}K) \qquad (1)$$

provided that the fraction of total enzyme molecules bound to starch is small. K is a partition coefficient and *n* is predicted to be 2/3 for enzyme adsorbed on a perfectly smooth face of a cube of molecular dimensions. For absorption on edges and/or into cracks in the surface, *n* is predicted to lie between 1/3 and 2/3[16].

## 2 EXPERIMENTAL

### 2.1 Preparation of Starch Suspensions

Starch granules of various botanical sources were suspended in 0.01M PBS, pH 7.4 to provide the required concentrations and agitated gently by swirling the mixture for 20 minutes in a conical flask. This was carried out either at room temperature and so designated 'native' or in a heated water bath for experiments where the effects of heat-treatment were to be investigated. The flasks were sealed with glass marbles to restrict water loss by evaporation during heating but in addition, the flask and contents were weighed before and after the 20 minute period so any losses could be made good.

### 2.2 Preparation of Guar Galactomannan Solutions

Guar gum flour (M90 Meyhall Chemical Co. Ltd) was suspended in PBS pH 7.4 and allowed to hydrate for at least 18 hours (overnight) at room temperature.

### 2.3 Digestion of Starch with Porcine Pancreatic α-Amylase

Aliquots (3 ml) of starch suspension were incubated at 37°C with porcine pancreatic amylase in sealed tubes with continuous end-over-end mixing. At timed intervals, aliquots of 200 μl were removed from each reaction tube and immediately spun in a microfuge for 30 sec to sediment undigested starch. The supernatant was used for analysis of reducing sugar content by a sensitive method[17,18].

In experiments with guar gum, baseline values were found to be unacceptably high and therefore the less sensitive assay using dinitrosalicylic acid[19] was used.

## 3 RESULTS AND DISCUSSION

A Freundlich plot for the action of a range of concentrations of amylase on native wheat starch is shown in Figure 1. The plot is linear with a slope of 0.69 that is significantly different from unity. Thus absorption of the enzyme to starch is kinetically important. The plot shows rates determined over 0.5-10.5 minutes of reaction but equivalent rate measurements taken over the initial 30 seconds are mostly interpretable by conventional kinetics i.e. the absorption step is not of kinetic significance. Data were obtained for a number of native botanical starches, both for the initial 30 seconds and for 10.5 minutes. Similar measurements were made on starches that had been subjected to 20 minutes of heat treatment at 100°C to gelatinise the starch. All results obtained are summarised in Table 1.

Apart from native waxy rice, the rate during the first 30 seconds provides Freundlich coefficients close to unity i.e. a kinetically-significant absorption step is not involved. For the longer time period, *n* values signify absorption and in most cases the values are less than 2/3. The granules are unlikely to be perfectly regular in surface but be pitted and cratered so that edge effects become significant[16]. Fully gelatinised starches have *n* values for the 0.5-10.5 minute assay that approximate more closely to the theoretical one of 2/3. The swollen gelatinised starch presumably presents a more even surface to which the enzyme may adsorb.

**Table 1** *Values of n obtained from the slopes of Freundlich plots. The values in A refer to rate measurements made for the first 30 seconds of the assay period and those in B are calculated from rates determined over the next 10 minutes of reaction. (Mean values calculated from several determinations).*

| *Starch Source & Type* | *A*<br>*n* | *B*<br>*n* |
|---|---|---|
| Wheat (native) | 0.9 | 0.7 |
| Wheat (gelatinised) | 0.8 | 0.7 |
| Potato (native) | 1.0 | 0.5 |
| Potato (gelatinised) | 0.9 | 0.7 |
| Normal Rice (native) | 1.0 | 0.5 |
| Normal Rice (gelatinised) | 0.9 | 0.7 |
| Waxy Rice (native) | 0.5 | 0.5 |
| Waxy Rice (gelatinised) | 1.0 | 0.7 |

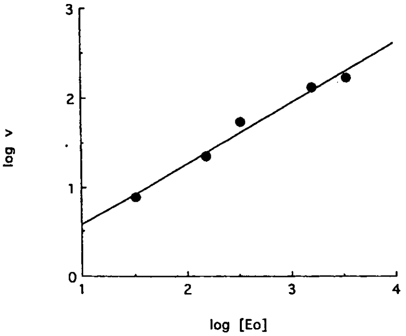

**Figure 1** *Freundlich plot for pancreatic amylase acting on 1% native wheat starch. The concentration of enzyme in the reaction mixtures ranged from 0.59 to 59.0 nanomolar.*

Product formed during the first 0.5 minute of the reaction probably arises from amylose fragments leached from starch granules during preparation of the suspension, and/or from short lengths of polysaccharide chain that protrude from the 'hairy billiard ball' structure of the compact granule[20]. In consequence amylase is acting on soluble substrate at this stage and so 'normal' enzyme kinetics apply. Similar conditions are met when amylase acts on small molecular weight artificial substrates such as p-nitrophenyl maltosides. The anomalous findings with waxy rice support these conclusions because this starch is composed of compact granules consisting almost entirely of amylopectin which solubilises less readily than amylose. Thus even in the early stages of the reaction, an absorption step is involved (*n* is less than unity) because of the paucity of leached material.

In experiments where substrate properties of different starches were compared, the enzyme concentration was carefully maintained at a constant concentration of 0.59 nanomolar throughout so as to minimise anomalies arising from the Freundlich relationship between v and [$E_0$]. Because amylase action on starch obeys Michaelis-

Menten kinetics, differences in measured Vmax values reflect true differences in $k_{cat}$ provided that $[E_0]$ is constant. Kinetic parameters were calculated from rate-substrate concentration data using non-linear regression . Relative catalytic efficiencies for action on the various substrates are calculated from $k_{cat}$ /Km ratios. The data are presented in Table 2.

**Table 2** *Kinetic parameters for porcine pancreatic α-amylase acting on starches. The kinetic parameters were obtained by fitting of experimental data to the Michaelis-Menten equation by weighted regression. The $k_{cat}$/Km values and hydrolysis rate of 1% solutions are relative to the values for native wheat starch of 0.27 x $10^5$ and 7.34μM.min$^{-1}$ respectively in the presence of 0.59 nanomolar amylase.*

| *Substrate* | *Km (%)* | *Relative $k_{cat}$ / Km* | *Hydrolysis rate* |
|---|---|---|---|
| Wheat (Native) | 0.51 | 1 | 1 |
| Wheat (100°C) | 0.13 | 46 | 15.5 |
| Potato (Native) | 0.58 | 0.6 | 0.67 |
| Potato (100°C) | 0.04 | 141 | 16.3 |
| Normal Rice (Native) | 0.70 | 4.8 | 6.0 |
| Normal Rice (100°C) | 0.03 | 126 | 10.8 |
| Waxy Rice (Native) | 0.68 | 7 | 8.3 |
| Waxy Rice (100°C) | 0.07 | 94 | 19.2 |

All reaction rates were determined over an incubation period of 10.5 minutes. Km is expressed in terms of percentage and so $k_{cat}$/Km values are of non-standard physical dimensions but are still useful for comparing the efficiency of the amylolytic process.

Calculations of kinetic parameters from data collected over the first minute of reaction give lower Km values and higher $k_{cat}$/Km values in general (data not shown). This again probably reflects the presence of leached material which is subject to preferential attack.

The Km values for native starches from the four different sources are all in the range 0.5 - 0.7 %. Heat treatment at 100°C in excess water decreases Km values for all starches suggesting that enzyme attack is facilitated by the disorder of structure induced by heating. The effects of heating on Vmax are more variable in that although wheat and potato starches show increases in Vmax , rice starches are hardly affected. Waxy rice starch is almost entirely amylopectin. Relative catalytic efficiencies are increased by the heat treatment, but again the effect is variable. For example, waxy rice increases by a factor of 13 fold whereas the increase is 235 fold for potato. Of the native starches, the rice varieties seem to be particularly good substrates for amylase. Amylopectin, present in high proportion in waxy rice, is itself a good substrate (data not shown). The presence of phosphate ester linkages in potato starch may aid swelling during hydrothermal treatments and thus accelerate the formation of molecular structures amenable to amylolysis[20,21]. In general, the relative rates of hydrolysis of 1% solutions of the starches follows the same trend as the $k_{cat}/Km$ ratios.

In a systematic study of the effect on catalytic efficiency of pre-heated wheat starch, it was found that the $k_{cat}/Km$ value increases greatly above 65°C but then falls again if the pre-treatment process is conducted above 75°C. Gelatinisation of wheat starch suspended in PBS occurs over the temperature range of 61-71°C. The fall almost certainly results from the formation of retrograded starch which is a poor substrate for amylase; amylose leached into solution during heating adopts an altered secondary structure (retrogrades) during cooling to room temperature[22]. We have shown that retrograded starch can have a direct inhibitory action on the enzyme (manuscript in preparation) which may come about through non-productive binding of the enzyme via the putative binding domain.

Guar galactomannan inhibits α-amylase action on starch non competitively with a Ki value of approximately 0.5% (Figure 2). Binding studies conducted in our laboratory reveal a direct interaction with amylase (manuscript in preparation) which we assume occurs through the putative binding domain, although interpretation of the inhibition is complicated because it is known that the guar gum can also bind to starch itself[10].

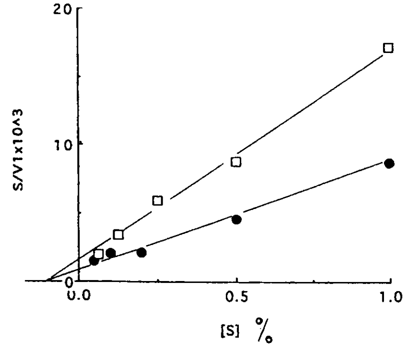

**Figure 2** *Hanes plot of the effect of guar galactomannan on α-amylase catalysed hydrolysis of gelatinised wheat starch. Guar gum was present in reaction mixtures at concentrations of 0 ● and 0.5 □ %*

Amylolysis is a complex process because of the many factors related to the physico-chemical properties of starch but we hope that as well as improving our understanding of the enzymology of starch digestion, our studies may advance knowledge of the mechanism of action of 'starch blockers' and amylase inhibition. This could lead to the development of effective pharmacological agents and novel 'functional foods' for slowing the post-prandial rise in blood glucose and insulin concentrations after carbohydrate-rich meals.

## Acknowledgements

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# HEALTH-PROMOTING FUNCTION OF WHEAT OR POTATO RESISTANT STARCH PREPARATIONS OBTAINED BY PHYSICO-BIOCHEMICAL PROCESS

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## 1 INTRODUCTION

Starch is a storage polysaccharide of most higher plants. Starch granules are composed predominantly of two polysaccharide macromolecules, amylose and amylopectin and remarkably little of any other substances. However the precise nature of both macromolecules varies from source to source e.g. degree of branching, phosphate substitution. The starch granules have a highly organised structure of concentric rings which corresponds with the dimensions of the constituent polysaccharides. It is stated that in plants amylose is present as both amorphous material and a double helix. At lower temperatures, molecular mobility and the overall granule swelling are associated with heat absorption. This process is microheterogeneous within the starch granule. During heating to about 100°C, starch granules are disrupted and form phase separated mixtures of amylose and amylopectin. Under low or no shear conditions, the system is probably bicontinuous in amylose and amylopectin. At high shear, the amylopectin becomes continuous, with inclusion of almost spherical amylose inclusions.

Starch is an organic polymer which is subjected to physical processes in food production or/and thermal and hydrothermal treatment. This processing changes the starch granules and their functional properties. Thus starch present in food has different rate and range of digestion in human gastrointestinal tract. According to the dietary carbohydrate classification,[1,2] there can be three categories of starch characterised by different digestion in the small intestine. Rapidly digestible starch (RDS) is rapidly and completely digested in the small intestine. This is associated with elevated plasma glucose and insulin, therefore it is linked with diabetes, coronary heart disease, and with the ageing process. Slowly digestible starch (SDS), is completely but slowly digested in the small intestine. It has a moderate influence on the plasma glucose and insulin levels but it is the most desirable form of starch from the nutritional point of view. Resistant starch (RS), is not digested in the small intestine and its increasing desirability requires further evaluation.[2] Definition of resistant starch (RS) was determined during the EU programme EURESTA as follows: "RS is a sum of starch and product of starch degradation not absorbed in the small intestine of healthy individuals".[3] With time this definition was supplemented with: "which may be fermented in the large bowel by microflora".[4] The dietary starch is potentially degradable by the action of alpha-amylase. However, certain factors may affect its hydrolysis and *in* *vivo* absorption. According to current nutritional classification, there are four categories of resistant starch in food[1,5]:

$RS_1$ - physically inaccessible, located in plant cell wall, occurring mainly in partly milled cereal grains, seeds or legumes;

$RS_2$ - resistant raw starch granules of B-type, occurring as native starch granules of raw potato, unripe banana, high amylose starch;

$RS_3$ - retrograded or crystalline non-granular starch, occurring in cooked and cooled potato, bread or corn flakes, retrograded amylose starch;

$RS_4$ - chemically modified starches, occurring in food containing chemically modified starches as chemically derivatised starch or linkage-altered dextrins.

The factors mentioned above, related to the starch itself, may be called "intrinsic factors". However, starch digestion is influenced by physiological, "extrinsic factors", including the degree of chewing, concentration of amylase in the gut and transit time through the stomach and small intestine.[2] For these reasons, the rate and extent of starch digestion *in vivo* vary both within and between individuals.

This work was aimed at obtaining preparations from commercial potato or wheat starches with higher amount of resistant starch and at determining by *in vitro* studies their ability to absorb some non-sugar substances, bile acids and cholesterol and to form short-chain fatty acids as the products preventing colon diseases.

## 2 EXPERIMENTAL

### 2.1 Materials

Wheat and potato starches were obtained commercially. For the analytical determinations, the following enzymes were used: thermostable alpha-amylase from *Bacillus licheniformis* (liquid) by Termamyl 120L, NOVO; amyloglucosidase (Fluka 10115, 70,7U/mg solid, from *Aspergillus niger*); alpha-amylase (Sigma A-3176 [EC 3.2.1.1], 28.6U/mg solid, from *porcine pancreas*). Other reagents used in the experiments were as follows: cholic acid (Sigma C-6445), deoxycholic acid (Sigma D-4297), taurocholic acid (Sigma T-9034), L-α-Lecithin (Sigma P-5394), reagent kits for the enzymatic determination of cholesterol (P.O.CH., Gliwice cat. No 178132140). They were prepared in solution of 0.05 mol phosphate buffer, composed of monobasic sodium phosphate and dibasic sodium phosphate at various pH (from 6.0 to 7.6). Glucose was determined using glucose oxidase-peroxidase (GOPOD) and chromogen kits from Cormay following the manufacturer's instructions.

*2.1.1 Experimental RS-preparations.* Resistant starch preparations were obtained from two different commercial starches: wheat or potato. These preparations were made on a laboratory scale by a physico-biochemical process using thermostable alpha-amylase according to the Scheme 1,[6,7] with some modifications.

## 3 METHODS

The resistant starch analysis was carried out using the interlaboratory method 'A',[8] as follows: 100mg of sample was incubated with 500U porcine pancreatic alpha-amylase at 37°C for 16h. The products of hydrolysis were extracted with 80% ethanol and the extract was discarded. Non-digested material was solubilised with 2 mol KOH and hydrolysed with amyloglucosidase (20U). Free glucose was finally analysed using the oxidase-peroxidase glucose test and measured spectrophotometrically at 500nm.

The *in vitro* digestibility of starch preparations was determined using 200U of porcine pancreatic alpha-amylase per 1 gram of sample. The enzyme solution was performed in 0.05 mol phosphate buffer pH 6.9, with the addition of 3 mmol $CaCl_2$. The sample (200mg) was suspended in buffer (20ml). After the addition of the enzyme solution, the alpha-amylolysis was performed for the following time intervals 1, 3, 6, 24 hours, at 37°C with permanent shaking. Before the hydrolysis, 100μl isopropanol was added to the sample to avoid microbial growth during incubation. At the determined time, 1ml sample was immediately mixed in capped centrifuge tubes with 4ml of 95% ethanol in order to inactivate the enzyme. The kinetics of alpha-amylolysis was measured as an equivalent of maltose read from the curve of maltose standard.

The cholesterol absorption was measured by *in vitro* analysis. The sample (100mg) was combined with 2ml emulsion composed of: 1% lecithin, 1.375% sodium salt of deoxycholic acid and 0.225% cholesterol prepared in 0.1 mol phosphate buffer of pH 6.8. Incubation for one hour at 37°C was performed in a shaking water bath. The kinetics of cholesterol absorption by 20μl emulsion was analysed at 10 minute intervals using reagent kits. The results were expressed as per cent of cholesterol adsorbed by sample at each time interval.

The absorption of bile acids (cholic, deoxycholic, taurocholic) was measured by *in vitro* analysis. The sample (100mg) was admixed with 10ml of solution of each bile acid. The solutions were prepared in 0.1 mol phosphate buffer pH 7.6 for each bile acid in 2 μmol/ml concentration. The samples and parallel blank samples were incubated at 37°C for 30 minutes. Centrifugation was performed at 2000g for 5 min. The sample (50μl) was combined with 5ml 70% sulphuric acid and 1ml of freshly-prepared furfural solution (2.3g/l) with careful mixing of the whole sample. Absorbance was measured at 510nm after 80 minutes. The results were expressed as per cent of bile acid absorption.

The estimation of the fermentability of experimental RS-preparations was performed *in vitro* using faeces of rats based on interlaboratory method.[9] Our own modification of this method concerned the preparation of inoculum (1:10 w/v ratio of rat faeces to carbonate-phosphate buffer) and the sample concentration (200mg of preparation in 10ml of inoculum). The fermentation was carried out for 6, 12 and 24 hours and with parallel control-blank sample of the medium. The pattern profile of short-chain fatty acids was determined by the gas chromatography method in a GC-14-Shimadzu Chromatograph with a FID detector and 10% FFAP on Diatomite C-AW-DMCS 100/120 column. The temperatures of column, injector and detector were 140, 200 and 240°C, respectively.

Scanning electron microscope (SEM) analysis was conducted on the native starches, RS-preparations from these starches and these after pancreatic alpha-amylase treatment for 24 hours. After drying by lyophilisation, the samples were mounted on aluminium stubs with double sided adhesive tape, held in a nitrogen stream to remove loosely stuck particles, coated with gold in a JEE 400 vacuum evaporator and viewed in JSM 5200 microscope at 10kV.

The light microscope (LM) analysis was carried out on 10% suspension of sample. It was heated to 90°C and kept for 15min. This water dispersion obtained was quickly smeared out onto an object glass and stained with iodine (0.33% iodine, 0.67% potassium iodine) for 2min. The OLYMPUS BX-60 microscope was used to examine the sample.

The statistical analysis of results was carried out with Statistica ver. 5 (StatSoft, USA) program using correlation and regression analysis.

## 4 RESULTS

The origin of resistant starch preparations were wheat or potato native starches (Figure 1A and Figure 1B). They have different crystallographic patterns (type A and type B), size and shape of granules, and chemical composition. In this work we tried to use these starches in order to obtain the preparations rich in resistant starch. During physico-biochemical process with thermostable alpha-amylase (Termamyl) at the temperature of 90°C (Scheme 1), the experimental resistant starch preparations were prepared.

**Scheme 1** *Preparation of RS from potato and wheat starch*

Suspension of starch
(starch to water; 1: 3.5)
    autoclaving (121°C / 1h)
Starch gel
    cooling (4°C / 12h)
Retrograded gel
    homogenisation
Mixture of gel with Termamyl
(1g of starch + 0.4 ml Termamyl 120 L)
    solubilised in hot phosphoric buffer
    (0.05 M, pH 6.0)
    incubation (90°C / 45min) and mixing
Starchy mixture
    centrifugation (1325xg / 10 min)
RS sediment
    washing:
    distilled water (18°C) 3x
    distilled water (90°C) 2x
    centrifugation (1325xg / 7min)
RS - suspension
    enzyme inactivation (autoclaving 121°C/20 min)
    cooling to room temp.
    centrifugation (1325xg / 10min)
Wet RS
    washing with
    distilled water (90°C)
    centrifugation (1325xg / 10min)
    lyophilisation
    milling to particles<400μm
RS preparation

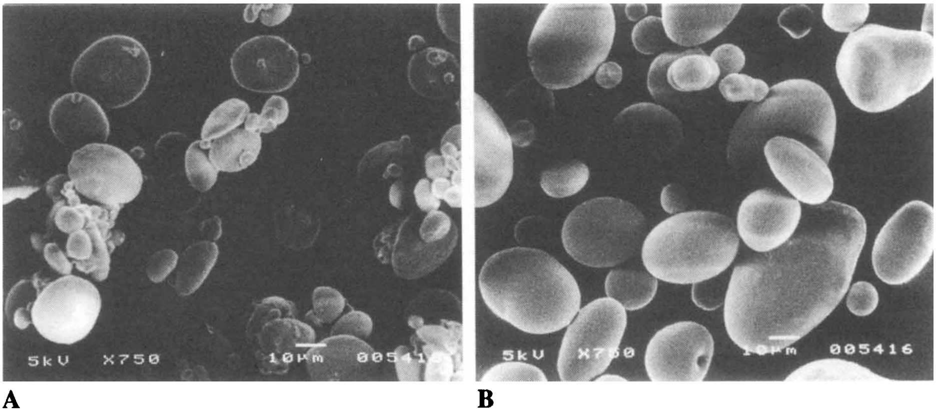

**Figure 1** *Scanning electron micrographs of native starches:* (A) *wheat;* (B) *potato*

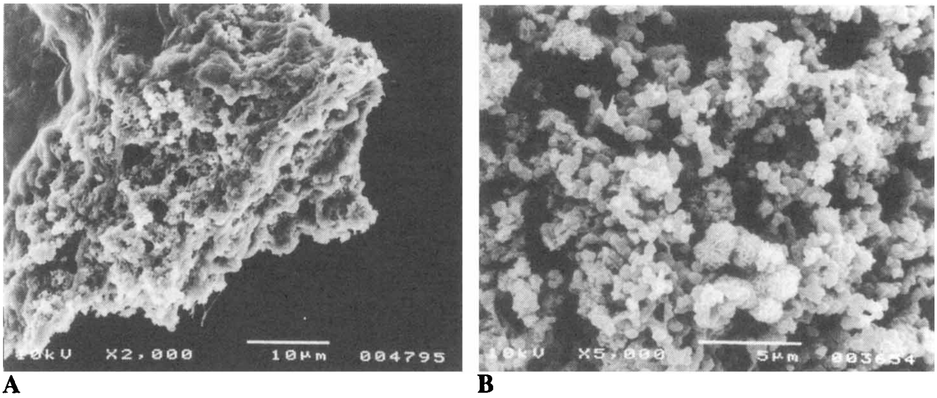

**Figure 2** *Scanning electron micrographs of RS-preparations from starches:* (A) *wheat;* (B) *potato*

The chemical characteristics of these preparations showed that, as well as a large amount of resistant starch (64% for potato and 58% for wheat), they contained certain amounts of mineral and nitrogen components (Table 1). A probable source of a part of these components was Termamyl, which can form a complex during the applied process. Microstructural view was presented on electronmicrographs (Figure 2A and Figure 2B). In these pictures we could observe fine-grained structures characterised by the size of 0.6 - 0.8μm and 0.6 - 1.0μm for potato or wheat, respectively. They had oval or spherical shape and a smooth surface. These structures form larger clusters and show a tendency to associate. Both RS-preparations show a significant development of structure similar to that of a cauliflower, however the wheat one is more dense (Figure 2A). In the potato one, a part of larger spherical forms reveals porous structure (Figure 2B). In pictures of light microscopy stained with iodine, spherical gel structures were obtained (Figure 3A and Figure 3B). They revealed the ability to form single chains, however no iodine complex was observed in any of the preparations investigated.

Taking into consideration digestibility in gastrointestinal tract, RS-preparations were subjected to 24-h hydrolysis by pancreatic alpha-amylase. Their digestibility *in vitro* was compared with that of native starches (Figure 4). The native granules of potato starch are a valuable example of $RS_2$ and in their case, the lowest availability was obtained for alpha-amylase during 24 hours of the enzyme activity. The investigated RS-preparations were attacked by this enzyme to a similar extent. Only in the case of native wheat starch, a considerably higher amount of released saccharides was observed in the tested periods during 24-h hydrolysis. The appearance of hydrolysed RS-preparations in a scanning electron microscope is presented in Figures 5A and Figure 5B. The electronmicrograph of wheat RS-preparation obtained after 24-h hydrolysis showed a clear cohesiveness and roughness of the structure (Figure 5A). In the case of potato RS-preparations, after the same time of hydrolysis, a more delicate structure was observed. Their image was characterised by spatially developed spherical forms which formed grainy, pumice-like clusters (Figure 5B).

**Table 1** *Chemical composition of native starches and their RS - preparations*

| *Samples* | *RS content [% d.m.]* | *Nitrogen [% d.m.]* | *Ash [% d.m.]* |
|---|---|---|---|
| Native starch: | | | |
| wheat | 65.1 ±2.5 | 0.04 ±0 | 0.2 ±0.02 |
| potato | 71.1 ±1.9 | 0.03 ±0 | 0.4 ±0.05 |
| RS - preparation from: | | | |
| wheat | 58.0 ±2.1 | 2.4 ±0.2 | 9.1 ±0.3 |
| potato | 64.2 ±2.8 | 4.3 ±0.3 | 13.2 ±0.7 |

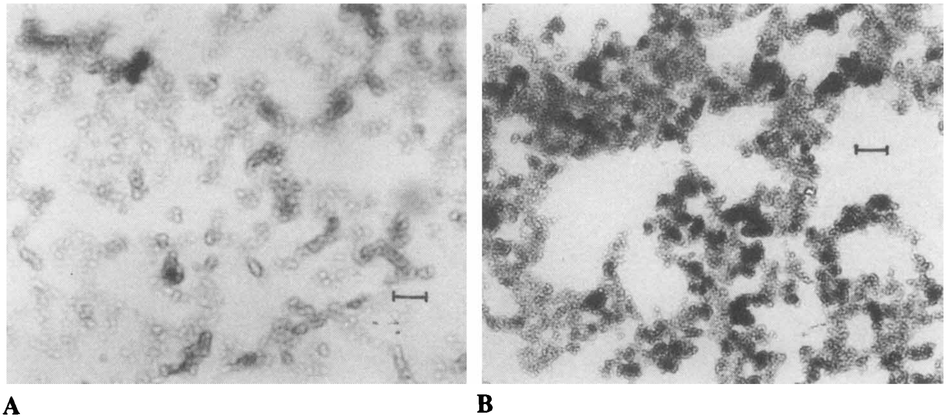

**Figure 3** *Light microscopy (LM) of RS-preparations from starches:* (A) *wheat;* (B) *potato. Bar = 4μm*

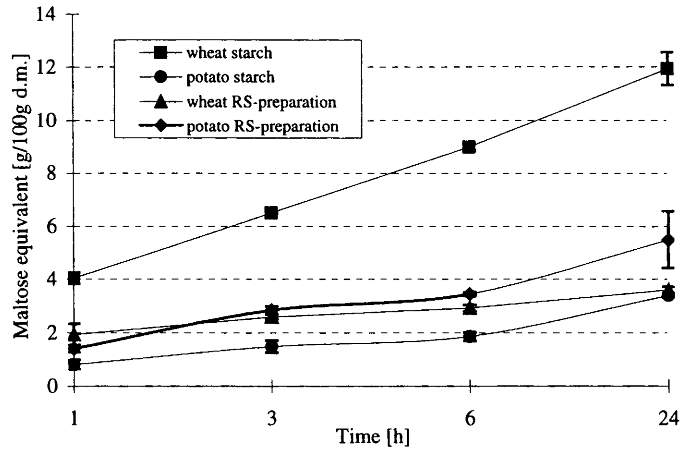

**Figure 4** *The kinetics of hydrolysis of native starches and their RS-preparations by pancreatic alpha-amylase*

The results of the research on the bile acids absorption are presented in Figure 6. They indicated a higher affinity to cholic acid of both RS-preparations analysed, as compared to native starches. It was especially significant in the case of RS-preparations which revealed also a higher absorption of deoxycholic acid. However, both RS-preparations investigated revealed similar absorption of taurocholic acid.

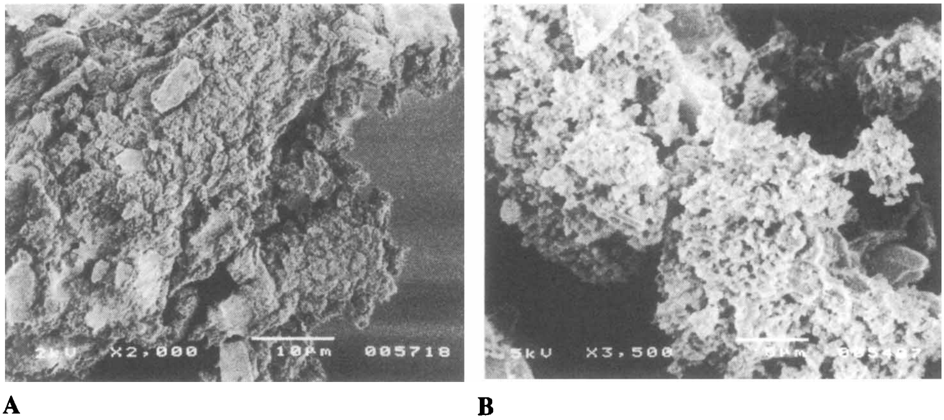

**Figure 5** *Scanning electron micrographs of wheat* (A) *and potato* (B) *RS-preparation after 24-h hydrolysis in vitro with pancreatic alpha-amylase*

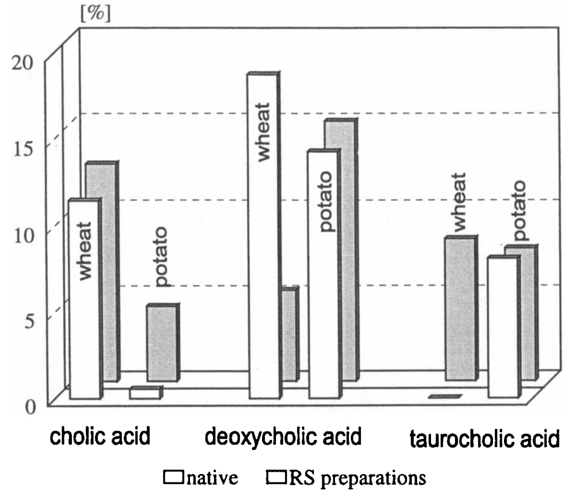

**Figure 6** *Absorption of the bile acids by native starches and their RS-preparations*

The results of the analysis of the cholesterol absorption are shown in Figure 7. The native potato starch ($RS_2$) had the biggest values of absorption during 1 hour of reaction. Measurements in every 10 minutes gave a picture of sinusoid oscillating near 20% of the cholesterol absorption. The same value of absorption (19%) was obtained for wheat RS-preparation, however, the remaining results ranged from 12 to 16%. The most interesting course of the cholesterol absorption was observed for potato RS-preparations during the first 10 min. After 40 min of measurements, the absorption was the lowest as compared to other preparations, but after 1 hour it reached 21%. The same value was obtained for native potato starch ($RS_2$) after 30 min.

The ability to form short-chain fatty acids using wheat and potato RS-preparations was studied by the *in vitro* fermentability method. The changes in pH were observed in medium fermenting for 24 h (Table 2). Basing on the results, it was found a more substantial decrease in pH in the fermenting medium with RS-preparations than in the control. Comparing the differences occurring after 6 h, pH was about 0.1 lower as compared to the control sample. However, after the next 6 h, there was observed a decrease in the pH of medium with RS-preparations, by about 0.2 pH for wheat and 0.35 pH for potato. After 24 hours of fermentation in medium with RS-preparations, there were no differences between potato and wheat preparations. However, the pH values of media with those preparations were different from that of the control samples (by 0.3 pH).

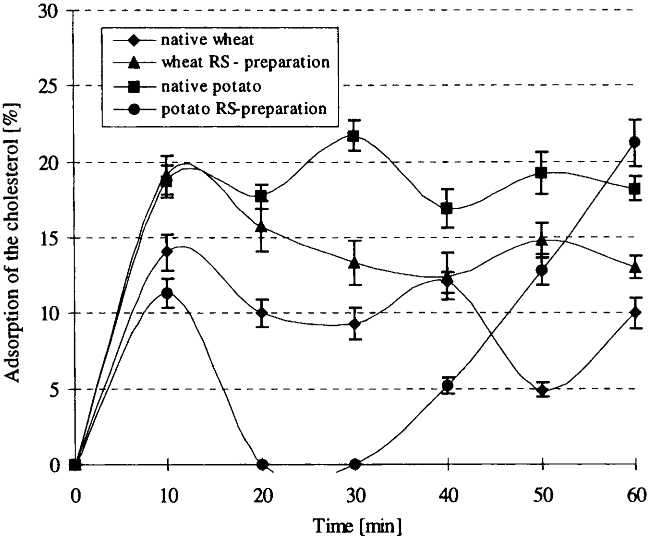

**Figure 7** *Absorption of the cholesterol by native starches and their RS-preparations*

**Table 2** *Changes of pH during in vitro fermentation for 24h*

| *Sample* | *pH of fermentation after* | | |
|---|---|---|---|
| | *6h* | *12h* | *24h* |
| Control | 7.62 ±0.18 | 6.92 ±0.07 | 6.57 ±0.04 |
| RS-preparation from: | | | |
| wheat | 7.51 ±0.10 | 6.74 ±0.06 | 6.26 ±0.05 |
| potato | 7.52 ±0.04 | 6.57 ±0.02 | 6.24 ±0.05 |

It is interesting to know how the experimental RS-preparations can affect the quantitative characteristics of acetic, propionic, butyric, isovaleric and valeric acids (Table 3). It was shown that after 6-h action of fermenting medium, a higher amount of short-chain fatty acids was obtained. For medium time with both preparations investigated, a higher amount (by 50%) of butyric acid was obtained. Wheat RS-preparation caused a higher amount (by 37%) of propionic acid to be obtained, while potato RS-preparation caused a decrease in the amount of acetic acid by about 17%. During further fermentation, it was observed that potato RS-preparation may be additionally a source of fatty acids, e.g. isovaleric and valeric acid after 12 hours of fermentation.

The correlation between pH and the content of total short-chain fatty acids is presented in Figure 8. The correlation coefficient was significant at the level $P< 0.10$.

**Table 3** *Content of short-chain fatty acids after in vitro fermentation of wheat and potato resistant starch preparations*

| *Sample* | *Short-chain fatty acids [mmol/sample*]* | | | | | |
|---|---|---|---|---|---|---|
| | *Acetic* | *Propionic* | *Butyric* | *Isovaleric* | *Valeric* | *Sum of acids* |
| **6h of fermentation** | | | | | | |
| Control | 0.158 ±0.002 | 0.088 ±0.001 | 0.035 ±0.001 | 0.015 ±0.002 | trace | 0.296 ±0.005 |
| RS-preparation from: wheat | 0.162 ±0.001 | 0.121 ±0.001 | 0.069 ±0.005 | 0.024 ±0.002 | trace | 0.376 ±0.020 |
| potato | 0.131 ±0.007 | 0.104 ±0.005 | 0.070 ±0.001 | 0.019 ±0.001 | trace | 0.324 ±0.003 |
| **12h of fermentation** | | | | | | |
| Control | 0.157 ±0.005 | 0.102 ±0.002 | 0.059 ±0.004 | 0.023 ±0.003 | trace | 0.341 ±0.010 |
| RS-preparation from: wheat | 0.156 ±0.004 | 0.120 ±0.002 | 0.082 ±0.002 | 0.025 ±0.003 | trace | 0.383 ±0.004 |
| potato | 0.128 ±0.002 | 0.095 ±0.003 | 0.081 ±0.003 | 0.046 ±0.004 | 0.039 ±0.003 | 0.389 ±0.020 |
| **24h of fermentation** | | | | | | |
| Control | 0.157 ±0.022 | 0.111 ±0.011 | 0.077 ±0.006 | 0.030 ±0.004 | trace | 0.375 ±0.043 |
| RS-preparation from: wheat | 0.164 ±0.008 | 0.119 ±0.014 | 0.099 ±0.014 | 0.035 ±0.004 | trace | 0.417 ±0.016 |
| potato | 0.106 ±0.001 | 0.090 ±0.003 | 0.091 ±0.008 | 0.061 ±0.005 | 0.081 ±0.013 | 0.429 ±0.028 |

* Sample: 200 mg preparation RS in 10 ml inoculum; Inoculum: rat faeces and nutritive buffer (carbonate-phosphate) in ratio 1:10 (w/v).

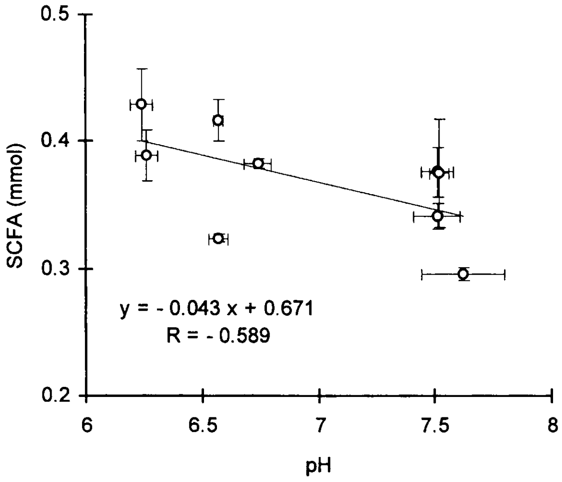

**Figure 8** *Relationship between pH of fermenting medium using RS-preparations and total short-chain fatty acids. P<0.10*

## 5 DISCUSSION

In this study, structural and physico-chemical changes in wheat and potato starches were observed. Application of such physical processes as autoclaving and then retrograding of gel structures with thermostable alpha-amylase indicated a strong influence on the native structure of both starches investigated. Apart from granular potato starch ($RS_2$), also RS-preparations, obtained after *in vitro* hydrolysis, were characterised by similar resistance to the activity of pancreatic alpha-amylase. Retrograded starch is composed of ordered structures which can be divided into two parts: (a) double helical amylose chains which form crystallographic structure of type B, and (b) amorphous part which is not accessible to pancreatic alpha-amylase.[10,11] The results obtained through the scanning electron microscopy and light microscopy indicate that, probably, this part consists of oligomers or a complex of 0.6–1.0μm size, which do not exhibit an affinity to iodine. It is stated that resistant starch recovered from exhaustive pancreatic alpha-amylase digestion had a chain length distribution between 15 and 80 glucose units,[12] but decreased to 15-65 glucose units when subsequently digested by Termamyl.[13] The fraction hydrolysed by thermostable alpha-amylase at 85°C is very likely an amorphous fraction of RS, which is hardly hydrolysed at lower temperatures.

It was interesting to obtain the information whether and how the obtained RS-preparations can play a potential role in helping to maintain or improve good health. It was our purpose to investigate the possibilities of their interaction with some non-saccharide but hydrophobic substances, such as bile acids or cholesterol, which are important in maintaining good health especially for elderly people or those after surgery, e.g. cholecystectomy. The previous studies concerning the microstructure and functional properties confirmed that these RS-preparations reveal similar hydrophilic and lipophilic properties,[14] but the crystallographic characteristics do not correspond with X-ray spectrum of either type A or type B. In this study, it was found a higher absorption of the bile acids analysed by RS-preparations. Since the role of RS and its ability to decrease the cytotoxicity of faecal water by inhibition of cholate conversion and decrease of soluble deoxycholate[15] are greatly emphasized, it can be assumed that also potato and wheat RS-preparation might reveal those properties. For those preparations, a significant affinity to deoxycholic acid was obtained, similar to buckwheat fibre, especially to acid detergent fibre.[16] In the studies conducted on volunteers consuming high-RS and low-RS diets, the authors maintained that the faecal concentrations of total and secondary bile acids were significantly lower during the high-RS than during the low-RS period.[17] The observed decrease reached 30% for total and 32% for secondary bile acids.

The investigations of the cholesterol absorption by RS-preparations showed a completely different kinetics of interaction for each of the preparations. The maximum amount (about 20%) of adsorbed cholesterol was obtained for wheat RS-preparation after 10 min of reaction, while potato RS-preparation adsorbed similar amount of cholesterol already after 1h. Previous comparisons of the ability of wheat preparations of different RS content to adsorb cholesterol proved that the preparation analysed in the present work reached the highest degree of absorption.[18]

The resistant starch compared with dietary fibre is a quantitatively important source of indigestible carbohydrates and starch, not dietary fibre, is the major substrate for fermentation in the human colon.[19] The main SCFA produced during colonic fermentation of indigestible carbohydrates are acetic, propionic and butyric acids. Butyric acid is preferred as the energy source by the mucosal cells in the colon[20], and has been shown to suppress the growth and doubling time of neoplastic cells.[21] Propionates are discussed in relation to their effect on the metabolism of carbohydrates and cholesterol, which are adsorbed and metabolised in the liver. In rats, a dietary supplementation with propionate appears to lower the serum and liver cholesterol.[22] Based on the fermentability of investigated RS-preparations by *in vitro* studies, a decrease in pH was found during 24 hours. Both preparations can be a source of butyric acid, considering the fact that it was generated already after 6 hours of fermentation. In general, the sum of fatty acids increased by 13% after 24 hours of fermentation. However, correlation coefficient between pH and total SCFA reached $r = -0.589$ and was lower than that obtained in caecum of rats fed starches of various origin.[23]

Summarizing the results of the presented studies, it can be stated that obtained wheat and potato RS-preparations, being a complex: resistant starch-minerals-nitrogen, can play a health-promoting function and be a component of so-called "functional food". The fibre-like character of RS-preparations suggests their potential preventing effect on the colon diseases.

## Acknowledgements

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# STARCH BIOSYNTHESIS IN THE SMALL GRAINED CEREALS: WHEAT AND BARLEY

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## 1 INTRODUCTION

A range of plants have been used as model systems for the investigation of starch biosynthesis because of either their diploid genetics (maize and rice), their suitability for use in mutagenesis programs (*Arabidopsis* and *Chlamydomonas reinhardtii*), their transformation efficiency (potato) or because of their historical use as a research model for genetic and biochemical studies (pea). Of these species, maize, rice and potato are economically important sources of starch while pea starch is of comparatively minor economic interest. In recent years, wheat and barley starch biosynthesis has attracted increasing interest because of the economic and social importance of these crops and because of the increased awareness of the importance of starch in determining the end-use properties products derived from these grains.

Barley is a very useful model system for studies of starch biosynthesis given its diploid nature and ease of transformation. Given the similarity in granule structure between wheat and barley, barley serves as an ideal model system for studying wheat starch biosynthesis. Two factors have slowed the study of wheat starch biosynthesis. Firstly, the hexaploid nature of the genome does not allow the ready identification of mutants through screening for variation in starch structure or properties. Secondly, the transformation efficiency of wheat has been much lower than model species. However, in recent years, solutions to both of these problems have been at least partially achieved.

The starch is produced in the amyloplast of the endosperm by the coordinated action of a number of enzymes that include ADP-glucose pyrophosphorylase (EC 2.7.7.27), starch synthases ( EC 2.4.1.21), branching enzymes ( EC 2.4.1.18) and debranching enzymes ( EC 3.2.1.41 and EC 3.2.1.68 ).[1,2] Each of these enzymes is present in the cereal endosperm in multiple isoforms. Some of these enzymes can be detected within the starch granule (GBSS, SSI, SSII, BEIIa, BEIIb)[3–6] and some are entirely stromal (plastidic ADP-glucose pyrophosphorylase, SSIII, BEI, isoamylase, pullulanase).

Unresolved questions in starch biosynthesis are why each of these enzymes is present in numerous isoforms in the cereal endosperm and what are the specific contributions of each enzyme to the synthesis of starch. Figure 1 shows one view of the starch biosynthesis pathway in the cereal endosperm. This paper will discuss the evidence for the presence of multiple forms of the starch biosynthesis pathway and the respective roles of the isoforms, and will present data showing the conservation of structure of genes between the dicot *Arabidopsis thaliana* and wheat.

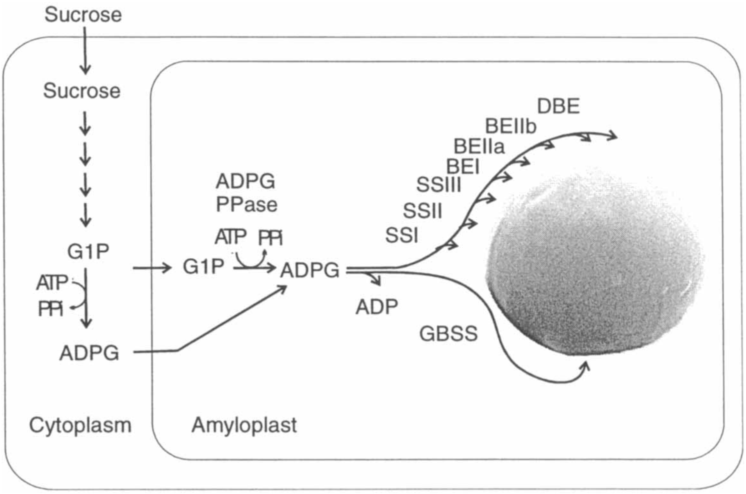

**Figure 1** *Proposed pathway for starch biosynthesis scheme in the cereal endosperm*

### 1.1 ADPglucose Pyrophosphorylase

Higher plant ADPglucose pyrophosporylases are composed of two subunit types, a large and a small subunit. In barley and maize there is strong evidence demonstrating that ADPglucose pyrophosphorylase is present in both the cytosol and the amyloplast (see Figure 1), with the bulk of the activity in the cytosol.[7] The cytosolic and amyloplastic enzymes are thought to be encoded by separate sets of large and small subunit genes. Mutations associated with ADPglucose pyrophosphorylase genes that reduce starch content (in maize, brittle-2 and shrunken-2) are found to be lesions within the large and small subunit genes expressed in the cytoplasm. It is likely that the distribution of enzyme activity in wheat is very similar to barley however this hypothesis has not been tested experimentally. The cytoplasmic location of ADPglucose pyrophosphorylase and the demonstration of its importance to starch biosynthesis, indicates that an active ADPglucose transporter is present in the amyloplast membrane.

### 1.2 Starch Synthases

In wheat and maize at least 4 classes of starch synthases are found, GBSS, SSI, SSII and SSIII.[8–13] The manipulation of GBSS in wheat has resulted in significant developments over the past decade. First, the combination of null alleles in each of the GBSS homologous loci has led to the production of zero amylose "waxy" wheats.[14] Secondly, the significance of the null 4A genotype in underpinning Udon noodle quality has been important in defining a major quality parameter.[15] Recently, waxy barley, lacking the predominant endosperm GBSS gene, has been shown to contain higher amylose contents than expected for a waxy mutant (about 8%) with starch granules that stain strongly blue with iodine being present in the aleurone and adjacent cells.[16] This may represent expression of GBSS from genes other than the major endosperm genes and the recent cloning of a second GBSS gene, located on chromosomes 2AL, 2B and 2D and expressed in the pericarp and other tissues but not in the endosperm,[17] provides strong support for this view.

The SSI homologous genes are located on chromosomes 7AS, 7BS and 7DS of wheat and encode 75 kDa proteins that are distributed between the starch granule and the soluble phase.[8] No mutants in any higher plant lacking SSI have yet been identified and so the role of SSI in starch biosynthesis remains undefined. In wheat, proteins encoded by SSII genes are present in the starch granule and have apparent molecular weights of 100, 108 and 115 kDa.[3] Yamamori[18] has recently described the generation of a triple null line lacking the 100, 108 and 115 kDa proteins in the starch granule and preliminary characterisation shows that starch granule morphology and starch structure are altered in the triple null line. The triple null line shows altered granule morphology, elevated amylose content (35%), an amylopectin with a shorter chain length distribution, and reduced granule crystallinity. The wheat homologue of the maize SSIII gene (*du1*) gene has recently been cloned and characterised and the homologous loci are found on chromosome 1.[10] No SSIII null lines have been described in wheat or barley, in maize the du1 mutation leads to an altered endosperm appearance and starch with altered chain length distribution.

Mutational studies have clearly established that GBSS is essential for amylose biosynthesis and that mutations in SSII and SSIII lead primarily to alterations in the synthesis of amylopectin. The reasons for the differences in the substrates selected by each isoform for extension, and the structures of the products made by each isoform, are unclear and the dissection of the biosynthetic genes through site directed mutagenesis provides a powerful mechanism to investigate structure/function relationships in the starch synthase genes.

### 1.3 Branching Enzymes

In plants, two classes of genes, known as BEI and BEII, encode starch branching enzymes. In wheat, Morell et al[19] partially purified the branching enzymes from the endosperm and showed that there were two classes of BE polypeptides present, designated BEI and BEII. The BEI genes were shown to be located on the long arm of chromosome 7.[19,20] Multiple copies of the BEI gene are present in the wheat genome, located as a tandem array.[20]

There is strong evidence in the cereals to demonstrate that the BEII class contains two genes, known in maize as BEIIa and BEIIb.[21] *Arabidopsis* contains two BEII genes however these do not appear to be direct analogues of BEIIa and BEIIb as they do not cluster into these grouping in sequence comparisons and show divergent gene structures.[6] In barley, BEIIa and BEIIb homologues have been cloned at the genomic and cDNA levels and fully sequenced at the cDNA level.[22,23] A wheat BEIIa type gene has been cloned and sequenced by Rahman et al [6] and it had been shown that this gene encodes the BEII form characterised by Morell et al.[19] Homologous genes encoding BEIIa and BEIIb are located on the long arm of chromosome 2. in wheat.

The individual roles of the three isoforms of BE found in the cereal endosperm have yet to be defined. Down regulation of BEI alone in potato and in wheat by antisense technology leads to only minor effects on starch structure and properties[24,25] and an impact of BEI down regulation is only seen in BEII deficient backgrounds.[26] The role of BEIIa in the cereal endosperm is unknown. However, in dicots, high amylose phenotypes are only obtained when BEII is down regulated, and in the cereal endosperm, high amylose (>50%) are only obtained when BEIIb is down regulated. The differences in the expression or properties of these isoforms that underpin the differential roles of the enzymes remains a subject for further research.

### 1.4 Debranching Enzymes

In maize, the *Sugary-1* mutation responsible for sweet corn has been demonstrated to be caused by a lesion in an isoamylase type debranching enzyme gene, *Su1*.[27] A similar mutation is known in rice. The requirement for debranching enzyme involvement in starch biosynthesis is clearly established by the analysis of mutations in a range of species however the specific roles of debranching enzymes in the starch biosynthesis process remain unclear.[2]

## 2 MATERIALS AND METHODS

### 2.1 Isolation of Clones from Genomic and cDNA libraries

Clones containing SSII and isoamylase were isolated from a genomic library prepared from *Triticum tauschii*, var strangulata, accession number CPI 110799. The library was screened using a PCR-derived fragments of the wheat cDNAs for these genes.

*DNA sequencing and analysis*

DNA sequencing was performed using the automated ABI system with dye terminators as described by the manufacturers. DNA sequences were analysed using the GCG suite of programs.

## 3 RESULTS AND DISCUSSION

### 3.1 Structure of Wheat Starch Biosynthetic Genes

The sequences of chromosomal DNA encoding GBSS, SSI, SSIII, BEI and BEIIa have been previously reported.[6,8,10,28] In the current research, genes encoding isoamylase and SSII have been cloned and sequenced. Figure 2 shows the exon/intron structures of genes cloned to date from the wheat starch biosynthesis pathway. In each case the genomic DNA sequence has been isolated from *Aegilops tauschii*, the D-genome donor of wheat.

It is striking that that there is little conservation of gene structure among the four starch synthase isoforms, suggesting that they diverged relatively early in the evolution of higher plant starch biosynthesis. At the amino acid level, a series of eight regions in the C-terminal domain has been conserved amongst starch synthases[10,11] indicating that there has been strong conservation of functional elements across the isoforms. The key regions defining the differences in substrate specificity and granular localisation of the starch synthases have yet to be defined. BE gene structures have been determined for BEI[28] and BEIIa[6] from wheat, and BEIIb from maize.[29] Comparison of these structures suggests that BEI and the BEII genes diverged earlier than BEIIa and BEIIb diverged. While BEIIa and BEIIb genes from cereals can be readily identified and assigned to classes through sequence comparisons, both BEIIa and BEIIb genes share an identical structure of 22 exons, with highly conserved locations of the exon/intron boundaries.

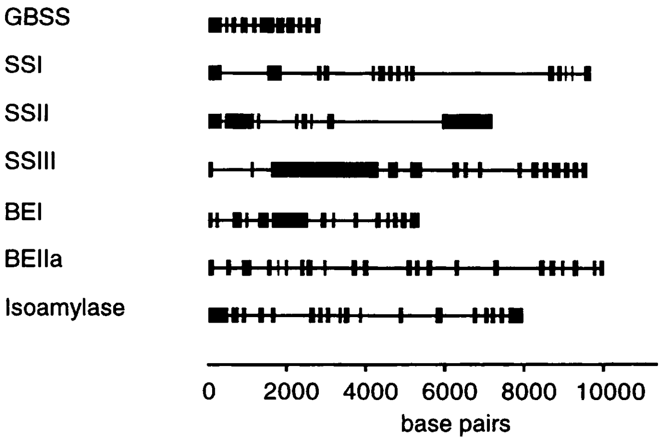

**Figure 2** *Exon/intron structures of wheat starch biosynthetic genes. Exons are shown as filled boxes and introns by a line. The structure shown encompasses the coding region of the gene, the 5' and 3' untranslated regions are not shown*

### 3.2 Comparison of Wheat and Arabidopsis Starch Biosynthetic Genes

Figure 3 shows comparisons of the exon structures of SSIII, BEIIa and isoamylase from wheat and Arabidopsis. This comparison indicates that the structures of these genes are essentially conserved across both dicots and monocots, adding further weight to the suggestion that the genes encoding isoforms of the starch biosynthetic genes diverged early

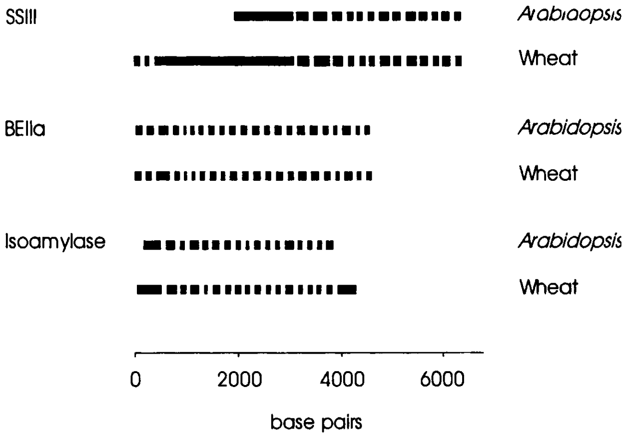

**Figure 3** *Comparison of the exon structures of Arabidopsis and wheat starch biosynthetic genes; SSIII, BEIIa and isoamylase. The Arabidopsis data was obtained from Genbank accessions AC007296, AL162506 and AF002109 respectively. The filled boxes show exon lengths, each intron has been replaced by a 100 base pair gap in order to facilitate comparison of exon lengths*

in higher plant evolution. The comparison of SSIII genes shows that while there are marked differences at the N-terminal end of the genes, the structure of the remainder of the gene is highly conserved between *Arabidopsis* and wheat. The functional significance of the extended N-terminal region of the wheat gene is unknown, although it appears that the wheat and *Arabidopsis* sequences are typical of a range of moncots and dicots respectively. Comparison of the isoamylase genes from wheat and *Arabidopsis* and wheat indicates that the structural organisation of this gene is also conserved across monocots and dicots.

### 3.3 Chromosomal Locations of the Wheat Genes

The wheat starch biosynthetic genes have been placed in the wheat genome (Figure 4) by the analysis of defined genetic stocks, either nullisomic/tetrasomic lines[30] or deletion lines. A cluster of wheat starch biosynthetic genes (GBSS, SSI, SSII, isoamylase) are found on the short arm of chromosome 7 while the BEIIa and BEIIb are found on chromosome 2 and SSIII on chromosome 1.

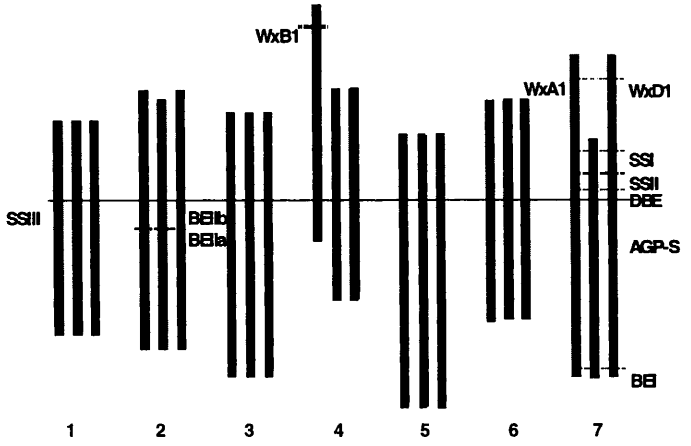

Figure 4 *Chromosomal locations of wheat starch biosynthetic genes. Chromosomal locations have been determined using the wheat chromosome engineered lines of Sears and Miller*[30]

## 4 CONCLUSION

The cloning and characterisation of the wheat starch biosynthetic genes will underpin the modification of wheat starch biosynthesis through either the isolation of natural mutants or through the use of genetic engineering approaches.

### 4.1 Identification of Natural Mutants

The identification of natural mutations in the GBSS genes has lead to the development of waxy wheats[14,31] and lines lacking one or two of the GBSS alleles. Yamamori et al[18] recently reported the generation and preliminary analysis of a line lacking the 100, 108 and

115 kDa starch granule proteins encoded by the SSII genes. The identification of natural or induced mutations in each genome in wheat will lead to the further development of useful genetic stocks for the manipulation of wheat starch biosynthesis. While genetic variation in the seed storage protein component of the wheat grain has been widely exploited to tailor the properties of wheat flour to specific end-uses, similar targeting and exploitation of variation in the wheat starch biosynthetic genes is in its infancy because of the technical complexity involved in identifying variation. Some simple calculations indicate the scope for the development of novel germplasm. If the complexity is constrained by only considering homozygous states, the range of complexity possible remains staggering. For example, for the seven genes shown in Figure 2, eight states (AABBDD, --BBDD, AA--DD, AABB--, ----DD, AA----, --BB--, ------) are possible for each gene, leading to eight to the power of seven ($8^7$) combinations (2,097,152). While many of these combinations may yield starches that are essentially wild type in structure and functionality, the experience with the single null 4A alleles of GBSS (leading to a marked starch functional phenotype, see Zhao et al,[15]) indicates that unexpected and useful phenotypes may arise not only from triple nulls but also from single and double null states.

## 4.2 Genetic Engineering Approaches

The possibility of generating sufficient numbers of transgenic wheat or barley plants to test hypotheses regarding the roles of specific genes is now feasible, given recent advances in transformation technology. General targets for genetic engineering are (a) the suppression of the activities of starch biosynthetic enzymes using technologies such as antisense, co-suppression or duplex mRNA-mediated suppression, or (b) the over-expression of starch biosynthetic or starch modifying genes from heterologous sources. A key technical challenge in the utilisation of genetic engineering technologies will be to identify and deploy gene expression elements that provide stable expression of the transgenes. The key hurdle to the utilisation of the technology remains the attitude of a range of societies to the commercial use of the technology. However, genetic engineering will in the short term yield much useful knowledge about the starch biosynthetic process through the ability to target specific alterations in the expression of individual genes in the pathway.

The cloning and characterisation of the genes from the wheat starch biosynthetic pathway provides key tools for the manipulation of starch biosynthesis by either mutational or genetic engineering routes in both wheat and barley that will lead to an enhanced knowledge of the roles of individual isoforms of starch biosynthetic enzymes.

## Acknowledgements

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## Abbreviations

SS: starch synthase; GBSS: granule bound starch synthase; BE: branching enzyme.

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# TRANSPORT OF METABOLITES INTO AMYLOPLASTS DURING STARCH SYNTHESIS

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## 1 INTRODUCTION

The biosynthesis of starch depends upon a supply of ADPglucose which is the immediate soluble precursor for the production of amylose and amylopectin. In storage tissues such as developing seeds and tubers, starch is synthesised within specialised plastids termed amyloplasts. Like all plastids, these organelles are surrounded by a double envelope. The outer envelope provides the selective barrier to the import of large molecules, particularly proteins, into the organelle. The inner envelope provides a selective barrier to soluble, small intermediary metabolites. The production of starch in heterotrophic storage tissues is dependent upon the supply of sucrose, via the phloem, from the leaf. Once sucrose has been imported into the cytosol it is broken down via sucrose synthase, UDPglucose pyrophosphorylase and fructokinase to give rise to phosphorylated sugars. In order for starch to be made within the amyloplast, precursors must be imported from the cytosol. As the substrate for granulebound or soluble starch synthases is an activated sugar, ADPglucose, then both the sugar moiety and the mechanism and source of energisation need to be considered. The focus of this paper will be the forms in which carbon and energy are supplied to the amyloplast which lead to the provision of ADPglucose within the organelle, and their influence on starch synthesis.

## 2 AMYLOPLAST METABOLITE TRANSPORT

### 2.1 Entry of Carbon into Amyloplasts

The biochemical characterisation of metabolite transport into plastids has been studied much more extensively in chloroplasts in comparison to amyloplasts. Nonetheless the combination of improved fractionation procedures for heterotrophic tissues, coupled with a molecular characterisation of gene expression has revealed a picture which implies that there is probably greater diversity of metabolite movement across the inner envelope of amyloplasts and that there may be significant differences between species[1]. This is hardly surprising as chloroplasts are net exporters of carbohydrate, whilst amyloplasts are net importers. By far the best characterised transporter in any plastid is the triose phosphate translocator[2], which has been extensively studied in green tissues. This inner envelope protein has 12 membrane spanning domains and catalyses the stoichiometric counter exchange between triose phosphates (dihydroxyacetone phosphate or glyceraldehyde 3-phosphate), phosphoglyceric acid and inorganic orthophosphate. In chloroplasts it functions to export photosynthate to the cytosol. Whilst amyloplasts are capable of catalysing a similar counter-exchange[3], there is disagreement over whether the same gene is expressed in green and non-green tissues[4,5]. However there is now ample evidence from labelling studies[6] and investigation of the enzymic capacity of amyloplasts[7] to render the likelihood that import of triose phosphate is necessary to sustain starch synthesis as negligible.

Consequently, other metabolites have been considered as cytosolic precursors for starch synthesis in amyloplasts, principally hexose phosphates. A number of groups working with purified preparations of plastids have shown that, depending on the species, glucose 6-phosphate (Glc6P) or glucose 1-phosphate (Glc1P) can enter amyloplasts and support starch synthesis. Almost invariably the synthesis of starch has depended on the simultaneous supply of ATP (see [1] for a review). Recently Kammerer *et al*[8] have cloned cDNAs coding for a Glc6P/Pi translocator from a number of species. The authors were successful in heterologous expression of the cDNA for the pea root plastid protein and showed that it has a higher affinity for Glc6P than any other phosphorylated intermediate, and shares only 36% identity to the triose phosphate transporter of leaves. Interestingly, there is little sequence similarity to the inducible Glc6P/Pi exchanger of *E. coli*. Whilst the pea root protein is clearly specific for Glc6P, it has been demonstrated that the hexosephosphate transporter from wheat endosperm amyloplasts is able to catalyse the counter-exchange of either Glc6P or Glc1P with Pi[9]. Nonetheless, in contrast to the situation for activated sugars which will be discussed shortly, it appears that all amyloplasts can import hexose phosphates.

## 2.2 Provision of ATP for Starch Synthesis

As outlined above, amyloplasts require uptake of ATP to energise starch synthesis. ATP cannot penetrate biomembranes, and all plastids analysed so far have been shown to possess an inner envelope protein that mediates ATP/ADP exchange at some stage during their development. Whilst mitochondria also possess an adenylate translocator which, in essence, catalyses the same activity, it is now clear that there are substantial differences between the two. The mitochondrial transporter is very strongly inhibited by compounds like bongkrekic acid and carboxyatractyloside, whereas the plastidic protein is not[1], and there is minimal immunological cross identity. The sequence of the plastidic ATP transporter has closest similarity (66%) to the homologue found in the gram-negative bacterium *Rickettsia prowazekii*[10], and has subsequently been expressed functionally in *E. coli*[11].

What has proved particularly intriguing about the amyloplast adenylate transporter, is the considerable influence it exerts over starch synthesis. The cDNA for the Arabidopis ATP/ADP transporter (AATP) was introduced into the genome of potato. Transgenic plants were also made in which the activity of the endogenous gene was reduced[12]. It transpires that the activity of the plastidic AATP has a profound influence over starch synthesis. In comparison to control lines, tubers of plants in which the activity had been decreased showed a significant decrease in starch content and a decrease in the amylose:amylopectin ratio. Even more striking was the observation that an increase in AATP activity in transgenic lines led to a substantial increase in yield, and an increase in the ratio of amylose to amylopectin. This clearly demonstrates that the activity of the plastidic AATP has a marked effect on starch synthesis in potato tuber amyloplasts, probably exerting its effect by controlling the size of the ADPglucose pool within the amyloplast, since ATP is a substrate for ADPglucose pyrophosphorylase (AGPase) which catalyses the synthesis of the starch precursor. Storage tissues of potato and other species possess several isozymes of starch synthases, which can be broadly classed into the granule bound (GBSS) and soluble starch (SSS) synthases. SSS isoforms are responsible for amylopectin elongation and have a higher affinity for ADPglucose than GBSS, which is responsible for amylose synthesis[13]. Altering ATP supply affects GBSS disproportionately and produces a greater change in amylose content compared to amylopectin, as well as influencing the overall rate of starch synthesis.

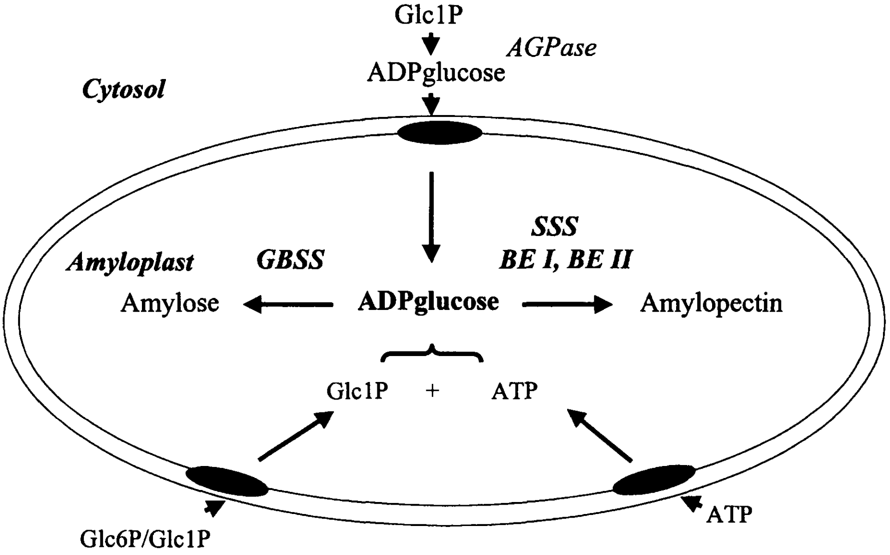

**Figure 1** *Labelling of amyloplast envelope polypeptides with $^{32}$P-azido ADPglucose. Amyloplast envelopes were purified from wheat endosperm and incubated with either 25μM $^{32}$P-azido ATP or 100μM $^{32}$P-azido ADPglucose and cross-linked under UV light. Protein was stained with Coomassie Blue*

## 2.3 Transport of Activated Sugar from the Cytosol

Until recently, AGPase the enzyme which catalyses the synthesis of ADPglucose, had been thought to be confined to plastids in all species.

$$\text{Glc1P} + \text{ATP} \underset{AGPase}{\leftrightarrow} \text{ADPglucose} + \text{PPi}$$

Studies of the endosperm of monocotyledenous species suggest that, in contrast to dicots, the bulk of AGPase activity is associated with the cytosol[14, 15, 16]. The enzyme is a heterotetramer made of large and small subunits. Immunobiochemical studies of subcellular fractions showed that both large and small subunits were detected in amyloplast preparations. Additionally, larger forms of each subunit were found to be associated with the cytosolic compartment. Thorbjørnsen et al[17] have shown that in barley, the small subunit which contains the catalytic site, is coded for by a single gene which is differentially spliced to give rise to two isoforms. One exon, which is transcribed primarily in leaf tissue, contains the N-terminus and a sequence coding for the transit peptide which directs the subunit to plastids. A second exon, which is transcribed in endosperm tissue contains an alternative N-terminal sequence which lacks any targeting sequence. The remaining 7 exons are common to both cytosolic and plastidic isoforms. As a consequence of this, AGPase in leaf tissue is localised exclusively in chloroplasts, whereas in endosperm activity is present in both compartments, with 90% or more found in the cytosol. Recent studies of wheat endosperm from our own laboratory have confirmed that there are distinctive cytosolic and plastidic forms of AGPase in this tissue and that the cytosolic subunits appear at around 10 days post anthesis, at the onset of the major period of grain filling.

Given that it seems likely that the bulk of the ADPglucose synthesised in the developing endosperm of the gramineae, is made in the cytosol this necessitates the import of the activated sugar into the amyloplast. The AATP referred to earlier is highly specific for ATP and ADP and will not readily translocate sugar nucleotides[11]. We have recently purified an ADPglucose transporter from amyloplasts of developing wheat endosperm. The protein has a molecular mass of 38 kDa compared with 62 kDa for the AATP. It also has broader specificity for adenylates and will counter-exchange ATP, ADP, AMP and ADPglucose. The two proteins can be visualised and distinguished from one another by cross-linking with $^{32}$P-azido-labelled adenylates (Figure 1). After pre-incubation and UV-induced crosslinking with either $^{32}$P-azido-ATP or $^{32}$P-azido ADPglucose, envelope proteins of wheat amyloplasts were separated by one-dimensional SDS electrophoresis. Autoradiographs of the membrane proteins crosslinked with azido-ATP demonstrate the presence of two proteins at 62 and 38 kDa. However crosslinking with azido-ADPglucose revealed interaction only with the 38 kDa protein. It can therefore be concluded that endosperm amyloplasts possess two types of adenylate translocators. The first catalyses the classical counter-exchange of ATP with ADP. By contrast the second has much broader specificity and will transport ADPglucose as well as ATP.

The observation that most of the AGPase activity in cereal endosperm is cytosolic emphasises two important aspects of the ADPglucose transporter. First, during grain filling, it is probably the major portal of entry of carbohydrate (as activated sugar) into the organelle for starch synthesis. Second, its activity may contribute significantly to the determination of the amyloplast ADPglucose content and as a consequence, as discussed earlier, would have a bearing not only on the rate of starch synthesis, but also the proportions of amylose to amylopectin.

## 3 CONCLUSIONS

Starch synthesis and the synthesis of amylose and amylopectin are strongly influenced by the pool of ADPglucose within amyloplasts where starch is made. This pool, in turn, is a function of the import of the precursors of starch synthesis. In storage tissues of dicotyledenous species, the synthesis of ADPglucose in amyloplasts draws on the import of hexose phosphates and ATP. In addition, it appears that endosperm of graminaceous species possess an alternative mechanism, whereby ADPglucose made in the cytosol is imported by a separate transporter. These tissues therefore possess the capacity for either .

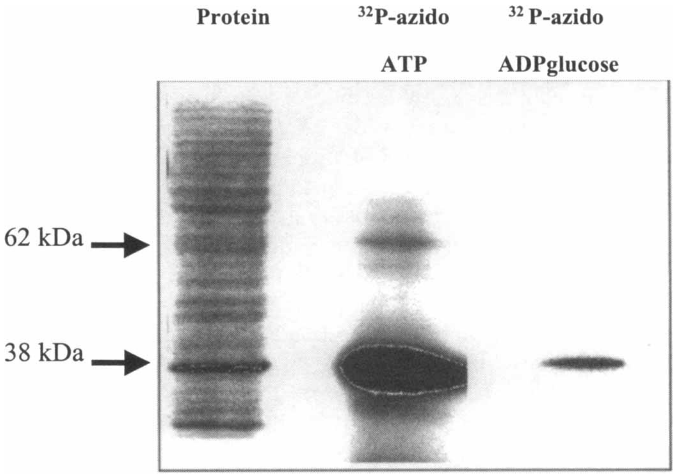

**Figure 2** *Pathways of starch synthesis in amyloplasts. Transporters on the inner envelope are shown by a solid elipse. GBSS = granule bound starch synthase, SSS = soluble starch synthase, BE = branching enzyme, AGPase = ADP glucose pyrophosphorylase*

route (Figure 2), although the localisation of AGPase suggests that the import of hexosephosphates and ATP into amyloplasts may be less important in controlling starch synthesis in cereals

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# THE SYNTHESIS AND DEGRADATION OF STARCH IN *ARABIDOPSIS* LEAVES: THE ROLE OF DISPROPORTIONATING ENZYME

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## 1 WHY USE *ARABIDOPSIS* TO STUDY STARCH METABOLISM?

The study of starch is normally associated with the starch-storing organs of commercially important crop species. However, starch accumulates in a large number of plants and in a wide variety of tissues in these plants. While there are notable differences in the fine structure and composition of starches from different sources, many of the fundamental aspects of starch metabolism are likely to be similar in all higher plants. The *Arabidopsis* leaf is an excellent model system in which to study starch biosynthesis for a number of reasons. First, starch accumulates in these leaves at a high rate. Roughly half of the carbon assimilated through photosynthesis is stored in the leaf as starch[1] and, on a gram-for-gram basis, *Arabidopsis* leaves synthesise starch as rapidly as a potato tuber. Secondly, the use of *Arabidopsis* enables a wide range of approaches to be taken in the study of starch metabolism, which are not yet possible using other species, including crops. This is due largely to the imminent completion of the *Arabidopsis* genome sequence. Using this resource allows the identification of all the genes encoding isoforms of starch metabolising enzymes, through homology to known DNA and protein sequences. The existence of populations of *Arabidopsis* that have been mutagenised by insertion mutagenesis makes it possible to screen for mutants in which specific genes encoding starch-metabolising enzymes have been knocked out. This knock-out strategy is a good approach for revealing the roles of each of these enzymes.

In contrast with the situation in Arabidopsis, our ability to decrease or eliminate the expression of specific enzymes in most other species relies on the discovery of conventional mutants or the expression of antisense RNA. Conventional mutants are typically discovered when the mutation results in a gross alteration in either the amount or structure of starch. Screens for mutant phenotypes are unlikely to detect subtle changes. On the other hand, antisense methods usually fail to eliminate expression completely and the contribution of the residual gene product is difficult to assess. While significant progress has been made using these approaches, they have their limitations.

*Arabidopsis* offers another major advantage over crop species in that starch is made during a single photoperiod. In crop species, starch accumulates over a considerable length of time, during which there are changes in cellular conditions, substrate supply and the activities and isoform complement of starch metabolising enzymes. Thus, starch extracted from a developed starch-storing organ is the product of a varying combination of enzymes acting in a varying environment. As a result, relating the starch phenotype of a mutant or transgenic plant to the decrease in a given enzyme isoform has been problematic. These sorts of developmental changes are not an issue in *Arabidopsis*. Thus, a difference in starch structure between a mutant and wild-type *Arabidopsis* plant can more readily be related to the action of the enzyme affected by the mutation than when the conditions are varying. Only with the knowledge gained from such a relatively straightforward system can we begin to understand what is happening in more complex systems.

The value of Arabidopsis as a means of studying starch metabolism is illustrated by our recent work on disproportionating enzyme. This enzyme was believed to be involved in starch metabolism but conventional mutants and antisense plants had produced conflicting results and failed to resolve its role.

## 2 THE ROLE OF DISPROPORTIONATING ENZYME IN STARCH METABOLISM

Disproportionating enzyme (D-enzyme) is a plastidial 1,4-α-D-glucan:1,4-α-D-glucan, 4-α-D-glucanotransferase (EC 2.4.1.25) found in many different starch-containing organs of plants[2,3,4]. Although probably involved in starch metabolism, the precise role of D-enzyme is yet to be established. D-enzyme transfers maltosyl units from one 1,4-α-D-glucan to another. Short malto-oligosaccharides (MOS) are the preferred substrates for D-enzyme *in vitro*[5], although the enzyme is capable of catalysing a range of reactions. The disproportionation reaction does not result in a change in the total number of α-1,4 bonds but does change the size distribution of MOS molecules (Figure 1), potentially generating suitable substrates for other enzymes of starch metabolism. For example, the creation of larger MOS molecules from small ones would provide substrates for starch-degrading enzymes such as starch phosphorylase and β-amylase which both act preferentially or exclusively on larger MOS molecules. Based on these considerations, it has been proposed that D-enzyme is involved primarily in starch degradation[5,6].

**Figure 1** *The action of disproportionating enzyme on maltotriose*

Prior to our work, attempts had been made to study the role of D-enzyme using an antisense approach in potato and by studying a mutant of *Chlamydomonas rienhardii* (a unicellular green alga) lacking D-enzyme activity. Elimination of more than 98% of D-enzyme activity through antisense in potatoes did not result in any detectable change in tuber starch[7]. However, delayed tuber sprouting and shoot growth suggested that starch is less effectively mobilised in such plants, consistent with the proposal that D-enzyme has a role in starch breakdown[7]. However, analysis of a mutant of *Chlamydomonas* lacking D-enzyme activity (*sta11*) led to a very different proposal for the role of D-enzyme in starch metabolism. Colleoni and colleagues[8] discovered that the mutant had a reduced starch content, altered amylopectin structure and high levels of MOS when grown under limiting nitrogen to promote starch accumulation. The authors suggest that D-enzyme transfers soluble MOS onto chains of the amylopectin component of starch[9], thus contributing to starch synthesis.

The conclusions from the study of *Chlamydomonas* are different from those from potato, and have radical implications for our understanding of starch synthesis. To gain definitive information about the role of D-enzyme in higher plants, we isolated a mutant of *Arabidopsis* that completely lacks D-enzyme.

## 3 PHENOTYPE OF A MUTANT OF *ARABIDOPSIS* LACKING D-ENZYME

The gene encoding D-enzyme in *Arabidopsis* (*DPE1*) is located on chromosome 5 (Genbank accession AB019236). Analysis of available genomic DNA sequence and Southern blotting experiments did not reveal any other D-enzyme genes in *Arabidopsis*. We used PCR with multiple primer combinations to screen for T-DNA insertions in the *DPE1* gene[10]. One line from the DuPont collection[11] was found to contain an insertion at the beginning of exon 2 in the gene. By raising an antibody to the *DPE1* gene product it was possible to show that the insertion in the mutant line (*dpe1*) abolished expression of this gene.

We assayed D-enzyme in crude extracts of *dpe1* using maltotriose as a substrate and measuring the glucose produced, and found approximately 5% of the wild-type activity. However analysis of the reaction products of the assay revealed that this residual activity was in fact α-glucosidase, which can cleave maltotriose into maltose and glucose[12]. D-enzyme activity in extracts of wild-type leaves converted maltotriose into glucose and larger MOS, primarily maltopentaose and maltoheptaose. We were thus able to conclude that there was no D-enzyme activity in the mutant line *dpe1*.

The loss of D-enzyme has marked effects on the starch metabolism of plants grown in a diurnal cycle. In wild-type *Arabidopsis* grown in a 12h/12h light and dark cycle in a controlled environment room, starch is accumulated throughout the day at a constant rate[13]. During the subsequent night the starch is degraded to provide carbon for sucrose synthesis and export, respiration and growth, such that at the start of the next day it is almost completely gone[1]. In *dpe1*, starch was still present at the start of the day. Starch was then accumulated during the day but at a lower rate than in the wild type and by the end of the day, mutant and wild type had similar amounts. During the night less starch was degraded in dpe1 than in the wild type. Thus both starch accumulation and starch synthesis appeared affected by the loss of D-enzyme.

We found that the amylose content of the mutant starch was higher than in the wild type. However, analysis of the chain length distribution of the amylopectin revealed no difference in the mutant and wild type. This was not the case in the *sta11* mutant of *Chlamydomonas*, where the amylopectin chain length distribution was altered considerably, leading to the suggestion that D-enzyme was participating in amylopectin synthesis[8].

The third aspect to the phenotype of *dpe1* was a massive accumulation of MOS during the night. At the onset of the dark period, the level of MOS was low in both mutant and wild type (0.05 mg/g FW). In the wild type this doubled transiently in the first four hours of the night, while in the mutant there was a fifteen-fold increase. This high level of MOS was maintained throughout the rest of the night and further analysis of this MOS pool revealed it to be primarily maltotriose; the preferred substrate of D-enzyme *in vitro*. This contrasted to the MOS pool in the wild type, which was almost exclusively maltose. These results indicate that D-enzyme is involved in the metabolism of maltotriose during starch degradation (Figure 2); a role that had been proposed based on the consideration of the enzymes kinetics and substrate preference[5]. At the onset of the day the amount of MOS in *dpe1* declined and after four hours the level of MOS was similar to the wild type.

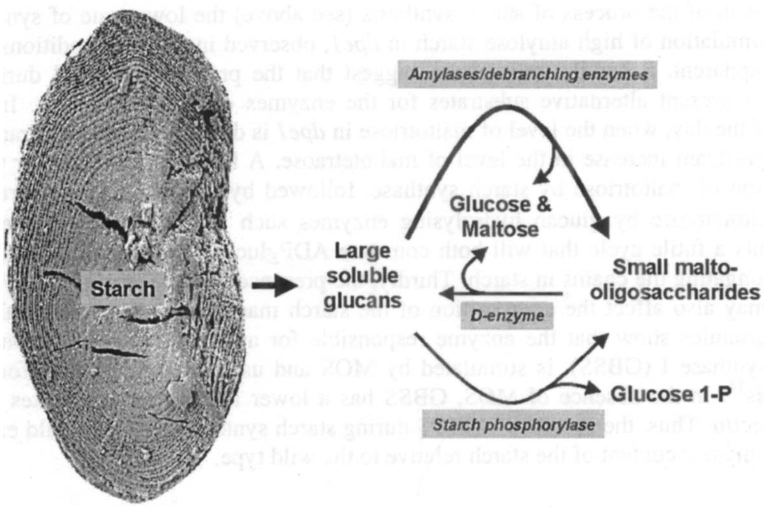

**Figure 2** *The role of D-enzyme during starch degradation in Arabidopsis leaves*

## 4 DISSECTING THE COMPLEX PHENOTYPE OF *DPE1*

In order to explain how the loss of D-enzyme was causing the complex phenotype observed in the mutant we experimented with the growth conditions of the plants to enable us to make a more meaningful comparison of the process of starch synthesis in wild type and mutant plants. In the 12h/12h growth conditions the mutant retained both starch and MOS at the end of the night whereas the wild type did not (see above). Thus, at the start of the day, the conditions in which photosynthesis and starch synthesis are taking place is different in the mutant and wild type. By placing the plants in the dark for 36h instead of the normal 12h, all the starch and MOS in the mutant was degraded. This meant that, when placed back in the light, starch accumulation proceeded under similar conditions in both wild type and mutant: the only difference being the lack of D-enzyme in the mutant. Under these conditions we found that both lines synthesised starch at the same rate, which was similar to the normal, wild type rate of synthesis in diurnal conditions. This indicates that D-enzyme is not important in determining the rate of starch synthesis. This was observed using two different light intensities. Furthermore, the amylose content of the starch synthesised under these conditions was similar in the two lines, and similar to that of wild type grown in normal diurnal conditions. Thus D-enzyme is not directly influencing the accumulation of amylose in starch. However, when placed back in the dark, the mutant plants accumulated MOS and had a slower rate of starch degradation. This indicates that the primary role of D-enzyme is in the recycling of MOS during starch degradation and that without this activity, starch degradation is impaired.

We suggest that the effect of the mutation on the rate of starch accumulation and the composition of the starch are secondary effects arising from the presence of MOS during the day when starch synthesis is occurring. There is evidence to support this view. First, when mutant and wild type plants were kept in the dark for 36h to allow a more direct comparison of the process of starch synthesis (see above) the lower rate of synthesis and the accumulation of high amylose starch in *dpe1*, observed in diurnal conditions, were no longer apparent. Secondly, our results suggest that the presence of MOS during starch synthesis present alternative substrates for the enzymes of starch synthesis. In the first hours of the day, when the level of maltotriose in *dpe1* is declining, there is a transient, but very significant increase in the level of maltotetraose. A likely explanation for this is the elongation of maltotriose by starch synthase, followed by the subsequent degradation of this maltotetraose by glucan hydrolysing enzymes such as β-amylase (Figure 3). This represents a futile cycle that will both consume ADPglucose and distract starch synthase from elongating the chains in starch. Thirdly, the presence of MOS during the synthesis of starch may also affect the composition of the starch made. *In vitro* studies with isolated starch granules show that the enzyme responsible for amylose synthesis, granule-bound starch synthase I (GBSS), is stimulated by MOS and uses them as primers for amylose synthesis[14]. In the absence of MOS, GBSS has a lower activity and elongates chains of amylopectin. Thus, the presence of MOS during starch synthesis in *dpe1* could explain the higher amylose content of the starch relative to the wild type.

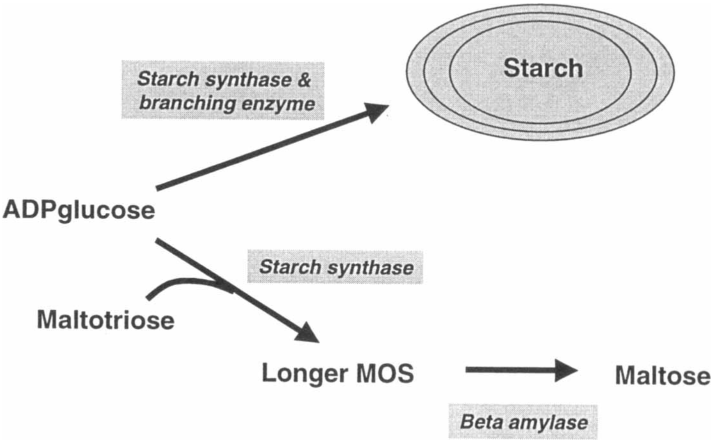

**Figure 3** *A possible futile pathway resulting from the presence of maltotriose during starch synthesis. Maltotriose elongation wastes ADPglucose and diverts starch synthase from the synthesis of the starch granule*

## 5 CONCLUSIONS

Working with *Arabidopsis* has enabled us to select a knock-out mutant deficient in D-enzyme activity and demonstrate clearly the role of this enzyme in MOS metabolism during starch degradation (Figure 2). The phenotype of this mutant also suggested that D-enzyme was involved in determining the rate of starch accumulation and the amylose content of starch. However, by altering the conditions of starch synthesis we were able to demonstrate that D-enzyme is not required for the synthesis of normal starch in *Arabidopsis*. This is consistent with the results obtained with potato[7], but not with those found with *Chlamydomonas* in which D-enzyme is proposed to play a direct role in amylopectin synthesis[9]. The differences between the effects of the *dpe1-1* and the *sta11* mutations may reflect the very different physiological conditions in which starch is synthesised in *Arabidopsis* and *Chlamydomonas*. Taken together, the phenotypes of the two mutants indicate that D-enzyme is not necessary for the synthesis of amylopectin, but it can in some conditions influence the structure of amylopectin during its synthesis.

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# THE SYNTHESIS OF AMYLOSE

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## 1 INTRODUCTION

One of the most intriguing challenges in understanding starch synthesis is to explain the apparently simultaneous synthesis of two different types of glucose polymer, which become integrated into the granule in very different ways. About 70% of the material in starches from storage organs is the branched polymer amylopectin. The remaining 30% is an unbranched, or only lightly branched polymer, amylose. The polymodal distribution of chain lengths within amylopectin molecules allows them to become ordered to form the matrix of the granule. The matrix consists of alternating, concentric, amorphous and semi-crystalline zones, with a repeat distance of several hundreds of nanometres. One amorphous and semi-crystalline repeat is known as a growth ring. Within the semi-crystalline zones, double helices formed by adjacent amylopectin chains are packed in a regular fashion to form lamellae with a repeat distance of 9 nm[1]. The amylose component does not participate in the formation of the ordered part of the matrix. Although amylose molecules in solution in vitro crystallise extremely readily, amylose molecules within the granule appear to be interspersed in a single-helical or random-coil form between amylopectin molecules[2,3]. Although amylose may be present within the semi-crystalline zone of the growth ring, it is likely that much of it lies within the amorphous zones in which amylopectin is in a loosely-packed and much less ordered form. The notion that amylose is not evenly distributed within the granule is supported by the its distribution in granules from plants with a reduced capacity to synthesise amylose. Iodine-staining of such granules reveals that amylose is to a large extent confined to particular zones within the growth ring[4,5] (Figure 1).

The most recent ideas on the synthesis of the amylopectin component of the granule are described elsewhere in this book. In this chapter, we will consider our present understanding of the synthesis and integration of the amylose component of the granule. We will discuss models to explain the mechanism of amylose synthesis, and factors which may determine how much amylose is synthesised and hence the amylose to amylopectin ratio of starch. One of the main themes to emerge will be the close integration of amylose and amylopectin synthesis. It is increasingly apparent that amylose synthesis is dependent on and controlled by amylopectin synthesis.

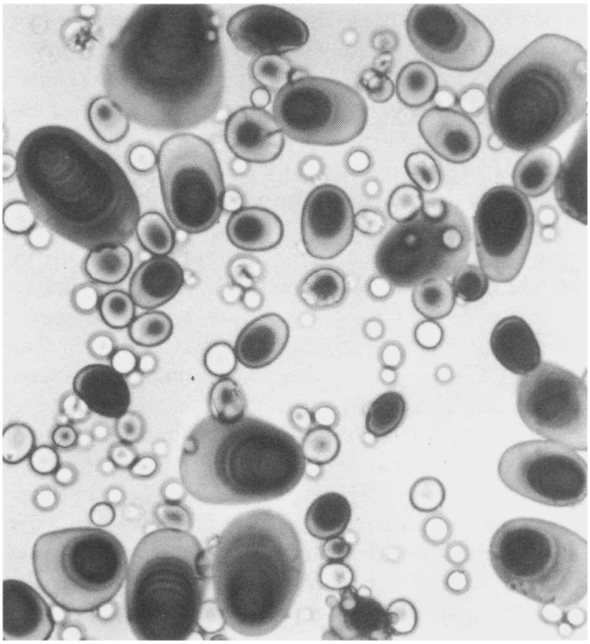


**Figure 1** *Iodine stained starch granules from a potato with drastically reduced activity of GBSSI. Starch was extracted from tubers of approximately 1 g fresh weight of a line expressing antisense RNA for GBSSI*[4]*, stained with dilute iodine-potassium iodide solution, and viewed with a light microscope. The large granules are approximately 60 μm in length. Note that amylose (dark-staining material) is concentrated in concentric rings within the granule, and is at the centre but not the periphery of the granule*

## 2 THE MECHANISM OF AMYLOSE SYNTHESIS

### 2.1 The Location of Synthesis

It has been known for many years that amylose synthesis is associated with one specific class of starch synthase known as granule bound starch synthase I (GBSSI). This understanding comes from analysis of mutants from many species of plant which lack the amylose component of starch. All such mutants thus far studied carry mutations affecting expression of the GBSSI gene. These include the *amf* mutant of potato[6], the *lam* mutant of pea[7], and *waxy* mutants of various species of cereals[8-10]. Expression of sense and antisense RNA for GBSSI in potato has confirmed that GBSSI is responsible for amylose synthesis. Reduction of GBSSI activity brought about by expression of antisense RNA in a wild-type background reduces the amylose content of starch, while restoration of GBSSI activity by expression of sense RNA in the *amf* mutant restores amylose synthesis[11,12].

The mechanism by which GBSSI synthesises amylose is by no means obvious. Starch-synthesising organs contain at least three other isoforms of starch synthase, yet all of these contribute to the synthesis of amylopectin and cannot replace the function of GBSSI in amylose synthesis[13,14]. As a first step to understanding the differences between GBSSI and other isoforms which allow them to synthesise different products, it is important to establish the location in which amylose is synthesised. There are two main possibilities (Figure 2). Synthesis could occur at the surface of the granule, in the same location as amylopectin synthesis. Amylose would then become incorporated into the granule matrix as amylopectin crystallised around it. Alternatively, synthesis could occur within the granule matrix. In this case, GBSSI protein would bind to amylopectin at the surface and be carried into the matrix as the amylopectin crystallised. Amylose synthesis would then occur where sufficient space and substrates were available inside the matrix.

There is now good reason to suppose that amylose synthesis occurs within the matrix rather than at the granule surface. First, GBSSI protein and activity are within the matrix rather than at the surface of purified starch granules. Treatments such as washing with salt, detergent and chaotropic reagents and incubation with proteases, which remove or inactivate surface proteins, have little effect on GBSSI protein and activity[15-17]. Second, evidence that amylose synthesis actually occurs within the matrix *in vivo* comes from studies of potatoes in which GBSS activity and hence amylose content have been reduced via expression of antisense RNA. Iodine-staining of the starch granules from tubers of these transgenic potatoes reveals that amylose is confined to the core of the granule and essentially absent from the periphery[5] (Figure 1). During tuber development, the volume of the core region that contains amylose increases as granule volume increases. This implies that amylose synthesis occurs inside the matrix as the granule grows, but that it lags behind amylopectin synthesis so that the outer part of the granule is amylose free[4]. Thus it seems likely that in a normal granule with a full complement of GBSSI amylose synthesis occurs at a rate which keeps pace with the synthesis of amylopectin, so that matrix spaces are filled with amylose very shortly after they are formed.

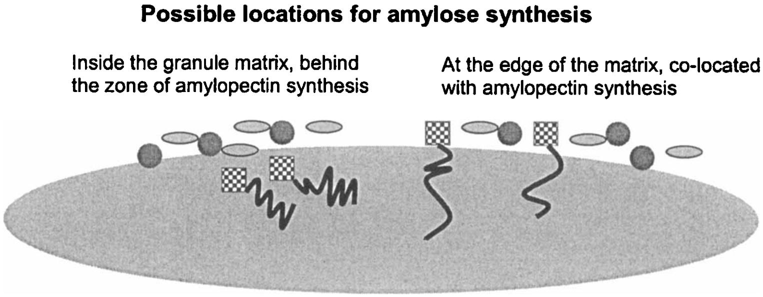


**Figure 2** *Possible locations for amylose synthesis. Starch-branching enzymes and isoforms of starch synthase other than GBSSI (solid circles and ovals) are presumed to synthesise amylopectin in a zone at the surface of the growing granule. Amylose synthesis via GBSSI (chequered square) could occur either in this zone (right), or beneath this zone in spaces within the matrix (left)*

The different location of amylose and amylopectin synthesis potentially provides an explanation for the difference in branching between the two polymers. It has been proposed that the product of GBSSI remains unbranched because its location within the matrix renders it inaccessible to starch-branching enzyme[18]. However, the presence within the matrix of isoforms of starch synthase other than GBSSI indicates that location alone cannot explain the unbranched nature of amylose. All starch granules so far examined contain a percentage of some or all of the isoforms responsible for amylopectin synthesis at the granule surface: presumably the proteins become trapped within the matrix as their product crystallises around them. These isoforms can be purified in an active form from isolated starch granules, and retain activity within the matrix in granules lacking GBSSI and amylose[19-21]. They are thus incapable of amylose synthesis even though they are active within the granule matrix. This suggests that GBSSI possesses some property lacking in all other isoforms of starch synthase which renders it capable of amylose synthesis.

## 2.2 The Properties of GBSS and Models for Amylose Synthesis

To gain information about the unique properties of GBSSI, we studied starch synthases in starch granules isolated from developing pea embryos. By supplying granules with radioactive ADPglucose, we established that GBSSI incorporated glucosyl units from ADPglucose mainly into the amylopectin rather than the amylose fraction of the starch[22]. This is also the case in granules isolated from sweet potato[23], potato[24] and leaves of *Arabidopsis* (S. Zeeman, unpublished). It appeared from this result that some component necessary for amylose synthesis – perhaps a small, soluble glucan necessary as a primer – is removed during isolation of starch granules. Consistent with this idea, addition of malto-oligosaccharides (of two to seven glucose units) as well as ADPglucose to isolated granules resulted in elongation of the malto-oligosaccharides by addition of glucosyl units from ADPglucose to produce amylose within the granules[22,24]. This ability to elongate malto-oligosaccharides to make long amylose chains is unique to GBSSI. We showed that in granules from the *lam* mutant of pea, embryos of which lack GBSSI but retains an amylopectin synthesising isoform of starch synthase (SSII) within their granules[7], glucosyl units from ADPglucose were incorporated only into amylopectin whether or not malto-oligosaccharides were present[22].

The ability of GBSSI but not SSII to elongate malto-oligosaccharides to produce amylose within the granule stems from a fundamental difference in the reaction mechanisms of the two enzymes. When granules from normal and *lam* mutant peas were supplied with ADPglucose and malto-oligosaccharides, small, soluble products were synthesised in both cases. In incubations of *lam* granules, the soluble product was almost exclusively a malto-oligosaccharide one glucosyl unit longer than the supplied malto-oligosaccharide. Thus when maltotriose and radioactive ADPglucose were supplied, the only radioactive product was maltotetraose. In incubations of normal granules, soluble products included maltotetraose, but longer malto-oligosaccharides up to nine or ten glucosyl units in length were also synthesised[24]. This chain length is probably the largest which can diffuse out of the granule: it seems likely that chains longer than this would remain trapped at the site of synthesis in the granule to become the amylose product observed in these incubations. Overall, these results indicate that SSII has a distributive mode of action: it adds a glucosyl unit from ADPglucose to a malto-oligosaccharide, then dissociates from the product. In contrast, GBSSI has a processive mode of action. After adding a glucosyl unit from ADPglucose to a malto-oligosaccharide it does not necessarily dissociate, and instead may use its product as the substrate for the addition of one or more further glucosyl units. It can thus build up long chains in conditions where SSII cannot.

These experiments suggest a mechanism for amylose synthesis *in vivo*. We propose that the processive mode of action of GBSSI allows it to synthesise long glucan chains from small soluble malto-oligosaccharides which diffuse into the granule matrix from the soluble fraction of the plastid. Once these chains exceed seven to ten glucosyl units in length, they can no longer diffuse freely within the granule[25]. Further elaboration of these trapped chains produces amylose. The distributive mode of action of other isoforms of starch synthase means that they cannot synthesise chains of sufficient length to become trapped inside the granule, hence they do not contribute to amylose synthesis.

Recent experiments have revealed a second potential mechanism for the synthesis of amylose, in which the processive mode of action of GBSSI is also important. Working with starch granules isolated from the unicellular green alga *Chlamydomonas*, van de Wal and colleagues found that radioactivity from ADPglucose was incorporated mainly into amylopectin rather than amylose, as in starches isolated from higher plants[26]. However, if the radioactive ADPglucose was replaced with non-radioactive ADPglucose during the incubation, radioactivity in the amylopectin fraction subsequently moved into the amylose fraction. Over prolonged periods of incubation in the presence of ADPglucose (one to two days) there was a large increase in the amylose to amylopectin ratio of the granules. These results are consistent with a mechanism in which chains within the amylopectin fraction of the starch are greatly elongated by GBSSI and then cleaved off - by an unidentified activity - to form amylose[27].

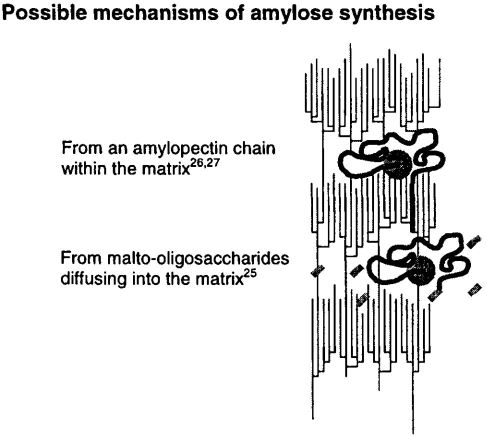


**Figure 3** *Possible mechanisms of amylose synthesis. Amylose (thick line) may be synthesised either by elongation of an existing amylopectin chain (top), followed by cleavage, or by elongation of short malto-oligosaccharides (bottom) which diffuse into the granule from the stroma of the plastid. Note that the depiction of GBSSI (circle) in amorphous lamellae within the semi-crystalline amylopectin matrix is for illustrative purposes only. The actual location of GBSSI within the matrix is not known*

Experiments with isolated granules thus reveal two possible mechanisms for amylose synthesis – elongation by GBSSI of either small, soluble malto-oligosaccharides, or chains within the amylopectin fraction followed by cleavage (Figure 3). It is not yet known which of these mechanisms operates *in vivo*, and it remains possible that both may operate, either in different circumstances or side by side.

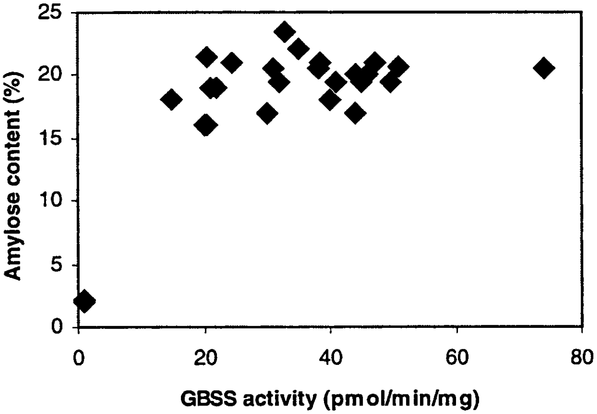
A further property of GBSSI of potential importance in the synthesis of amylose by either of the above mechanisms was identified by expression of the protein in soluble form in the bacterium *E. coli*[28]. In cell extracts, GBSSI elongated malto-oligosaccharides only in a distributive manner, and had a much lower affinity for these compounds than the enzyme on starch granule. However, addition of amylopectin to assays of the soluble enzyme greatly increased its affinity for malto-oligosaccharides and caused it to act in a processive manner. SSII expressed in *E. coli* was not rendered processive by the addition of amylopectin. Thus the properties of GBSSI critical to its ability to synthesise amylose may be conferred upon it by its association with the amylopectin matrix of the starch granule.

## 3 CONTROL OF THE AMYLOSE CONTENT OF STARCH

Although the precise mechanism of amylose synthesis is not yet known, it is nonetheless possible to draw some important conclusions about factors which control the amount of this component in starch granules. We will consider two obvious factors – the amount of the GBSS protein/activity and the amount of the substrate, ADPglucose – and a third, more complex factor: the nature of the GBSS and the granule matrix.

### 3.1 The Activity of GBS

There is unequivocal evidence for potato tubers and maize and wheat endosperm that the activity of GBSSI is not a significant factor in controlling the rate of amylose synthesis and hence the amylose to amylopectin ratio of the starch. The relationship between GBSSI activity and amylose content has been studied in all three organs using plants differing in the dosage of mutant alleles of the GBSSI gene[29-31], and in potato using plants expressing antisense RNA for GBSSI[11,32]. At wild-type GBSSI activities, amylose content is only dependent to a small extent upon activity (data for potato are displayed in Figure 4). This indicates amylose content is primarily controlled by factors other than the amount of GBSSI in all three cases. It seems likely that this conclusion will apply to storage organs generally. One exception, however, is the endosperm of rice seeds. The *japonica* and *indica* subspecies of rice differ in average amylose content, *indica* cultivars generally having a higher content than *japonica* cultivars. Many *indica* cultivars also have considerably higher GBSSI protein and activity than *japonica* [33-37]. The low GBSSI content of *japonica* and some *indica* cultivars is due to a single base-pair change in the regulatory region of the gene which results in inefficient splicing during mRNA maturation and hence low levels of mature mRNA[36,38]. The relationship between amylose content and GBSSI protein content for high amylose, *indica* cultivars shows that, as for potato, wheat and maize, GBSSI is not the main factor controlling amylose content. However, in *japonica* and low-GBSSI *indica* cultivars GBSSI is of considerably greater importance in determining amylose content (Figure 5).



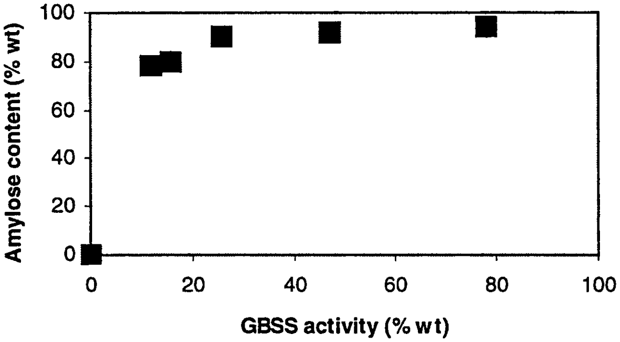


**Figure 4** *Relationship between GBSS activity and the amylose content of starch in potato tubers. The upper and lower graphs are derived from data from potatoes in which GBSSI activity was varied by altering the gene dosage of the amf allele*[29]*, or by expressing antisense RNA*[32]*, respectively. On the upper graph, the average activity in normal, wild-type potatoes is 35-40 pmol.min*$^{-1}$*.mg*$^{-1}$*. Note that significant reductions in amylose content occur only when GBSS activity is strongly reduced from wild-type levels. An increase in GBSS activity above wild-type levels (upper graph) has no effect on amylose content*

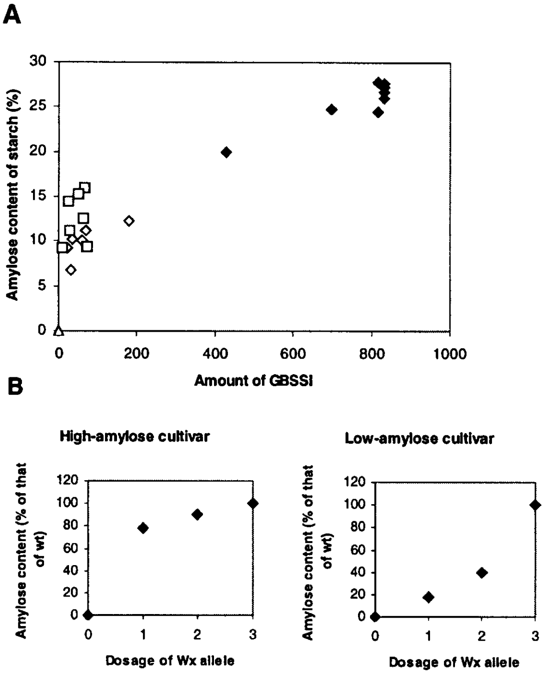


**Figure 5** *Relationship between amount of GBSSI and the amylose content of starch from rice endosperm. A: data taken from a study of high-GBSSI indica cultivars (filled diamonds), low-GBSS indica and japonica cultivars (open diamonds and squares, respectively) and a waxy cultivar (triangle)*[36]. *Note there is an overall hyperbolic relationship between GBSSI amount and amylose content. It can be predicted from this curve that in high-GBSSI indica cultivars an increase in GBSSI will have little or no effect on amylose content, and a large reduction in GBSSI content will be required to bring about a significant decrease in amylose content. In contrast, in low-GBSSI indica and in japonica cultivars, it can be predicted that increasing GBSSI content will increase amylose content, and relatively small decreases in GBSSI content will cause a significant decrease in amylose content. B: data taken from a study of a high-GBSSI indica cultivar (left) and a japonica cultivar (right) in which GBSSI content in the endosperm was varied by changing the dosage of mutant and wild-type (Wx) alleles at the waxy locus*[37]. *The GBSSI content is expected to increase linearly with the number of Wx alleles present. Note that for the indica cultivar amylose content plateaus at three dosages of Wx (the normal, wild-type situation), suggesting that GBSS content is unimportant in determining amylose content in the wild-type. In contrast, in the japonica cultivar amylose content at three dosages of the Wx allele is three times greater than at two dosages. This suggests that the GBSS content of the endosperm is important in limiting amylose synthesis in the endosperm of japonica cultivars*

## 3.2 The Concentration of ADPglucose

The concentration of ADPglucose may be important in determining the relative rates of amylose and amylopectin synthesis and hence the amylose to amylopectin ratio of starch, under at least some circumstances. GBSSI has a much lower affinity for ADPglucose than the amylopectin synthesising isoforms of starch synthase, hence a change in ADPglucose concentration in the plastid could potentially alter the relative rates of amylose and amylopectin synthesis (Table 1). Evidence that this may actually occur come from low-starch mutants of *Chlamydomonas* and pea deficient in ADPglucose synthesis. Mutations drastically reducing the activity of plastidial phosphoglucomutase (*rug3* and *sta5* in pea[29] and *Chlamydomonas*[40] respectively), ADPglucose pyrophosphorylase (*rb* and *sta1* in pea[41] and *Chlamydomonas*[42] respectively) and sucrose synthase (*rug4* in pea[43]) have no direct effects on starch synthase activities, yet cause a substantial reduction in the amylose content of starch. For example in a *rug3* mutant of pea, the starch content of the seed is reduced by 88 % and the amylose content of the starch by 60%[44]. In the *Chlamydomonas* mutants, starch content is reduced by about 90% and amylose content falls from 15-30% to virtually undetectable levels[40,42]. A similar relationship between ADPglucose concentration and amylose synthesis has been proposed for transgenic potatoes with decreased levels of ADPglucose pyrophosphorylase, and with either decreased or increased levels of a plastidial adenylate transporter proposed to import ATP into the plastid for ADPglucose synthesis. Reductions in the activity of ADPglucose pyrophosphorylase in tubers were correlated with both a decrease in starch content and a decrease in the amylose to amylopectin ratio of the starch[45]. Similarly, variation in the level of the adenylate transporter was correlated with changes in both the rate of starch synthesis and the amylose to amylopectin ratio of the starch. Tubers with high levels of starch had high amylose to amylopectin ratios, and those with low starch had low amylose to amylopectin ratios[46].

**Table 1** *Affinity of granule-bound and soluble starch synthase activities for ADPglucose, and estimates of ADPglucose concentration in plastids*

| *Plant organ* | *Granule-bound starch synthase* "$K_M$" (mM) | *Soluble starch synthase* "$K_M$" (mM) | *Concentration of ADPglucose in plastid* (mM) |
|---|---|---|---|
| Maize endosperm[56-58] | 3.0 | 0.1 | - |
| Pea embryo[47,59] | 1.4 | 0.3, 0.5, 0.13 | 0.9 |
| Potato tuber[45,47,60] | 4.5, 1.9 | 0.13 | 0.04 |
| *Chlamydomonas*[26,42] | 3.5 | 0.6 | - |
| Amaranth perisperm[61] | 1.1 | - | - |
| Wheat Endosperm[62] | - | 0.06 | - |
| Pea leaf[63,64] | - | 0.09 | 0.3 |
| Arum spadix[65] | - | - | 0.4 |

*Data are taken from the references noted. Affinities are given as "$K_M$", the ADPglucose concentration needed for half-maximum activity. Values for the granule-bound enzymes were estimated from measurements made on isolated starch granules. Estimates of ADPglucose concentrations were made on the assumption that the plastid is 10% of the cell volume, and that the tissues had a relative density of unity.*

More detailed studies of pea have provided evidence consistent with the idea that the effects on amylose to amylopectin ratio in the mutant and transgenic plants are brought about by changes in the concentration of ADPglucose[47]. First, we estimate that the ADPglucose concentration in plastids of developing, wild-type pea seeds is higher than the $K_M$[ADPG] of the amylopectin-synthesising isoforms, but lower than the $K_M$[ADPG] of GBSSI (Table 1). The effects of a change in the concentration on the activity of the amylopectin-synthesising isoforms will thus be different from those on the activity of GBSSI. Second, we found a clear relationship between ADPglucose levels and the amylose content of the starch in developing embryos of mutant and wild-type pea seeds (Figure 6). Third, we established that incubation of detached embryos of wild-type pea seeds at low sugar concentrations resulted in a low level of ADPglucose and a low ratio of amylose to amylopectin synthesis (measured by incorporation of radioactivity from radioactive sugars), whereas incubation at high sugar concentrations gave high levels of ADPglucose (comparable with those in attached embryos) and a higher ratio of amylose to amylopectin synthesis.

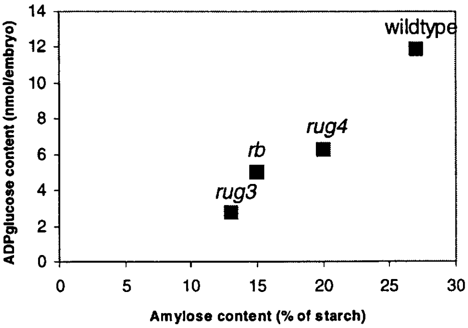


**Figure 6** *Relationship between ADPG content and amylose content for developing embryos of wild-type peas and pea carrying mutations at three loci affecting ADPglucose synthesis*[47]

### 3.3 The Nature of the GBSS Isoform and the Granule Matrix

There is now reason to think that the amylose content of starch can potentially be determined not only by the levels of GBSSI and of ADPglucose, but also by the nature of the GBSSI and the granule matrix in which it is located. Our first insights into this possibility came from studies of the GBSSI and amylose content of starches in different organs of the pea plant[48]. In the major starch-storing organ, the embryo, starch contains about 30% amylose. Mutations at the *Lam* locus, which lie in a gene encoding GBSSI, eliminate the amylose and the GBSS protein from the starch. A different picture emerged for other organs of the plant. The starch in leaves, pods and the root nodules containing the nitrogen-fixing bacterium *Rhizobium* has less amylose than embryo starch. The amylose content of leaf, pod and nodule starch was essentially unaffected by mutations at the *Lam* locus. The major granule-bound protein of these starches – of approximately the same size as the embryo GBSSI and immunologically closely related to it – was also not affected by mutations at the *Lam* locus. These findings indicated that pea has two different forms of GBSSI, one encoded at the *Lam* locus responsible for amylose synthesis in the embryo and the other encoded elsewhere and responsible for most of the amylose synthesis elsewhere in the plant. A second GBSS gene has subsequently been identified (our unpublished data).

A similar picture to that in pea is emerging for cereals. It has been known for some time that mutations at the *Waxy* loci eliminate amylose from the starch of the endosperm, but not from all other parts of the plant[49-53]. A second GBSSI gene has now been identified in wheat[54]. Analysis of wild-type and *waxy* mutant wheat shows that its product is responsible for synthesis of amylose in most parts of the plant other than the endosperm, including the pericarp immediately surrounding the endosperm[52-54].

The existence of two different forms of GBSSI, expressed in different parts of the plant in which the starches have different amylopectin structures and amylose contents, indicates that the forms have distinct characteristics which may be important in the regulation of amylose synthesis. This idea is strengthened by the fact that in both pea and wheat, the primary amino acid sequences predicted from the two GBSSI genes are more closely related to sequences from other species than to each other[54]. Attempts to discover whether there are differences between forms of GBSSI, via expression in *E. coli* and amylose-free mutants of plants, are under way. Published results thus far indicate that there may indeed be profound differences. Expression of a GBSSI gene from cassava in the *amf* mutant of potato resulted in only low levels of amylose synthesis in the transgenic plants[55], even though expression of the endogenous, potato GBSS in the mutant fully restores the wild-type amylose content[12]. The relationship between GBSS activity and amylose content in the starch of plants expressing the cassava gene was very different from that in the starch of plants expressing the potato gene. This result suggests that the cassava GBSSI functions differently from potato GBSSI in the potato starch granule. Cassava and potato amylopectins differ in structure and in organisation within the granule, and cassava granules are very much smaller than those in potato. All of these differences could potentially influence the activity of GBSSI.

Overall, we suggest that amount and nature of amylose synthesised by a particular GBSSI is determined by at least three factors: 1) The intrinsic properties of the enzyme, which probably differ from one GBSSI to another. 2) The structure of the amylopectin matrix in which the enzyme is located. The matrix may directly influence the properties of the enzyme, provide some of the glucan substrate on which GBSS acts, and limit both the availability of ADPglucose and malto-oligosaccharides as substrates and the space available for amylose accumulation. 3) The concentration of ADPglucose and malto-oligosaccharides in the stroma of the plastid in which the enzyme is located. These concentrations are likely to differ from one organ to another, and in response to changes in developmental and environmental conditions.

Further studies of plants expressing heterologous GBSS genes will enable the relative importance of these factors to be elucidated. Such experiments may shed light on what determines the structure as well as the amount of amylose in starch. Both the molecular size and the number and distribution of branches in amylose differ from one starch to another. This variation could be due to differences in the matrix space available for amylose synthesis, or to differences in the intrinsic properties of GBSS, or a combination of these factors.

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# NULL ALLELES AT THE WAXY LOCI IN WHEAT AND OATS: ORIGIN, DISTRIBUTION AND EXPLOITATION

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## 1 INTRODUCTION

Amylose is synthesised in cereal grains by the activity of the granule-bound starch synthase (GBSS, EC 24.1.21), also known as the waxy protein. In diploid grasses such as corn (*Zea mays*) and barley (*Hordeum vulgare*), the existence has long been known of naturally occurring mutations eliminating both GBSS synthesis and amylose production. Resultant starches, composed entirely of amylopectin, are known as "waxy" starches. In polyploid grasses such as wheat (*Triticum aestivum*) and oats (*Avena sativa*), multiple copies exist of the genes (*wx* loci) encoding GBSS. Hence, naturally occurring waxy mutations in wheat were long unknown, as the elimination of function of any one locus was masked by the presence of additional, functional alleles. In the early 1990's, Nakamura[1] and colleagues devised electrophoretic methods allowing the separation of the gene products of the three wheat *wx* loci. In addition, null, or non-functional alleles were found at each locus[2,3]. Two significant subsequent accomplishments include the development of amylose-free (waxy) wheats via classical breeding to combine the three null alleles[4], and the discovery that wheats with two, or even one, null allele may produce starch with reduced amylose contents[2]. Such wheats have been termed "partial waxy"[2,3].

## 2 PROPERTIES OF PARTIAL WAXY WHEATS

Amylose contents of partial waxy wheats range from approximately 15-25%[2,5], slightly reduced relative to typical wild-type levels (25-30%). The presence of two null alleles conditions a greater reduction in amylose content than does one null allele. Null alleles at the *wx-A1* locus were detected at a frequency of greater than 10% in samples of wheats of Japan, Korea and Turkey[3]. At the *wx-B1* locus, null alleles are common in wheats from India and Australia[3]. Null alleles at the *wx-D1* null allele occur only in two wheats BaiHuo and BaiHuoMai, both from China[3]. In the U.S. hard winter wheat gene pool, null alleles at both the *wx-A1* and *wx-B1* occur, albeit at a low frequency, among advanced breeding lines and cultivars[5]. Only one U.S. wheat, the cultivar 'Ike' has been found to carry both *wx-A1* and *wx-B1* null alleles. The *wx-A1* null allele present in U.S. hard wheats may be traced, by pedigree analysis, to a Korean wheat known as 'Seu Seun 27', introduced in the 1950's as a source of semidwarf genes. Likewise, the *wx-B1* null allele of most advanced U.S. hard wheats, was derived from a common source, the Australian cultivar 'Hard Federation'. The higher frequency of null alleles at the *wx-A1* and *wx-B1* null alleles among the world's wheats may not be indicative of a higher mutation frequency at these loci, but rather may arise from a type of genetic "founder" effect. Genes present in wheats making significant and early contributions to pedigrees may be appear common, merely due to their presence, in keystone or founder cultivars. If they confer no deleterious effect, they are not eliminated via selection. Alternatively, unconscious selection for some desirable quality trait, conditioned by the presence of null alleles, might have fixed them in certain breeding pools.

Aside from having, on average, lower amylose contents, functional properties of partial waxy wheats may differ from those of wild-type. One hundred and forty-three experimental lines, derived from four breeding populations, and grown at two Nebraska (USA) locations in 1998, were used to investigate these effects. The lines carried null alleles at the *wx-A1* locus (a null), the *wx-B1* locus (b null), both loci (2X null) or were wild-type. The cultivars Ike (2X null) and Vista (wild-type) were included as checks, but were excluded from the statistical analysis. The most dramatic effects (Figure 1) were observed with the 2X null lines. Mean starch peak viscosity (as measured by the Rapid Viscoanalyser) starch breakdown, and final viscosities of 2X null lines differed significantly from those of wild-type wheats. Starch pasting temperatures (not shown) did not differ. Thus, partial waxy wheats allow the development of high viscosity flours for a variety of end-uses.

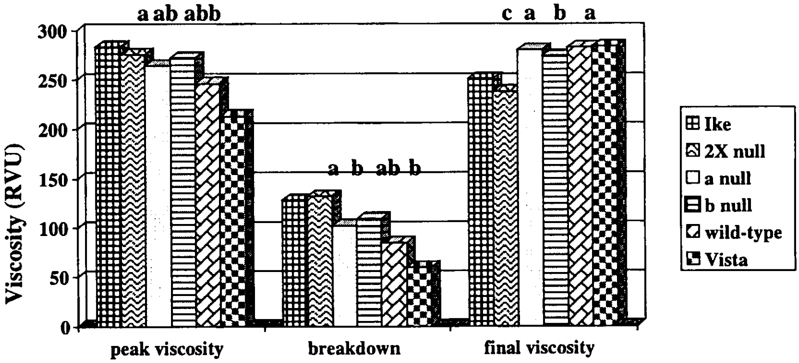

**Figure 1** *Mean starch pasting properties of partial waxy and wild-type genotypes. Values appended by the same letters did not differ significantly at p=0.05. Check cultivars, Ike and Vista, included for reference*

## 3 PROPERTIES OF WAXY WHEATS

Lacking amylose, waxy wheats have radically different functional properties than wild-type and partial waxy wheats. Waxy wheat starch attains peak viscosity at 80 C, rather than at the 95 C temperature typical of sound wild-type or partial waxy wheat. Waxy wheat starch also demonstrates a more rapid breakdown, and a much lower final viscosity, when analysed via typical Rapid Viscoanalyser pasting profiles[6]. Because of their unique pasting profiles, waxy wheats have abnormally low falling numbers that are independent of α-amylase activities[6].

The most common use of wheat flour, at least in the Western world, is in the production of bread. Experiments with waxy wheat flour demonstrate, however, that this likely will not be the most common use of waxy wheat. Bread baked from 100% waxy flour has coarse and open internal grain structure, similar to that observed with severely sprout-damaged wheats. In addition, loaves baked from 100% waxy wheat flour collapse, losing as much as 25% of their volume within 24 hours (Figure 2).

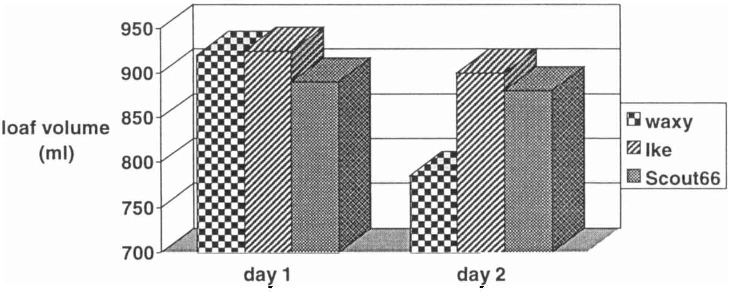

**Figure 2** *Loaf volumes from 100 gram bake tests of a waxy wheat line Ike (2Xnull) and Scout 66 (wild type)*

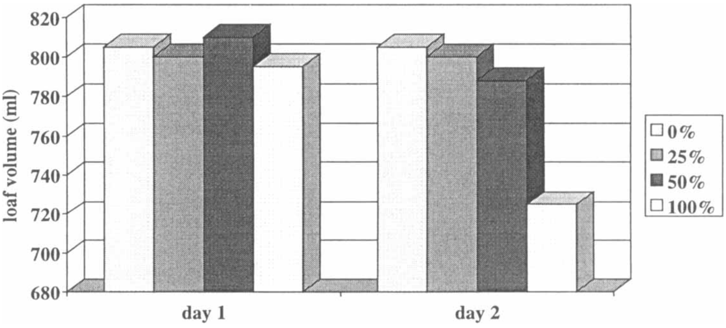

**Figure 3** *Loaf volumes of flours produced from 100% waxy*

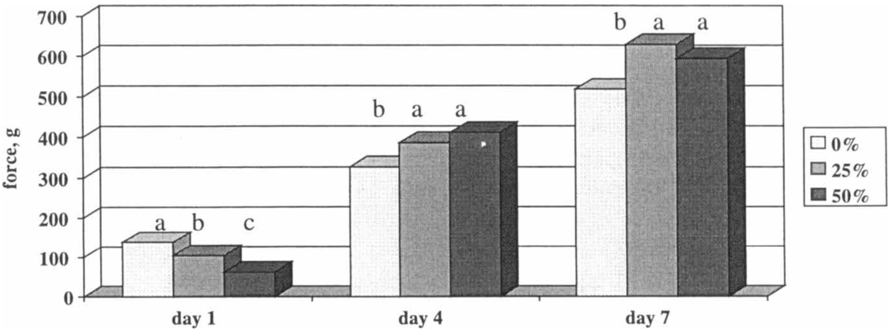

**Figure 4** *Crumb firmness of loaves baked with 0%, 25% and 50% waxy wheat flour. Values appended by the same letter did not differ significantly at p=0.05*

The poor baking qualities of waxy wheat may preclude the use of 100% waxy flours in commercial baking applications. However, blends of waxy/non-waxy flours might be acceptable. In addition, speculation exists[7] that waxy wheats might be useful in altering shelf-life duration of baked goods. To investigate these possibilities, 25% and 50% blends of waxy/Scout 66 flour were baked, and compared to 100% Scout 66 (wild-type) and 100% waxy flours. Loaf volumes, and crumb firmness, as determined by an Instron, were measured. Loaf grain and texture of the 50% waxy loaves still were unacceptable (not shown); the 25% waxy blend did produce loaves with acceptable internal appearance. In addition, the collapse of loaves after 24 hours was not detected (Figure 3) in the blended flours. Crumb firmness (Figure 4) could not be measured in the waxy sample due to the collapsed loaves. One day after baking, flours containing either 25% or 50% waxy flour had significantly softer crumb than the loaves from the wild-type flour. However, at 4 and 7 days post baking, the situation reversed, and the waxy blends had firmer crumb than the wild-type loaves.

Commercial applications of waxy wheat in modern baking may be restricted to use as a blending flour, and may be applicable only in loaves meant to be consumed within 24 hours. The unique pasting properties of waxy wheat do suggest, however, possible roles in food products such as gravies and soups, in which rapid cooking and a thickened, but not overly thick texture is desired. Additional experimentation is desirable to establish additional uses of this unique food product.

## 4 THE WAXY PROTEINS OF OATS

The genetic composition of oats is similar to wheat. Both are allohexaploid species, combining the genomes of three ancestral species. Chromosomal rearrangements among the three oat genomes must have occurred, as, unlike wheat, oat does not tolerate the loss of chromosomes easily. Triplicate factor inheritance in oats is likely. When electrophoretic techniques devised for the separation of wheat GBSS was applied to oats, extensive genetic polymorphism was detected. Among a selection of 80 oat cultivars ancestral to the U.S. oat gene pool[8], polymorphism was common. At least 4 isoforms

(Figure 5) were detected, and null alleles were common. The "a" isoform has been observed in all oats tested to date.

Genetic studies are necessary to establish the number of oat *wx* loci, and the loci of origin of each isoform and null allele. Working hypotheses are: 1) there are three loci, that GBSS-a, present in all oats tested to date, is the product of one locus, 2) GBSS-b and GBSS-c arise from independent loci, and 3) the loci encoding GBSS-b and GBSS-c also carry null alleles. To confirm, lines producing only GBSS-a (assumed genotype designated --/aa/--, where – designates a null allele) were intermated with lines producing GBSS-a, GBSS-b and GBSS-c. GBSS phenotypes were scored on $F_2$ seed. Results are given in Table 1.

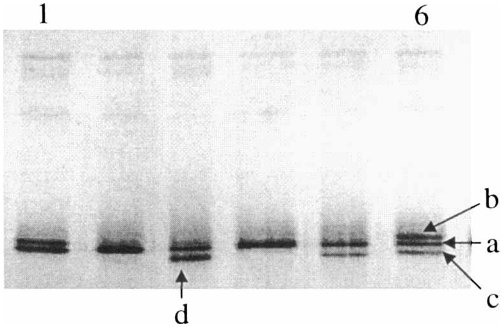

**Figure 5** *GBSS isoform variation in oats. Letters designate recognised isoforms. Lanes 1 = Ceal, 2 = Orbit, 3 = Trispernia, 4 = Calibre, 5 = Centennial, 6 = Cherokee.*

**Table 1** *GBSS isoforms (phenotypes) observed in $F_2$ seed from matings between oat lines producing GBSS-a only, and lines producing GBSS-a, GBSS-b and GBSS-c.*

| *Number of seed of each respective $F_2$ phenotype* | | | | | |
|---|---|---|---|---|---|
| bac | ba | ac | a | $X^2$ | p |
| 37 | 19 | 12 | 6 | 3.09 | >0.10 |

The chi-square analysis confirms that the observed ratios did not differ from the expected phenotypic segregation ratio of 9:3:3:1. Thus, assuming the ubiquitous GBSS-a is the product of one locus, three *wx* loci exist in oats. It remains to be determined if genetic complementation can result in the production of waxy oats. This may require the identification of a null allele at the locus encoding GBSS-a. Applying the nomenclature of wheats to oats, it is of interest to note that the majority of oats tested can be considered "partial waxy" as most carry at least one null allele.

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# EFFECT OF INTER- AND INTRA-ALLELIC VARATION ON STARCH GRANULAR STRUCTURE

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## 1 INTRODUCTION

Starch granules consist of ordered and disordered, or amorphous, regions. The ordered regions are composed of double helices of amylopectin, some of which are arranged in either A- or B-type crystallites, depending on the plant species. The disordered regions are composed partly of amylose and partly of amylopectin chains that have not formed double helices.[1] Differences in granular structure, therefore, can be due to changes in the proportion of amylopectin forming double helices, in the proportion of these double helices that form crystallites and in the type and distribution of crystallites. Granular structure can also change according to differences in the amorphous regions of the granule.

Understanding the control of granular structure is complex, since it depends on the synthesis of amylose and amylopectin followed by the thermodynamic organisation of these polymers into the structural elements mentioned above. The biosynthetic pathways for amylose and amylopectin are reasonably well understood at a genetic and biochemical level.[2] How these molecules associate to form the structural elements of the granule, however, is not known. It is not possible, therefore, to predict the effect of biosynthetic changes on granule structure.

Although, at present, it has not been possible to relate polymer biosynthesis to granular structure and properties, it is evident that mutations in genes at specific loci produce predictable changes in granule structure.[3] It is possible therefore, to describe gene action in terms of structural characteristics associated with the starch granule and to use genetic variation to probe granule structure directly, without the need for understanding the underlying biochemical mechanisms.

In this paper we will outline which aspects of starch granular structure are affected by specific mutations of starch biosynthesis. This will cover three types of genetic action: the action of similar mutations in species that normally contain either A-, B- or C-type starch (homologous variation); the action of genes at different loci affecting starch biosynthesis within a single species (inter-allelic variation); and the action of different alleles at a single locus affecting starch biosynthesis (intra-allelic variation).

## 2 HOMOLOGOUS VARIATION

Several homologous mutations affecting starch biosynthesis have been identified in different species. For example, mutations affecting a major granule-bound starch synthase (GBSSI) have been described in maize (A-type starch), potato (B-type starch) and pea (C-type starch). In maize this mutation relates to the *wx* locus, giving the characteristic 'waxy' phenotype. The equivalent mutation in potato is at the *amylose free* (*amf*) locus and in pea at the *low amylose* (*lam*) locus. In each case these mutations result in starches that have very low, or no, amylose.

We have shown that in each species the presence of the low amylose mutation increased the proportion of double helices in the starch compared with starch from the equivalent wild type. This is not surprising since the double helices are believed to be formed only from amylopectin branches. The increase in the proportion of double helices, however, was much less than the increase in the proportion of amylopectin within these starches. This is because part of the amylopectin replaces amylose as the amorphous material within the starch granules of these lines.

The effect of the low amylose mutations, with regard to total crystallinity, was less clear. This is because crystallinity is affected by the moisture content of the starch and, as such, is not a good characteristic for comparative purposes.

During gelatinisation in excess water, the peak temperature for the low amylose A- and B-type starches was higher than for starch from the wild type. This was much less clear for the C-type pea starch, with starch from one *lam* mutant having a slightly lower gelatinisation temperature and the other *lam* mutant a slightly higher gelatinisation temperature than for starch from the wild type (Figure 1). It is likely that the different gelatinisation behaviour of low amylose pea starch, compared with maize and potato, is the result of this mutation affecting the ratio of A to B-polymorphs in the C-type starch.

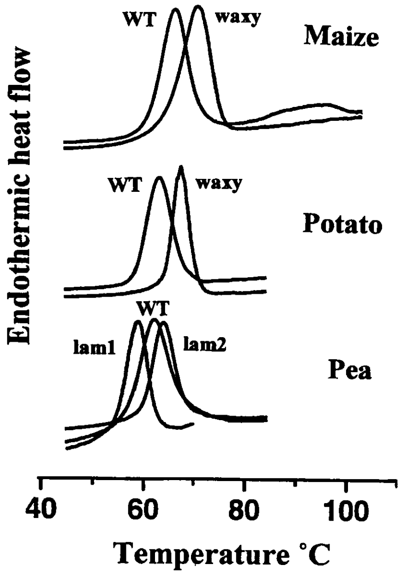

**Figure 1** *Proportion of A- and B-type crystallinity in starches from a range of pea mutants (see Table 1), compared with starch from the wild-type (WT)*

## 3 INTER-ALLELIC VARIATION

By definition, in order to study the effects of mutations at single loci it is essential to use near-isogenic lines. These are lines in which the genetic background is similar except for genes at the locus or loci being studied. The best example of such a set of lines has been developed in pea (*Pisum sativum*) at the John Innes Centre.[3-9] The near-isogenic series in pea consists of mutants with mutations at six independent steps in starch biosynthesis. The products from all of the genes have been biochemically characterised and shown to cover three different synthetic steps directly affecting starch synthesis and three steps affecting the provision of substrate for starch synthesis (Table 1).

**Table 1** *Enzyme activities and specific subunits/isoforms affected in the pea mutants*

| *Mutation* | *Activity* | *Subunit/isoform* | *Reference* |
|---|---|---|---|
| *r* | Starch branching enzyme | SBEI | 4 |
| *rb* | ADPglucose pyrophosphorylase | Large subunit | 5 |
| *rug3* | Phosphoglucomutase | Plastidial | 6 |
| *rug4* | Sucrose synthase | | 7 |
| *rug5* | Soluble starch synthase | SSII | 8 |
| *lam* | Granule-bound starch synthase | GBSSI | 9 |

The synthetic steps are starch branching enzyme (SBEI), granule-bound starch synthase (GBSSI) and soluble starch synthase (SSII), encoded by genes at the *r*, *lam* and *rug5* loci, respectively. The three steps affecting substrate provision are ADPglucose pyrophosphorylase, plastidial phosphoglucomutase and sucrose synthase, encoded by the *rb*, *rug3* and *rug4* loci, respectively.

Seeds from the mutants have a range of starch contents and the starches contain a range of amylose and amylopectin contents (Table 2). With regard to starch content, the most extreme mutant is *rug3*, while the most extreme mutants with regard to starch composition are *r*, which has a very high amylose content and *lam*, which has a very low amylose content.

**Table 2** *Allelic variation for starch and amylose content in the mutant pea lines*

| *Loci* | *Starch (% seed dry wt)* | *Amylose (% starch)* |
|---|---|---|
| *r* | 27-36 | 60-75 |
| *rb* | 30-37 | 23-32 |
| *rug3* | 1-12 | 12 |
| *rug4* | 38-43 | 31-33 |
| *rug5* | 29-35 | 43-52 |
| *lam* | 39-49 | 4-10 |

We have shown that the presence of the *lam*, *rb* and *rug4* mutations increased the proportion of double helices in the starch compared with starch from the wild type. The proportion of double helices in starch from the *rug3* mutant, however, was similar to the wild type and starch from the *r* and *rug5* mutants had a lower proportion of double helices. Although there was a tendency for starches with a reduced proportion of double helices to have reduced crystallinity, this was again unclear because of the dependence of this characteristic on the moisture content of the starches.

The effect of the mutations on the proportion of A- and B-type crystals found in the starches, however, was much clearer (Figure 2). The variation ranged from the starch produced by the *r* mutant, which contained only B-type crystals, to the starch produced by the *lam* mutant, which contained a high proportion of A-type crystals, compared with starch from the wild type.

Starches from the wild type and the *rb*, *rug3*, *rug4* and *lam* mutants showed normal gelatinisation peaks in excess water, the peak temperatures ranging from less than 60°C to about 70°C. Starches from the *r* and *rug5* mutants, however, did not show typical gelatinisation peaks.[10]

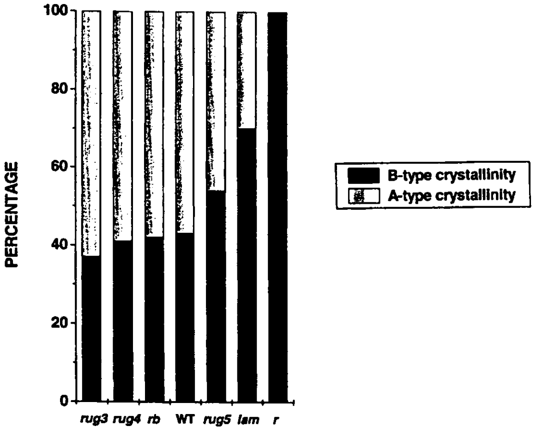

**Figure 2** *Effect of low amylose mutations in maize, potato and pea on starch gelatinisation peaks in excess water*

## 4 INTRA-ALLELIC VARIATION

The pea mutants comprise an allelic series of mutations at each of the six loci, each allelic series being near-isogenic except for the allelic differences at each locus.[3]

So far we have looked in detail only at the series of alleles identified at the *r* locus, which encode SBEI. The starch content of the alleles at this locus ranges from 37% for *r-g* to about 27% for *r-f*, compared with about 49% for starch from the wild type (Table 3). The composition of the starches from the various alleles ranges from about 60% amylose for *r-g* to about 70% for most of the other alleles (Table 3). From these compositional differences it could be assumed that the *r-g* mutant was less severely affected than the other mutants. This was confirmed by biochemical studies showing that this mutant had a low level of branching enzyme activity, whereas no enzyme activity could be detected in any of the other lines.[11]

**Table 3** *Allelic variation at the r locus for starch content and composition*

| *Allele* | *Starch content (% embryo dry wt)* | *Amylose content (% total starch)* |
|---|---|---|
| wild type | 48.8 | 36.3 |
| *r-d* | 27.5 | 70.7 |
| *r-e* | 32.1 | 69.4 |
| *r-f* | 26.5 | 70.3 |
| *r-g* | 37.0 | 60.2 |
| *r-h* | 30.3 | 72.0 |
| *r-j* | 26.7 | 70.1 |
| *r* | 32.5 | 72.3 |

With regard to the effect of this allelic variation on granular structure, we made a comparison between the starches from the *r-g* and *r-f* mutants, the latter having previously been shown to be a null mutation.[11] These two lines were also compared with the original *r* mutation and the wild type. We found that the proportion of double helices decreased progressively with increases in the severity of the mutation. A similar but less clear relationship was found for total crystallinity. More significant, however, was the change in the proportion of crystal types found. Starch from the most severe alleles, *r* and *r-f*, had only B-type crystals, while the less severe *r-g* had about 20% A-type crystals. Likewise, during gelatinisation starch from the two severe alleles showed no peak of transition, while starch from the *r-g* line showed a definite peak, although this was much broader than for starch from the wild type.

## 5 CONCLUSIONS

The approach of identifying and characterising genes in a single genetic background that have specific effects on starch granular structure, coupled with the study of homologous mutations in different species, is an extremely powerful method for studying the result of gene action. Future studies will utilise this material to determine how and when granule structure is affected during the development of the granule and, by developing double mutants, if, how and when genes interact to give new starches.

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# Poster Abstracts

# RAPID METHODS TO ASSESS STARCHES DURING PRODUCTION AND FOR QUALITY CONTROL

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The Rapid Visco Analyser (RVA) is a rotational cooking viscometer widely used to assess starch and starch containing products. The starch manufacturing industry has developed new methods for testing starch using the RVA. These methods have been optimised for different classes of food and industrial starches, including various native, substituted, cross-linked and thin-boiling types, providing rapid results that show good discrimination and repeatability. Results are provided in traceable units and the methodology has been automated to minimise errors and labour costs. These methods overcome many of the problems with the historical methods, providing the opportunity for improved process control during starch manufacture, and universally acceptable quality specifications for trade.

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# EVALUATION OF STARCH CONVERSION IN PROCESSED STARCH USING THE RAPID VISCO ANALYSER

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The Rapid Visco Analyser is being used extensively to study starch pasting characteristics for both starch based raw materials and processed food. However, we have found that the use of different grinders for sample preparation can exert a significant effect on the RVA profile when measuring the degree of starch conversion of processed samples even if measurements were made on a defined sieve fraction.

In this study, RVA profiles have been recorded for raw maize grits and two extruded half pellets based on wheat and maize. The material was milled, either with a disc mill or an impeller mill. Differences in the recorded RVA profiles were related to differences in particle properties of the ground products (particle size distribution, particle shape, protein content and starch damage).

Generally, milling the samples with the impeller mill resulted in greater starch conversion than milling with the disc mill as shown for raw maize grits by X-ray diffraction, DSC and alkaline viscosity measurements. Several other laboratory mills were tested and all produced particles, within the sieve range of 125 - 212μm that had substantially differing RVA profiles. Cooling the sample during milling did not nullify the milling effects. All the laboratory mills produced less than 20% of the particulates of the size range required for the RVA analysis.

# PREPARATION AND PROPERTIES OF MODIFIED BANANA STARCH PREPARED BY ALCOHOLIC-ALKALINE TREATMENT

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Granular cold-water-soluble (GCWS) starches were prepared from banana starch treating it with 40 and 60 % ethanol and two controlled temperatures (25 and 35 °C). GCWS starches prepared at 25 °C and with 40 and 60 % ethanol had the lowest cold-water-solubility and that prepared with 40 % ethanol at 35 °C and stored at room temperature showed low tendency to retrogradation, as assessed by transmittance. Solubility and swelling profiles were similar for GCWS starches and the freeze-that stability was increased with the modification. The apparent viscosity of GCWS banana starches was higher than its native starch counterpart.

# THERMOMECHANICAL PROPERTIES AND BAKING EXPANSION OF STARCH DOUGH

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"Polvilho azedo" (sour cassava starch), a natural lactic fermented cassava starch, presents an expansion during baking, which is still not understood. This study was focused on monitoring the baking behaviour of sour cassava starch dough in comparison to those of native cassava and corn starch doughs; dough thermomecanical properties were investigated. The expansion of the sour cassava starch began just after the gelatinisation temperature, whereas the weight loss increased sharply due to water evaporation. Temperature and weight loss of the cassava starches doughs were higher than for corn starch dough. Native cassava starch dough presented an intermediate behaviour. The weight loss during baking, and consequently, the driving force of the expansion mechanism in cassava starch can be attributed to water vaporisation. Thermomechanical measurements performed by Dynamic Mechanical Thermal Analyses showed a drop of the storage modulus for sour cassava starch dough just before the beginning of the expansion. This drop was dramatically lower for the native cassava starch dough and absent for the corn starch dough. This suggest that the expansion mechanism of sour cassava starch is related with the low viscosity dough.

## CHARACTERIZATION OF WATER IN STARCH POWDER BY NUCLEAR MAGNETIC RESONANCE

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The aim of this study is to correlate water activity and NMR data on a model of bound water, mimicking a common food product. Starch is humidified by absorption of water vapour, resulting in activities ranging from 0.25 to 0.96 (Novasina hygrometer). In these samples, water can be slightly or strongly bound to the polysaccharide. The NMR technique distinguish these two pools, through the broad and narrow water resonances of bound or slightly bound components respectively. Off-resonance irradiation partly saturating the protons of the bound water induces a decrease of the intensity of the slightly bound water peak. This approach measures the water exchange as well as the proportions of the two compartments. Comparison of this technique with the classical water activity measurement emphasises the interest of the NMR approach.

## INHERITANCE OF WHEAT STARCH PASTING VISCOSITY

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The presence of the null Wx-B1 allele (for granule bound starch synthase, GBSS) in wheat cultivars has been suggested by several workers as the basis for an increase in the peak pasting viscosity of the starch relative to the wild type. A population of doubled haploid progeny of a cross between parents with different Wx-B1 alleles has been used to study the relationship of starch viscosity to this allele. QTL analysis shows that high paste viscosity is highly significantly linked to markers associated with the Wx-B1 locus on chromosome 4A in this cross. However, there is a wide variety of starch viscosities shown in wheats grouped according to the presence of either the null or normal alleles, with considerable overlap between the two groups. This is true both for this doubled haploid set and for the range of cultivars commonly grown in Australia where the null Wx-B1 locus is common (found in approximately 40% of cultivars). Thus, there must be other genes involved that modify the primary effect of the GBSS allelic composition. Starch viscosities from a number of other wheat crosses have also been examined. The parents for these crosses were chosen based on GBSS allele and the parental starch viscosity. The crosses were between:

Low viscosity null-Wx-B1 X High viscosity null-Wx-B1
High viscosity null-Wx-B1 X High viscosity null-Wx-B1
High viscosity null-Wx-B1 X Low viscosity null-Wx-B1
Low viscosity null-Wx-B1 X Low viscosity normal-Wx-B1
Low viscosity normal-Wx-B1 X High viscosity null-Wx-B1
Moderate viscosity normal-Wx-B1 X Low viscosity null-Wx-B1
High viscosity normal-Wx-B1 X Low viscosity normal-Wx-B1 (2 crosses)

The viscosity of most progeny spanned the range of the parental viscosities with an approximately normal distribution. In the case of the high null X high null cross, the viscosities of almost all of the progeny were higher than the viscosities of the parents. These results suggest that there are other genetic influences on wheat starch viscosity besides the Wx genes. Identification of these genes may help in selecting wheats with starch of predictable viscosity.

## STARCH IN MAIZE

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Maize crops have an essential role as food and feed for human and animal consumption and as a source of industrial products. Products from the maize kernel, especially corn starch, are the basis for nearly all industrial uses of maize. The starch produced in nonmutant endosperms is composed of approximately 25 percent amylose (linear polymers of glucose linked by $\alpha$-D(1$\rightarrow$4 glucosidic bonds) and 75 percent amylopectin (branched polymers of glucose primarily linked by $\alpha$-D(1$\rightarrow$ 4) bonds as well as branches linked by $\alpha$-D(1$\rightarrow$6 bonds).

The advent of routine genetic manipulation of plants in the past decade has made it possible, in theory at least, to increase yield and to provide novel raw materials through alteration of the pathway of starch synthesis . In this context the endosperm mutants, *waxy (wx)* and *amylose extender (ae)*, are two extreme hereditary elements of maize that alter the distribution of amylose and amylopectin in endosperm starch.

The amylomaizes are used mainly for sizing of glass fibres prior to weaving, a component of gummed candies, preparation of a clear hot water dispersible, edible film for packaging foods, dyes, and other soluble materials; and coating paper to reduce water and fat absorption. Amylomaize starch sales are small but growing especially as "chemistry green" project for producing of biodegradable materials in alternative to raw materials derived from petro-chemistry.

As a consequence of the growing demand for maize with a high content of amylose it seems useful to develop more productive *ae* maize hybrids. In this context our Institute is carrying out a research programme for selecting new maize materials with high level of amylose in the kernel.

## HIGH FIELD NMR SPECTROSCOPY ANALYSIS OF BETA-LIMIT DEXTRINS FROM STARCH-LIKE POLYSACCHARIDES: AN APPROACH TO THE UNDERSTANDING OF AMYLOPECTINE CRYSTALLINITY

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Amongst all branched α-1, 4 glucans, amylopectin occupies a very special place. Its specific, well-ordered α-1, 6 branching pattern, originating from a complex enzymatic control, permits an efficient packing of short linear chains within the currently accepted cluster model and allows amylopectin to grow into exceptionally large macromolecules. This peculiar feature leads to unique physicochemical properties including reversible swelling, crystallinity of high water content complexes as well as several others. It is essential to uncover this branching organisation to understand the unique architecture of amylopectin and its relations to some of starch's macroscopic properties. In order to study the branching sequences we have used the beta-amylase degradation on structurally related glucans : glycogen, phytoglycogen, waxy maize amylopectin as well as native wheat and potato starches. The beta amylase degradation trims off all branches by removing maltose residues from the non-reducing ends until it reaches a branching-off point, leaving a short stem of only a few glucose residues. The digestion removes half the mass of the starting glucan. The limit beta-dextrins so obtained are depleted in short chains and proportionally 'enriched' of branched points. The polysaccharides were digested in a frontal stirred ultrafiltration cell equipped with a 1000 MWCO cellulose membrane. The dextrins were washed, concentrated, lyophilised then -OH protons were replaced with deuterium by exchanging twice in $D_2O$. NMR spectra were recorded in a deuterated methyl sulphoxide/ $D_2O$ mixture at 500 MHz. On this poster we will show the changes observed in the proton and 13C carbon resonance frequencies corresponding to the transformation of the native polysaccharides into the beta-limit dextrins. The structural implications will then be discussed.

## RESULTS OF THE NEW TYPE MICRO VISCO-AMYLO-GRAPH

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Presented is the new member of the VISCOGRAPH and AMYLOGRAPH family. The advantages of this new model are the increased heating and cooling capacity to shorten the test time, and the small sample size. The instrument is controlled by comfortable Windows software which also evaluates and documents the tests. Some examples show the comparison of test results done with different starches and flours between the previous models and the MVAG and between shorter and longer test times.

## GENETIC, ENVIRONMENTAL, AND PROCESSING EFFECTS ON PROPERTIES OF HARD WHITE WHEAT STARCH: RELATIONSHIP TO NOODLE QUALITY

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Asian noodles are typically made of hard red varieties of common (hexaploid) wheat, unlike European-style pasta which is made of durum wheat. Physical properties of starch play an important role in determining suitability of a wheat variety for particular types of noodle product. For example, Japanese *udon* noodles (which contain flour, sodium chloride and water) require a high-swelling starch, whereas Cantonese yellow alkaline noodles (which contain flour, alkaline salts, and water) require a lower-swelling starch. There is increasing interest in marketing hard white varieties of wheat from the western United States to Asian noodle producers. We studied the pasting properties of flour of 30 elite hard white wheat genotypes from the University of California, and adapted to Californian production conditions. Two genotypes exceeded the high-swelling check variety, Klasic, in peak viscosity. Others showed particularly low viscosity. Final viscosity was highly negatively correlated to gel chewiness, and peak viscosity was correlated with gel hardness and adhesiveness. A subset of 9 genotypes were grown in three different locations (environments) in California. Klasic showed high variability in pasting behaviour across environments, explaining its reputation for inconsistency in noodle quality. Several genotypes were extremely stable across environments. The effect of several combinations of NaCl, NaOH and $Na_2CO_3$ on pasting of purified starch of 10 genotypes was examined. The relative effects of different treatments were generally similar across genotypes, but there were notable exceptions. Some genotypes showed high resistance to breakdown in the presence of added salts. These effects are important in assessing the use of a variety for different types of noodles (e.g. salted vs. alkaline types).

## CONTROL OF STARCH GRANULE SWELLING: ROLE OF SURFACE MINOR COMPONENTS AND AMYLOSE IN A RANGE OF STARCHES

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Two of the most important features of starch behaviour are (i) the very different swelling properties (rate and extent) exhibited by starches from different botanical origins and (ii) the persistence of granule remnants (ghosts) following complete disruption of polysaccharide order during gelatinisation (Stark *et al.*, 1983; Prentice *et al.*, 1992).

Starches swelling rapidly on heating (cassava, potato, waxy maize) tend to be more shear sensitive and contain less protein (and lipid) than starches displaying a more controlled swelling (wheat, maize). Ghosts of high structural integrity could be readily obtained from protein (and lipid) rich barley, wheat and maize starches but not so easily from protein (and lipid poor) waxy maize or potato starches (Prentice *et al.*, 1992). There is therefore a correlation between lipid/protein content, granule swelling type and ability to yield robust ghosts.

The role of minor components in the swelling behaviour (and granule integrity) was investigated by monitoring the effect of a range of selective extractions of starch samples varying in botanical origin and amylose content.

Extensive extraction by SDS (2% w/v, 20°C) showed that the influence of surface components on starch swelling is highly dependent on starch type. Rapid swelling types and (slow swelling) high amylose starches were largely unaffected, whereas other slow swelling starches were converted to rapid swelling types. SDS and other extracts were characterised by elemental analysis and SDS polyacrylamide electrophoresis and were found to contain proteins and lipids characteristic of the relevant storage organ. Yields of ghosts from gelatinised maize and wheat starches were not significantly affected by prior SDS extraction, but were more fragile.

Mechanisms are proposed to account for the control of starch granule swelling and ghost integrity, based on specific roles for surface minor components and amylose retrograded within swollen granules.

Prentice, R.D.M., Stark, J.R. and Gidley, M.J., Granule residues and "ghosts" remaining after heating A-type barley starch granules in water. *Carbohydr. Res.*, 1992, **227**, 121-130.
Stark, J.R., Aisien, A.O. and Palmer, G.H., *Starch*, 1983, **35**, 73-76.

## USE OF BARLEY MUTANTS TO UNDERSTAND STARCH SYNTHESIS

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The aim of this work is to understand the molecular basis of mutations that cause a decrease in starch accumulation in barley endosperm. Barley mutants with a reduced starch content in their seeds have been generated in several plant-breeding programs in the past (e.g. those at the Risø Institute in Denmark). In some cases, mutants were originally isolated because they had a high lysine content. Subsequent analysis showed that they had a low starch content as well. The low-starch and high-lysine phenotypes proved to be genetically inseparable. Analysis of the phenotypes of double mutants showed that the change in lysine content was a secondary effect of the mutations and that their primary effect was most probably on starch synthesis. Thus, it is highly likely that many, if not all, of the mutations in the low-starch mutants of barley lie within genes encoding components of the starch biosynthetic machinery.

A limited genetic analysis of some of the barley mutants showed that the mutations affect different enzymes in the pathway of starch synthesis. To pin-point the site of action of each of the mutations, we have compared the activities of starch biosynthetic enzymes and the concentrations of certain metabolites in the mutants with those in the wild-type, parental lines. In this poster, we will show the results of a preliminary characterisation of two types of low-starch mutant. One of the mutants has a reduced activity of the plastidial ADPglucose transporter and the other lacks isoamylase activity and accumulates phytoglycogen as well as starch in its endosperm. Analysis of these and other low-starch mutants is furthering our understanding of starch synthesis in barley.

## FUNCTIONAL PROPERTIES OF NON-TRADITIONAL STARCH SOURCES FROM SOUTH AMERICA

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This study was undertaken to evaluate the possibility of using starches from non-conventional sources as alternative solution when specific functional properties are non-satisfied by conventional starches. Twenty two non-cereal starches, among which 17 were from roots and tubers from South America, were evaluated and compared to common starches from cereals, potato and cassava.

Several functional properties, mostly sought by industrial users, were determined: hot paste viscosity, clarity of the gel, resistance to acidic medium (pH 2.4) and to sterilisation (121°C for 2 hours) and stability of paste at -20°C. Several starches presented promising results like starch from *Canna indica* that has very high hot paste viscosity and resistance to sterilisation and acidic condition and starch from *Arracacia xanthorrhiza* that gave clear paste with low syneresis during freezing storage conditions.

The functional properties were related to starch physico-chemical characteristics. Amylose content was strongly correlated to resistance to acidic medium and degree of syneresis. On the other hand, hot paste viscosity was mainly related to starch granule size: the larger the granule, the higher the viscosity. In addition gel clarity appeared positively correlated to hot paste viscosity that reflected the predominance of starch swelling and disintegration during cooking on gel clarity.

## EXPRESSION AND CHARACTERISATION OF POTATO STARCH SYNTHASE ISOFORMS IN E. COLI

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The starch synthases of storage organs exist as multiple isoforms which have different roles in the synthesis of the two components of starch, amylose and amylopectin. An investigation into the properties of the individual isoforms is essential to further the understanding of how the physical properties of starch are determined and how they can be changed.

We are studying the roles of the three main starch synthase isoforms in the potato tuber. Granule-bound starch synthase I (GBSSI) is a protein of 60-kD. It has a low specific activity and is associated with the starch granules. Starch synthases II and III (SSII and SSIII) are 79-kD and 130-kD respectively. They have high specific activity and are found in the stroma as well as associated with the granules. These two isoforms differ from GBSSI primarily in the possession of serine-rich, highly flexible N-terminal domains.

It has long been known that GBSSI activity is required for the synthesis of amylose, whereas SSII and SSIII contribute to the synthesis of amylopectin. To study the properties of the three isoforms individually, we have expressed them in E. coli. Significant differences in activities of the isoforms are apparent which may be important in determining their specficities *in vivo*. To define regions of the isoforms determining these characteristic traits, truncated and chimaeric proteins have been produced. These experiments reveal that the C-terminal region of GBSSI confers most of the specific properties of this isoform and may be important in defining the specificity of GBSSI in producing amylose *in vivo*.

## CHEMICALLY MODIFIED STARCHES AND THEIR APPLICATIONS

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A range of chemically modified starches has been prepared from potato starch by esterification with fatty acid chlorides using a novel homogeneous solubilisation technique.

Degrees of substitution were controlled by metered addition of acylating agent and verified to be typically within the range 0.5 to 2.8. A number of so-formed starches readily formed water-resistant films. Self-plasticising to a degree, these materials are undergoing investigation as potential packaging components with particular application interest in the food sector. Other starches prepared by the same route have vegetable-oil gelling and thermoplastic properties. The preparation of the starches and some physical properties of the films will be reported.

## BIO-NANO-COMPOSITE MATERIALS: MATERIALS MADE OF INORGANIC PARTICLES HOMOGENEOUSLY DISPERSED INTO A BIOPOLYMER (STARCH) MATRIX

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Research in the area of biological renewable materials has shown that the main biological building blocks of life – carbohydrates, fats, proteins, and their derivatives – could substitute products from traditional non-renewable sources. However its realisation will need significant and continuous improvement of those materials. Problems of applications of bioplastics arise not only from the competition with the highly developed synthetic polymers but also from their inadequate properties. One of the big problems connected with the use of most of the natural polymers especially of the carbohydrates is their high degree of water uptake, water permeability or swelling in water. All this contributes to a considerable loss in mechanical properties. Possibilities to decrease the hydrophilicity and to increase the values of mechanical properties so far are: i) application of coating(s) of the processed bioplastic material with hydrophobic materials; ii) blending with different, hydrophobic, biodegradable synthetic polymers (polyesters) and iii) new ways of reactive extrusion of natural polymers (graft- and co-polymerisation, esterification during extrusion process). A new possibility in this direction is seen in the creation of composite materials of thermoplastic organic biopolymer and nanoscopic inorganic particles incorporated on a molecular scale.

## APPLICATION OF RHEOLOGICAL TECHNIQUES TO THE STUDY OF STARCH FUNCTIONALITY IN FOOD SYSTEMS

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Starches used as thickening ingredients in food systems are chosen with functional properties tailored to suit particular processes and product formulations. Chemically modified starches, for example, are often selected to confer a specific functionality *e.g.* shear stability.

In this study we investigate shear stability and the methodology associated with measuring this parameter. The rheological properties of a selection of cereal starches have been studied. Starch pastes were prepared and characterised using a Rapid Visco™ Analyser and Rheometrics controlled strain rheometer.

The sensitivity of the starches to different shear histories has been demonstrated and attempts are currently being made to quantify stability in terms of the energy input during the application of mechanical shear. It is hoped that this work will lead to a better understanding of the influence of shear history on starch functionality and relate more closely to what happens during the food manufacturing process.

## THE ROLE OF GBSSI IN STARCH BIOSYNTHESIS

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It has been known for sometime that GBSSI is the isoform of starch synthase responsible for the biosynthesis of amylose, whereas the soluble isoforms (mainly SSII and SSIII) are involved in the biosynthesis of amylopectin in potato tubers. However labelling experiments with isolated starch granules using ADP [$^{14}$C] glucose have demonstrated that GBSSI can also elongate amylopectin chains. In transgenic potato tubers with very low soluble starch synthase activity (antisense SSIII and SSII/SSIII) starch content surprisingly is unchanged suggesting that GBSSI may be able to compensate for the lack of soluble activity. However amylose content appears to be unchanged suggesting that GBSSI might be making an important contribution to amylopectin synthesis in these tubers. We will present analyses of new transgenic lines which will provide novel information about the role of GBSSI *in vivo*.

## EFFECT OF STARCH PROPERTIES ON PASTA QUALITY OF DURUM WHEAT

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Durum wheat (*Triticum durum* L.) semolina is considered to be the best material for making high quality pasta products. Starch is the major component of semolina. Separation, fractionation and blending of flour components have been successfully used in bread wheat (*T. aestivum*) to analyse the effects of different flour components on bread-making quality, but only a few attempts have been made with durum wheat semolina. In this paper, we describe the reconstitution studies carried out to evaluate the effects of starch properties on pasta quality. The functional properties of reconstituted "semolinas" were evaluated by Mixograph, Extension Tester and RVA parameters and by preparation of pasta using a small-scale pasta extruder. Cooking quality of these pastas was assessed using a Texture Analyser (TA.XT2). Cooked pasta diameter, firmness and stickiness were measured. The effectiveness of the reconstitution procedure was tested by using the components isolated from durum semolina. The effect of other starches was measured by replacing the durum starch from semolina with starches from different origins (*T. aestivum*, *Hordeum vulgare* and *Zea mays*).

## BIODEGRADATION OF 'MATER BI' IN SEA WATER

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The synthesis of new biodegradable polymers for commodities application are developed to realise low environmental impact when disposed of.

'Mater Bi' products is the family of biodegradable thermoplastic materials, based on starch, developed and marketed by Novamont S.p.A. Italy. They are a new generation of bioplastics from natural sources, which retain their properties while in use, and when disposed properly, completely biodegrade. It is known that living microorganisms transform Mater Bi products into water, carbon dioxide, and/or methane[1]

The Mater Bi samples made of starch and poly(ε-caprolactone) and cellulose derivatives were the object of this work.

The degradation of Mater Bi samples in sea water in natural environment and in sea water with sodium azide ($NaN_3$) in laboratory, to eliminate the influence of microorganisms, has been studied.

The characteristic parameters of sea water (temperature, pH, salt and oxygen contents) were measured during the period of degradation and their influence on degradation of Mater Bi has been discussed.

The changes in weight, the tensile strength and morphology of Mater Bi samples were tested during experiment.

After 24 weeks incubation in sea water the weight of Mater Bi samples with cellulose derivatives decreased about 26%, but in sea water with sodium azide18%.

Mater Bi samples made of starch and poly(ε-caprolactone) were completely assimilated after four weeks incubation in sea water in natural environment. But, surprisingly, after the incubation in sea water with sodium azide the weight of Mater Bi samples with poly(ε-caprolactone) decreased much less because of the absence of micro-organisms.

In conclusion we can state that the Mater Bi samples made of starch and poly(ε - caprolactone) are more susceptible to biodegradation in sea water in natural environment than Mater Bi samples made of starch and cellulose derivatives.

1. Information from Novamont S.p.A., Italy

## A POLYMER SCIENCE APPROACH TO RETROGRADATION KINETICS

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The retrogradation of non-expanded extruded waxy maize starch in the rubbery state was studied using proton NMR relaxometry as a function of water content (between 20 and 65 g of water per g of starch) composition and storage temperature.

The Lauritzen-Hoffman[1] theory of crystallisation of chain folded synthetic polymers was combined with:

the equation proposed by Ten-Brinke and co-workers[2] to describe the plasticisation of the amorphous polymers by e.g. water;

the Flory[3] equation (1953) relating the melting temperature of polymer crystallites to the solvent concentration.

This approach has been shown[4] to enable the successful modelling of the retrogradation results in both the temperature and the water content domains over a wide range of composition and storage conditions.

1. Lauritzen, J I and Hoffman, J D *Journal of Applied Physics,* 1973, **44**, 10, 4340-4352.
2. Tne Brine, G Karasz, F E and Ellis, T S *Macromolecules,* 1983, **16**, 244-249.
3. Flory, P J *Principles of Polymer Chemistry*, Cornell University Press, Ithaca, New York, 1953, **541** 594.
4. Farhat I A, Blanshard J M V and Mitchell J R, *Biopolymer*, 2000, in press.

## STRUCTURAL CHARACTERISATION BY NMR OF BRANCHED GLUCOSE OLIGOSACCHARIDES DERIVED FROM AMYLOPECTIN OR GLYCOGEN

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A knowledge of the structures of the smallest dextrins produced by α-amylase action on amylopectin or glycogen is useful in determining the specificity of the enzyme, in particular the restrictions imposed on hydrolysis of α(1-4) linkages by a neighbouring α(1-6) linkage are revealed. Characterisation of larger, multiply-branched dextrins can be a source of information on the distribution of branch points in the original biopolymer. It could be used to compare or screen amylopectins and glycogens from a variety of sources for structural differences, or be of value in fundamental studies of starch biosynthesis. The enzymes used were BLA (bacterial liquefying α-amylase) and BSA (bacterial saccharifying α-amylase).

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## CRYSTALLINE ORIENTATION IN POTATO STARCH GRANULES USING X-RAY MICROFOCUS DIFFRACTION

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Wide angle microfocus X-ray diffraction experiments carried out at the European Synchrotron Radiation Facility have revealed crystalline orientation in potato starch granules analogous to a hedgehog disclination in liquid crystals. Orientation was determined using the (100) WAXS peak characteristic of B-type starch in a series of scans taken at 5 μm steps across the entire granule area. These results show that the amylopectin helices are radially oriented and point towards a non-central location within the granule. This location implies the observations are not an artefact of the scattering geometry. The current hypothesis is that the directors are pointing to the hilum, as inferred by birefringence[1], but further work correlating the data with microscopy is required to confirm this. Both native and a genetically modified potato starch exhibited the same type of radial orientation. We will additionally be confirming the generality of the observation for other starches

1. Blanshard. JMV *'Starch Granule Structure & Function'* in *'Starch: Properties and Potential'*. Ed. T. Galliard. Chichester: Wiley, 1987 p. 117

## EXPRESSION AND COMPLEX FORMATION OF SELF-GLUCOSYLATING PROTEINS

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In mammalian systems, a self-glucosylating protein has been identified as a primer for glycogen biosynthesis. Likewise, starch biosynthesis is thought to be initiated on a protein primer. In our search for starch protein primers, wheat and rice cDNA clones were isolated encoding proteins homologous to a previously isolated self-glucosylating protein, termed reversibly glycosylating polypeptide (RGP1). Based upon their amino acid sequences these proteins can be divided into two classes, RGP1 and RGP2. RGP1 has been implicated in biosynthesis of cell wall polysaccharides. Activity tests with UDP-glucose and plant extracts showed that both proteins have self-glucosylating activity. Our aim is to obtain indications about the role of these proteins in polysaccharide biosynthesis by studying their localisation and interaction with other proteins.

RGP1 and RGP2 were detected in all tissues of wheat, except for leaf where no RGP2 was detected. In young seeds the expression was high and gradually decreased during development. The protein patterns corresponded to the steady-state mRNA levels. Subcellular localisation of RGP1-GFP and RGP2-GFP fusion proteins showed that both proteins are mainly localised in the cytoplasm and not in amyloplasts or cell walls. This suggests that RGP proteins are not directly involved in priming of starch or cell wall polysaccharides.

Gel filtration revealed that RGP1 and RGP2 are present in high molecular weight protein complexes. Within one multimer both RGP1 and RGP2 may be present, since yeast two-hybrid analysis showed that these proteins form homo- as well as hetero-dimers. We performed yeast two-hybrid screening and antibody affinity purification to find other proteins present in this complex. However, we did not obtain indications that this complex contains other proteins besides RGP1 and RGP2.

## STARCH-LIPID INTERACTION STUDIED BY ISOTHERMAL MICROCALORIMETRY AND SURFACE TENSION

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The interaction between starch and lipids that is of great importance in many applications has been studied. The interaction influences the ageing properties of starch. In order to understand more of the interaction the starch-lipid system has been studied with two different methods: isothermal microcalorimetry (IMC) and surface tension measurements according to axisymmetric drop shape analysis (ADSA). The interaction between lipids and the different polymers of starch, amylose and amylopectin, was compared. Each starch polymer was investigated at three different concentrations.

Isothermal microcalorimetry measures the heat produced when gelatinised starch solution interacts with lipids at different concentrations, while ADSA measures the amount of lipid interacting with the starch at the same conditions. The two methods give a detailed understanding of the starch lipid interaction. The interaction between lipid and the different starch polymers has both similarities and differences. The total amount of lipid that will bind to the starch polymer samples is the same but the binding isotherm for the different samples differs. The interaction between amylopectin and lipids is similar to the interaction between amylose and lipids but the energy produced with amylose is higher than with amylopectin. The starch lipid interaction has no or very low critical association concentration (cac). The absence of cac indicates that the added lipid starts to interact already at low concentrations.

## FURTHER OBSERVATIONS ON THE POSSIBLE INVOLVEMENT OF DEBRANCHING ENZYMES IN STARCH BIOSYNTHESIS

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During the last decade, there have been several suggestions that debranching enzymes (DBEs) are involved in the biosynthesis of amylopectin, and play a role in converting pro-amylopectin into amylopectin by a trimming mechanism, in which about one-half of the outermost branch points in the precursor are hydrolysed. In higher plants, two types of DBE have been found. The first is limit dextrinase (incorrectly described as pullulanase by certain authors), which has a high affinity for α-dextrins and a much lower affinity for amylopectin. The second is isoamylase, which can completely debranch amylopectin and phytoglycogen, but has a low affinity for α-dextrins. A partial deficiency of one of both of these DBEs in certain maize and rice mutants is believed to cause the synthesis of phytoglycogen rather than starch. This idea is erroneous, since limit dextrinase has no action on phytoglycogen type structures, whilst isoamylase is the most effective DBE known and is widely used for chain profile analysis; it is not suitable for the selective hydrolysis of only a proportion of the branch points in a polysaccharide substrate. Moreover, extracts of ungerminated oats contain a much higher level of limit dextrinase than other cereals, but still produce a normal two component starch.

Some of the recent evidence for DBE involvement is based on genetic analysis for genes that code for DBEs during the early development of starch synthesising plant cells. By itself, this is not proof that the enzymes are active during this stage. Most starch degrading enzymes are produced in inactive forms by proteolysis.

The overall conclusion from this, and much other work, is that the degree of branching in amylopectin is controlled by the interactions of various isoforms of starch synthase and branching enzyme, and that DBEs do not play a part in this process.

## SOME ASPECTS OF THE STRUCTURE AND BIOSYNTHESIS OF STARCH - BACK TO BASICS

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The current literature contains several examples where authors have exercised considerable licence in their interpretations of experimental facts, and the resulting diagrams could give rise to misleading conclusions. Examples include (a) the depiction of the outer chains of amylopectin containing several helices, whereas the analytical results show an average outer chain length of ca. 15 glucose residues, equivalent to only 2-3 helices; (b) molecular structures for amylopectin showing many outer chain lengths; (c) diagrams of cluster models for amylopectin which do not show that a proportion of the outermost B-chains carry more than one A chain; (d) discussions of starch granule structures on the basis that the major part is crystalline, although experimental measurements show the degree of crystallinity of many A-type starches is 33-45% and for B-type starches, only 15-28%; (e) models for starch biosynthesis showing roughly equal numbers of amylose and amylopectin molecules, whereas in fact, there are ca. $10^2$ molecules of amylose per molecule of amylopectin. It follows that the biosynthesis of amylose is numerically, a much more frequent process than that of amylopectin.

Whilst it is well known that amylose has an inherent tendency to form double helices in solution, it is not generally realised that amylopectin shows a related but more complex tendency to alter its conformation when in an environment of high protein content, and form granules, as shown by K. Doi and A. Doi in Japan as long ago as 1969 (Denpun Kogyo Gakkaishi, 17, 89-97). These granules can show either A- or B-type X-ray diffraction patterns, depending upon the temperature. This observation has implications for the biosynthesis not only of amylopectin, but also of the two-component starch.

## DIFFERENTIAL SCANNING CALORIMETRY STUDY OF THE GELATINISATION OF WHEAT STARCH IN THE PRESENCE OF SUGARS

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The theory of starch gelatinisation is that the structural complexity of starch gives rise to a non-equilibrium melting process. The native granule of starch is a layered structure of amorphous and crystalline regions. Melting of crystalline domains is limited by the kinetically controlled plasticisation of the predominant amorphous domains. The concept of glass transition which immediately precedes the true melting of starch crystallite has been established[1].

We have previously suggested[2] that the effects of varying starch concentration and maltose content of the temperature of melting of native potato starch crystallites reported by Lelievre[3] could be understood in terms of the role of water in depressing the gelatinisation temperature of starches. In his study, Lelievre followed melting through the loss of birefringence using a cross polarised light microscope with a heating rate of 1°C/h. The objectives of this present study was to assess: (1) the applicability of this observation to wheat starch - sucrose on the gelatinisation temperature and (2) the effect of heating rate on the gelatinisation thermograms.

As anticipated, the native wheat starch suspension in excess water showed a typical gelatinisation peak at 57°C when heated in DSC pans at 10°C/min. The position of which showed little dependence on water content in the range water/starch 1:1 to 4:1.

While, at 10°C/min, the addition of sugar increased the gelatinisation temperature (peak temperature of DSC endotherm) by up to ~14 K at water/solid (solid=starch+sugar) ratio of 1:1 and a sugar/starch ratio of 1:8, the increase was less than 5K at higher water contents (water/solid~4.5:1). The gelatinisation temperature was also affected by the heating rate and the effect was related to the well known timescale dependence of the glass transition temperature.

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## EFFECTS OF LIPIDS ON THE GELATINISATION AND RETROGRADATION OF RICE STARCH IN RELATION WITH COOKED RICE

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The cooked rice, Japanese staple food, has a plain taste. Recently, Japanese consumers are concerned with quality of cooked rice, and want to buy high quality rice.

It is well known that textural properties especially stickiness and hardness are important factors for palatability of cooked rice. Usually textures of cooked rice are changed by rice variety and growing district. Since there are many components in rice, texture of cooked rice is affected by the interaction of starch and other components.

Fat content is 3% and fat-by-hydrolysis content ranges from 0.5% to 0.7% in the brown rice. Since the fat is a minor component in rice with comparison to starch and protein, there have been very few studies on the effect of fat on the physical properties of cooked rice. Effects of fat on the gelatinisation of rice grains and rice starch were studied by differential scanning calorimetry (DSC) in the present work.

Two varieties of 90% polished rices, Koshihikari and Matsumae were used. Koshihikari was estimated to be highly palatable, whilst Matsumae was not. At Isihkawa Agricultural Research Centre, rice grains of Koshihikari and Matsumae, and rice starch were defatted by methanol-chloroform. Ten rice grains samples with different defatting times were prepared : 0,0.5,1,2,4,6,8,10,12,24 hour. Ten rice grains of each sample and water of 1.4 times of rice grain's weight were sealed in the DSC cell of 1.5 ml, hermetically. The temperature was kept at 25°C for 10 min and then raised at 0.5°C /min to 120°C.

Heating DSC curves for rice grains-water mixture showed two endothermic peaks Peak 1 and Peak 2 around 60°C ~ 90°C, in addition to other two endothermic peaks Peak 3 and Peak 4 around 90°C ~ 120°C. Peak 3 and Peak 4 were attributed to the disintegration of amylose-lipid complex.

In both varieties of defatted rice and rice starch, Peak3 and Peak4 shifted slightly to lower temperatures, but Peak 1 and Peak 2 shifted remarkably to lower temperatures. Endothermic enthalpy of Peak increased whilst that of Peak 2 decreased with increasing defatting time.

## THE EFFECT OF ENDOGENOUS AMYLASES ON THE MOLECULAR WEIGHT AND PASTING PROPERTIES OF SWEET POTATO STARCHES

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The molecular weight and pasting properties of starches, from freshly harvested and commercial sweet potatoes imported from Uganda and Israel respectively were investigated. Starches were extracted with and without 0.01% mercury chloride, as an enzyme inhibitor. Total amylase ($\alpha$ - and $\beta$ -) activity in the sweet potato roots was determined by high performance size exclusion chromatography (HPLC-SEC) coupled to a multi angle laser light scattering refractometer detector, viscometry (intrinsic viscosity), and final viscosity as one of the Rapid Visco Analyser parameters. Starches (Kawogo variety) with the lowest amylase activity, 0.199 μmol/min had high molecular weights as indicated by the high HPLC-SEC and intrinsic viscosity results in comparison to starches (Soroti variety) with high amylase activity, 0.212 μmol/min. Starches treated with mercury chloride had higher molecular weight $12.09 \pm 0.05$ and $8.986 \pm 0.2 \times 10^5$ g/mol compared to starches treated without mercury chloride for Kawogo and Soroti starches, respectively. Flours from sweet potato will be extruded and the effect of initial high amylase activities on the performance of the product is being assessed.

## FUNCTIONAL PROPERTIES OF THERMOPLASTIC SAGO STARCH

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Sago starch was plasticised in a twin-screw fully intermeshing counter-rotating extruder at 15% moisture content in the presence of plasticisers (glycerol and urea). Temperature, rotor speed and urea concentration were varied accordingly. The effects of urea concentration were examined. Tensile properties of the melt, aqueous solubility and water binding capacity varied with urea addition. The melt exhibited pseudoplastic behaviour at 0.12 phr urea content. However, at 15 phr urea content, departure from pseudoplasticity was observed. Urea addition to sago starch led to browning of the extrudate and variation

in the water binding capacity, aqueous solubility and tensile properties respectively. Moisture sorption in all the formulations increased with increase in equilibrium relative humidity. Scanning electron microscopy (SEM) showed good disintegration and evidence of brittle failure of thermoplastic starch.

## EFFECTS OF SOME POLYSACCHARIDES ON THE GELATINISATION AND RETROGRADATION OF CORN STARCH

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Effects of the addition of curdlan on gelatinisation and retrogradation of corn starch were examined by rheology and DSC. Complex Young's modulus E* and the stress-strain curves of cylindrically moulded gels of corn starch with and without curdlan were observed as a function of the concentration of curdlan and the storage time at 5°C. Both fracture stress and E* increased whilst fracture strain decreased with increasing storage time as has been observed for many starch gels. Fracture stress and E* of gels containing curdlan increased faster than those of gels without curdlan at the initial stage (after more than 10 days). From the temperature dependence of E*, it was found that both storage and loss moduli increased with increasing storage time at 5°C, but both moduli of gels stored for a long time decreased dramatically with increasing temperature up to 55°C which is lower than the gelatinisation temperature. These results suggest that the main molecular forces which are responsible for the network formation are hydrogen bonds. Once the structure formed during the storage is broken by heating, the structure was not recovered by lowering the temperature within the time scale of the experiment. Differential scanning calorimetry for corn starch suspensions with curdlan of various concentrations was carried out. The temperatures of gelatinisation and re-gelatinisation and the endothermic enthalpy as a function of concentration of curdlan and storage time were examined. The gelatinisation temperature shifted slightly to higher temperatures by the addition of curdlan, however, the gelatinisation enthalpy per unit mass of starch did not change so much. Effects of curdlan on the syneresis were also observed. These experimental results are compared with those observed for corn starch systems with other hydrocolloids such as konjac glucomannan and xyloglucan using the same methods.

## CHARACTERISATION OF THE SUBSTITUENT DISTRIBUTION IN HYDROXYPROPYLATED POTATO STARCH

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Starch is often chemically modified to obtain new products with specific properties for use in different industrial areas. The chemical and functional properties achieved when modifying starch depend on e.g., reaction conditions and molar substitution, but are also affected by the distribution of substituents. It is essential to study the relationships that exist between modification procedure, structure, and final properties, which requires reliable methods for determination of the substitution pattern in these derivatives.

In this work, hydroxypropylated potato amylopectin starch modified in *i)* a granular slurry (HPPAPg), or *ii)* a polymer "solution" (HPPAPs), was investigated. The substituent distribution in the anhydroglucose unit (AGU) was determined by gas chromatography and mass spectrometry (MS). In order to investigate the location of substituents along the polymer chain, HPPAP was hydrolysed by enzymes with different selectivities. The hydrolysis products obtained were characterised by e.g., chromatographic and mass spectrometric techniques. Comparison between the products obtained from enzymic hydrolysis of HPAPP and those obtained from hydrolysis of unmodified, native starch made it possible to get some information about the substituent distribution.

There was no significant difference between the molar distribution of hydroxypropyl groups in the AGU in the two derivatives. Enzymic debranching of the HPPAP samples showed that the enzyme action was hindered by the hydroxypropyl groups. This hindrance was more pronounced in HPPAPg, indicating differences in the localisation of substituents around the branching points. Simultaneous exo- and endohydrolysis of HPPAPg liberated more unsubstituted glucose units than did HPPAPs, indicating a more homogeneous distribution of substituents in HPPAPs. In addition, matrix assisted laser desorption/ionisation MS and high-performance anion-exchange chromatography coupled to electrospray MS were shown to be useful techniques for identification of both unmodified and modified mono- and oligosaccharides in the hydrolysates.

## CRYSTALLINITY AND MORPHOLOGY OF AMYLOSE AND AMYLOPECTIN BLENDS

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Starch is an interesting raw material for a wide variety of renewable and biodegradable plastic materials. Applications ranging from films to microporous materials can be made from starch or its components, amylose and amylopectin. The roles of the different components and their influence on each other in films are under investigation.

Films of starch, amylose, amylopectin and blends thereof were made by solution casting and examined using X-ray diffraction, microscopy and differential scanning calorimetry. Amylopectin films were found to be amorphous while amylose films had a relative crystallinity of 33%. Blends of amylose and amylopectin showed an increasing degree of crystallinity with an increasing amount of amylose, up till 25% amylose, and were thereafter constant at around 34%. We believe that the amylose present in the blends influenced the amylopectin to start crystallising, possibly in part by a co-crystallisation mechanism. The appearance of an endotherm from crystallised amylopectin was found in DSC scans at around 110°C. The enthalpy of the endotherm increased with an increasing amount of crystallised amylopectin in the amylose-amylopectin blends. The endotherm also increased in enthalpy and peak temperature as the water content decreased. Light microscopy showed phase separation between amylose and amylopectin when the amylose content was low, but no phase separation was observed when the amylose content was 25% or higher. This was probably a result of amylose forming a continuous network and preventing phase separation when the amylose concentration was sufficiently high. The influence of amylose on amylopectin thus affect several properties in the films.

## STRUCTURAL PROPERTIES OF NOVEL STARCHES

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In this study we investigate the granule structure and gelatinisation behaviour of natural and mutant starches. Analytical techniques include small and wide-angle x-ray scattering (SAXS and WAXS), differential scanning calorimetry (DSC) and environmental scanning electron microscopy (ESEM). A broad selection of natural starches from many different botanical sources is examined along with a series of waxy maize starches with an additional mutation to the amylose extender gene. It is hoped that this work will lead to a deeper understanding of the causes of the variations in both structure and behaviour of different starches, and help in the process of finding natural starches with interesting and useful functionality.

Results show that the mutation to the amylose extender gene in waxy maize starch, which increases the length of the amylopectin branch chains, also results in a B-type WAXS pattern, and a severe disruption to the inter-lamellar SAXS peak. This corresponds with an increase in the gelatinisation temperature and a broader gelatinisation endotherm. Results from some of the starches from different botanical sources reveal the many possible variations in granule shape and size, x-ray scattering profiles and gelatinisation temperatures.

## CHARACTERISATION OF SBE-A AND SBE-B FROM POTATO

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Starch branching enzymes (SBEs) are responsible for making the alpha 1-6-branch points in the growing amylopectin molecule. They are probably responsible for the chain-length distribution of the starch together with starch synthesising enzymes and debranching enzymes. We have characterised SBE-A and SBE-B from potato using *in vitro* methods and a novel substrate. The enzymes used have been over-expressed and purified from *E.*

*coli*. The *in vitro* methods used are iodine assays with amylose and amylopectin as substrate; gel permeation chromatography (GPC) and high performance anion exchange chromatography (HPAEC) with the novel substrate. The novel substrate consists of linear dextrins with a quite narrow molecular weight distribution as opposed to amylose and amylopectin.

We have been able to show that the enzymes differ significantly in their activities; SBE-A is more active with amylopectin as a substrate and SBE-B is more active with amylose. Both enzymes are stimulated by the presence of phosphate. After incubation with SBE-B the enzyme products of the novel substrate were analysed by HPAEC which revealed two populations of chains.

## CHARACTERISATION OF STARCH GRANULAR FRACTIONS FROM DIFFERENT WHEAT FLOURS

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Multivariate regression (PLS2) has been used to relate the starch granule size distribution to the response surface coefficient matrices for bread characteristics loaf volume, weight, form ratio (height/width) and crumb firmness. The models obtained explained 55% and 48% of the total variance in the response surface coefficients for bread weight, and form ratio respectively. No models were obtained for crumb firmness and loaf volume. The correlations obtained indicated that high weights and form ratios were promoted by A-granules and, further studies to investigate the relationship between the bread loaf characteristics and the properties of starch and starch fractions of wheat flours were undertaken.

In the present study starch and starch fractions were purified from 6 different wheat flours with the same protein content. For each starch fraction the starch granular size distribution, swelling properties and paste viscosity was measured. In addition the chain length of amylopectin was obtained using high performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD). The results from the analyses of the properties of the purified starches were related to the properties of the bread loaves by multivariate statistical methods.

## PRODUCTION OF HEAT-STABLE, BUTYROGENIC RESISTANT STARCH

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Butyrate formed by glucose fermentation in the large intestine is both substrate of the energy metabolism and important signal metabolite for colonocyte growth. Butyrogenic resistant starch d type III (RS) preparations require a distinct polymer chain length of 10 - 35 glucose units. Such α 1,4-D glucans were synthesised *in vitro* with amylosucrase in aqueous solution with different sucrose concentrations. The content of RS could be increased from 34 to 94% in dependence on the retrogradation conditions. The procedure was optimised in respect to various parameters like temperature, polyglucan concentration in the gel, polymer chain length distribution and fermentability. High concentrations and relatively high recrystallisation temperatures (about 25°C) favour the formation of heat-stable RS with DSC-To and -TP values 110°C. The fermentation of these RS led to *in vitro* short chain fatty acid levels (acetate, propionate, butyrate) of 2000-25000 μmol/g faeces dry weight with butyrate contents of 30-60-%. Readily fermentable heat-stable RS, which can be used in various "functional foods," can be produced from poly-1,4-1-D-glucan of optimal chain length.

## TOWARDS UNDERSTANDING THE INVOLVEMENT OF PHOSPHORYLASES IN WHEAT STARCH METABOLISM

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Major uncertainties compromise the understanding of the physiological function of plant α-1,4-glucan phosphorylases (EC 2.4.1.1). Although the activities and transcript levels of especially plastidic phosphorylases often appear to increase upon starch synthesis and thus suggest a role for the enzyme in starch synthesis, antisense studies have not shown reduced expression to significantly affect leaf starch accumulation. The functions of cytosolic phosphorylase forms can only be speculated upon.

These uncertainties highlight our interest in the hitherto neglected phosphorylases of the important starch producing crop plant wheat. Wheat leaves contain a single plastidic and two cytosolic phosphorylase isoforms, which are also evident in roots and seeds. We are purifying these enzymes and isolating the respective genes to explore the role of phosphorylase in seed starch synthesis and degradation. A partial sequence of the gene encoding the plastidic isoform is being used as a probe to monitor transcription levels in developing, resting and germinating seeds. The plastidic wheat form shows strong substrate inhibition in starch degradation assays, and its presence in only developing seeds indicates it to be indeed involved in reserve starch synthesis. Since the cytosolic isoforms are particularly evident in germinating seeds, they may complement amylases in effecting reserve starch breakdown.

# A STUDY OF THE GELATINISATION OF WHEAT STARCH USING ATTENUATED-TOTAL-RELECTANCE (ATR) FOURIER TRANSFORM INFRA-RED (FTIR) SPECTROSCOPY

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The structural changes of native starch during heating, in excess and limited water content, are a complex range of phenomena occurring at different levels of order: short range order (molecular level) or long-range order (packing of the polysaccharide chains). Methods used to study the gelatinisation of starch are typically applicable at one of these levels of order. DSC (Differential Scanning Calorimetry) widely accepted as the standard method to follow the gelatinisation of starch, monitors the energy required to alter the starch during the heating process. However, the manner in which the disruptions of the different levels of order within the starch the starch granules are reflected by the DSC endotherm is questionable.

Loss of short-range order during starch gelatinisation can now be followed , *in-situ*, by an ATR-FTIR (Attenuated Total Reflectance - Fourier Transform Infra-Red) method and the results can be directly compared with DSC results. A starch suspension can be heated, without loss of water, while structural changes relating to the loss of short-range order are quantified using FTIR spectra. The attachment of an IR polarizer to the ATR-FTIR cell enable to concurrently follow the loss of short-range order plus the loss of longer-range order that give rise to the birefringence properties of starch granules.

Wheat starch was heated (1°C / minutes) at several moisture contents (70%, 55%, 45%, 35% water(W/W) and structural changes followed by ATR-FTIR and DSC. The ratio of the absorbances of the IR bands at 1045 and 1022 cm $^{-1}$ was used to quantify the loss of short-range order[1,2]. In excess water (70% water), the loss of short-range order was recorded in the exact range of temperatures where the single DSC endotherm, called G, occurs. At intermediate moisture contents (55% to 33% water) the DSC profile exhibited a double endotherm, with a tail M1, added to the single endotherm G. In these conditions, the loss of short-range order was observed to take place in the range of temperatures where G but also M1 were detected. Results obtained using the IR polarizer suggest that for wheat starch suspension, the DSC endotherm recorded during the gelatinisation was more dependent on the loss of short-range order than on the loss of long-range order.

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# ANALYSIS OF D-ENZYME FUNCTION IN POTATO

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D-enzyme (4-α-glucanotransferase, EC 2.4.1.25) acts upon 1, 4-α-D-glucans, breaking and reforming α-1,4 links. Malto-oligosaccharides, amylose and amylopectin can serve as donor or acceptor molecules for this reaction and glucose can also serve as acceptor, but maltose is not a substrate for D-enzyme[1]. D-enzyme is found in numerous tissues, but is particularly abundant in potato tubers[2]. There are several possible functions for D-enzyme in starch metabolism. It could create malto-oligosaccharides to serve as substrates for starch synthase or branching enzyme. It could participate in recycling of products of debranching activity[3] or it could be involved in facilitating the phosphorylitic breakdown of starch. Potentially D-enzyme could be involved in starch synthesis, breakdown or both.

We have transformed potato with sense and antisense copies of the D-enzyme cDNA and created plants with a range of D-enzyme activities, some as low as 1% of the amount in wild type (Takaha et al., 1998). Plants with lowered D-enzyme grow slower than controls and the yield of tubers is often lower. Despite this, tuber starch content and composition appear normal. Recent work on Arabidopsis D-enzyme mutant (Critchley et al., unpublished) has shown plants lacking D-enzyme grow slower and the leaves show a reduced capacity to degrade starch in the dark, thus linking D-enzyme to a role in starch breakdown.

Current experiments are aimed at investigating effects of lack of D-enzyme on activities of other enzymes of starch metabolism, and at measuring starch breakdown in the leaves of potato plants with lowered D-enzyme.

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# PHYSICO CHEMICAL CHARACTERIZATION OF AMAZ, A NEW THERMOREVERSIBLE GELLING STARCH PRODUCT

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New or enhanced functionalities of starch may be obtained by enzymatically changing structural properties of this macromolecule. For this, glucosyltransferases can be used such as phosphorylases (elongation of amylopectin side chains) and branching enzymes (increase number of branching points). In this study the effect of enzymes able to change the side-chain distribution of amylopectin was investigated. α-1,4-α-1,4 glucosyltransferases (amylomaltase: AMase) may be suitable to achieve this type of modification.

The effect of AMase treatment was studied comparing the structure of the original material (potato starch) and resulting product (AMase modified potato starch: AMAZ). The molecular properties of AMAZ were characterised with anion exchange chromatography with a pulsed amperometric detector (HPAEC) and size exclusion chromatography with both light scattering and refractometric detection (SEC-MALLS-RI). Results obtained with debranched material show that amylose has disappeared, and that the side-chain length distribution of the amylopectin is modified into a more homogeneous one.

As shown by further physico-chemical characterisation of AMAZ, these changes in the molecular composition of starch are accompanied by distinctly changed gelation characteristics. Repeated heating and cooling of an AMAZ suspension resulted in changes in the storage modulus (G') markedly different from that observed with the unmodified material: conversely to normal starch, AMAZ shows a gelatin-like thermoreversible behaviour.

These results demonstrate that changes in the amylopectin molecular structure brought about by incubation with α-1,4-α-1,4 glucosyltransferases convert starch into a product with new functionalities: a high molecular weight material, with low viscosity at high temperature, that behaves as a thermoreversible gel (WO 9815347).

# EXTRACTABILITY AND SIZE DISTRIBUTION STUDIES ON WHEAT STARCH USING FLOW FIELD FLOW FRACTIONATION

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Although starch is one of the most studied components of wheat flour, its molecular mass and size, which are fundamental physical characteristics, are not easily determined. Thus it has been difficult to develop an understanding of the relationship between starch structure and its functional properties in end-use products such as bread and biscuits. Three problems had to be overcome. First, a method had to be found to solubilise starch polymers without degrading or altering their polymeric characteristics. Secondly, appropriate calibration standards, with defined molecular mass of the same order of magnitude as starches, had to be found. Thirdly, reliable methods had to be developed to measure the size and shape of these very large molecules. This work aimed to solve these problems. FFF (Field flow fractionation) was applied as the method of choice for molecular size determination of the macro-polymers. Optimum solubilisation was achieved by varying sonication conditions, extraction times and solvents. Four minutes sonication in a water/ethanol solution (1.8 mL distilled water + 50 µl of 95% ethanol) gave complete solubilisation of starches with 0 - 22% amylose content. The standard carbohydrates pullulan, beta-glucan, polyethylene oxide and dextran, with molecular weight up to 40 million Daltons, were used to develop reliable standard curves for the analysis of starch size and mass. Each standard set of carbohydrate polymers had a different linear relationship between molecular mass and retention time. This result demonstrates that carbohydrates having different bonds between glucose units give different retention time and thus it is the first evidence that FFF could distinguish differences in shapes of biopolymers as well as sizes. The standard curves were used to estimate the molecular weights of starches with different amylose content. Molecular sizes determined by the FFF were 7.6 - 8.3 (in retention time units), equating to molecular weights of 4.7 - 19.7 million by comparison with the dextran standards.

## GENETICS OF WHEAT STARCH GRANULE SIZE DISTRIBUTION

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Wheat endosperm starch granules have a bimodal distribution. Type A granules are around 25 µm in diameter and B granules around 5 µm, with the separation at 10 µm. B-granule content adversely affects efficiency of harvest of starch in starch/gluten manufacture and increases water absorption of flour so may affect end-use quality, including biscuit baking times, bread loaf staling and noodle texture, in various ways. The genetics of this trait was therefore investigated.

Crosses within *Triticum turgidum*, tetraploid wheat, and *Triticum aestivum*, hexaploid wheat, showed that B-granule content was determined by endosperm genotype and largely by additive gene action. Dominant gene action was also significant and could act either to increase or to decrease B-granule content, depending on the source. Epistasis was significant and the effect of cytoplasm was minor but significant.

Molecular marker studies, breeding initiatives and end-use tests are in progress.

## STARCH GRANULE DISTRIBUTION IN WHEAT AND ITS WILD RELATIVES

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Wheat endosperm starch granules are initiated in two phases during development, leading to a bimodal distribution with peaks at around 25 μm in diameter (A granules) and around 5 μm (B granules). The evolution of this trait was investigated.

A survey of over 1000 genotypes of 20 *Triticum* and *Aegilops* species showed a wide range in B-granule contents (5 - 60% of starch) and A-granule sizes (13 - 40 μm modal diameter). *T. monococcum*, a diploid species, had the smallest A granules and the greatest content of B granules, and the primitive tetraploid *T. turgidum* var. *dicoccoides* was almost as extreme. Other subspecies of *T. turgidum* and also triticale had similar starch granule size distributions to hexaploid breadmaking wheat. Many *Aegilops* species had similar peaks at 5 μm and 25 μm but had a slightly lower content of B granules than most domesticated wheats. Rye and Ae. *tauschii* had similar modes but some larger A granules. Ae. *crassa* and Ae. *peregrina*, tetraploid species, had large A granules and few B granules.

## GLASS TRANSITION BEHAVIOUR OF AMORPHOUS POTATO STARCH FORMED BY BALL-MILLING

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It is known that the ball-mill treatment converts native potato starch to amorphous state, however, the physical properties for the converted amorphous state have not been understood. For example, there is no report about the existence of glass transition, the transition temperature. In this study, by using DSC (Differential Scanning Calorimetry) and WAXD (Wide Angle X-ray Diffraction), it was confirmed that the ball-milled amorphous starch exhibits a glass to rubber transition accompanying with the recovery phenomena of relaxed enthalpy. Such transition behaviour was different from that of the glassy starch formed by heat and quenching method. Further, it was suggested that the ball milling process for potato starch progresses the enthalpy and volume relaxation with breaking the crystalline inner native potato starch

## PROBLEMS AND SOLUTIONS OF MEASURING THE MOLECULAR WEIGHT OF RICE STARCHES

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Molecular weight is one of the most important physical properties underpinning amylose and amylopectin polysaccharide behaviour. A requirement of most methodologies for determining molecular weight is the complete solubilisation of both amylose and amylopectin. A combination of solvents and the use of enzymes facilitate this for rice starches. Debranching enzymes have been used as a first step in determining the amylopectin structure of three rice starches. The solubilised material was separated by size exclusion chromatography and detected by light scattering and concentration detectors (SEC/MALLS). The absolute molecular weight of amylose ($4.8x10^5$ - $5.1x10^5$ g/mol) and the relative $M_w$ (weight average molecular weight) of the chains of amylopectin from three rice starches were estimated.

If the samples were not debranched but dissolved in dimethysulphoxide (DMSO), it was difficult to obtain sufficient separation using SEC/MALLS. This was even more difficult if processed starches were investigated using this technique. The same solvation technique was used for samples studied by analytical ultracentrifugation (AUC). The results with high rotor speed showed very good separation of starch components. The sedimentation coefficient ($s^o_{20,w}$), for amylose, which is related to its molecular weight, was 5.3 - 5.7 Svedberg. The sedimentation coefficient of amylopectin was found to be time dependent. The sedimentation coefficient ($s_{T,b}$) (at a particular concentration) obtained from the extrapolation at time = 0. We found that it is possible to obtain $M_w$ of amylose from whole rice starch by using low speed sedimentation equilibrium: ~5 x $10^5$ g/mol, was obtained for native rice starch.

The AUC method has also been applied to rice starches subjected to different durations of thermal treatment or acid hydrolysis. In both these cases the treatment was seen to have an effect on the amylopectin. The apparent sedimentation coefficient of amylopectin shifted towards that of amylose as the processing conditions were increased.

## AMYLOPECTIN STRUCTURE AND AN ACTIVITY OF STARCH METABOLISING ENZYME IN RICE ENDOSPERM ARE CONTROLLED BY THE *ALK* LOCUS

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The differences in chain-length distribution of amylopectin have been observed in endosperm starch of rice. The amylopectin in most of *japonica* rice varieties has shorter chains (DP6 to 11) than *indica* varieties.

The locus which controls the chain-length distribution was determined by gene mapping analysis using 98 backcross inbred lines (BILs) derived from a cross between a *japonica* variety, Nipponbare and an *indica* variety, Kasalath. The gene was mapped at the *alk* locus on chromosome 6. The *alk* locus has been known as one of the qualitative trait loci of rice and is responsible for the varietal difference, especially between *japonica* and *indica* rice varieties, in the disintegration property of endosperm against an alkali (KOH) solution. It has been reported that the differences in alkali disintegration is correlated with the gelatinisation temperature of rice starch and the cooking property of rice.

Zymogram analyses for starch metabolising enzymes in endosperm were conducted with the same BILs to find out the responsible enzyme for the structural alteration of amylopectin. Polymorphisms in the mobility or the intensity of activity bands were detected with 6 isoforms of starch metabolising enzymes. The genes which regulate the polymorphisms were mapped with one of the genes mapped at the *alk* locus. The presence or absence of the starch metabolising enzyme in BILs corresponds to the patterns of chain-length distribution of amylopectin. Where the activity was present, amylopectin from the BIL showed chain-length distribution similar to Kasalath and when the activity lacked, amylopectin structure was similar to that of Nipponbare, enriched in shorter chains.

It is highly possible that *alk* gene, the gene that controls the chain-length of amylopectin, and the gene that controls the activity of one of starch metabolising enzymes mapped at *alk* locus, are identical and the property of alkali disintegration is regulated by the starch metabolising enzyme via the structural alteration of amylopectin.

## INTRODUCTION OF ACETYL GROUPS IN WAXY MAIZE STARCH DOES NOT AFFECT ITS SUSCEPTIBILITY TO ANNEALING

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Crystallinity, gelatinisation behaviour and susceptibility to annealing of acetylated waxy maize starches, with molar substitutions (MS) ranging from 0.00 to 0.19 were studied. Waxy maize starch crystallinity decreased in a non-linear way with increasing MS. At lower levels of substitution, the crystalline register was relatively unaffected by the chemical derivatisation, suggesting that, under such conditions, substitution primarily occurred at the amorphous amylopectin branching points. At higher MS, semi crystallites were increasingly destabilised, as indicated by the rapidly decreasing overall crystallinity.

Differential scanning calorimetry (DSC) analyses in excess water showed lower onset ($T_{onset}$) and peak temperatures of gelatinisation and lower enthalpy values for the more heavily substituted starches. Under limited moisture conditions, the G-endotherm of starches with higher MS had a lower enthalpic content and occurred at lower temperatures.

The acetylated waxy maize starches were annealed (at temperatures 8-16°C below $T_{onset}$). Analysis of variance revealed that: (1) the gelatinisation temperature of the annealed starches was clearly affected by MS and by the annealing temperature (or more precisely: $T_{onset} - T_{annealing}$); (2) the influence of MS and temperature of annealing were mutually independent. The first observation implies that the position of glass transition of the acetylated starches is clearly a function of MS, suggesting an internal plasticisation mechanism. The second observation indicates that the introduction of acetyl groups, predominantly in the amorphous phase, does neither hamper, nor facilitate the annealing of waxy maize starch.

When DSC and X-ray results for acetylated waxy maize starch were combined, it became clear that both $T_{onset}$ and gelatinisation enthalpy decreased more than would be anticipated based on the changes in crystallinity. Apart from diminishing the crystallinity of the raw starch and lowering of the glass transition temperature, acetylation thus also reduces the thermal stability (melting/dissociation temperature and enthalpy) of either the remaining semicrystallites or the non-crystalline amylopectin double helices.

## CORRELATION BETWEEN GELATINISATION BEHAVIOUR AND STRUCTURAL PROPERTIES OF RICE STARCHES

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Rice starches were isolated out of 15 milled rice samples with genetically widely differing backgrounds using a pronase based isolation procedure. The gelatinisation behaviour of the purified starches was investigated in excess water by differential scanning calorimetry; wide angle X-ray data were collected; relative double helix content was examined using CP/MAS $^{13}$C-NMR; degree of polymerisation (DP) was studied by high performance anion exchange chromatography; amylose contents were determined.

A large variation in gelatinisation temperatures and enthalpies was observed, as expected. Waxy and low amylose starches exhibited larger relative crystallinities than regular starches. Similar observations were made for relative double helix contents. The gelatinisation enthalpies correlated more strongly to the relative crystallinity than to the relative double helix content. When dissociation enthalpy of the amylose-lipid complexes was subtracted from the gelatinisation enthalpy an even stronger correlation between enthalpy and relative crystallinity was observed.

Factor analysis revealed that the corrected (as above) gelatinisation enthalpy, gelatinisation temperature, and the ratio of the fraction with DP 6-12 to the fraction with DP 13-24 were the most important variables to explain variance among rice starches. This analysis again indicated that gelatinisation enthalpy was correlated stronger to relative crystallinity than to relative double helix content.

## EXPRESSION AND CHARACTERISATION OF THE POTATO R1 PROTEIN IN *E. COLI*

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Potato starch is characterised by its high content of covalently bound phosphate as compared to e.g. cereal starches. The phosphate groups are monoesterified to the hydroxyl groups at carbon C-6 (70%) or C-3 (30%) in the glucose moiety of the amylopectin molecules in starch. The biosynthetic pathway leading to phosphorylated starch is still not known.

Recently a protein called R1 believed to be involved in starch metabolism and phosphorylation has been identified[1]. We have inserted the cloned gene into a thio-fusion expression vector for *E. coli* expression. *E. coli* cells expressing R1 store more glycogen than wild-type cells. The expression of R1 also leads to an increase in phosphate content and changes in the branching pattern of the resulting glycogen. Activities of several of the glycogen biosynthetic key-enzymes are altered in the cells harbouring R1 in comparison to wild-type cells. Also activities of amylolytic enzymes are altered in these cells.

The obtained results will be discussed in relation to glycogen and starch phosphorylation and carbohydrate storage and mobilisation.

1. Lorberth R, Ritte G, Willmitzer L, Kossmann J, *Nature,* 1998, **16**, 473-477.

## GENETICS OF STARCH PRODUCTION

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Peas (*Pisum sativum* L.) are an excellent system for studying the genetics of starch synthesis - there are many conventional and molecular markers and it has a large seed, making it extremely useful for chemical and biochemical studies. Using chemically-induced mutants, six loci have been identified that encode genes affecting the content and composition of starch. The mutants have all been purified by backcrossing to the original line used for the mutagenesis and re-selecting through 6 generations, so that they now represent a unique set of isogenic material differing at single loci. Such material permits comparisons to be made in the starch between lines and the differences to be attributed to changes at a single locus. Furthermore each locus is represented by multiple alleles representing subtle changes in starch composition. The mutants are of both substrate-supplier and starch-synthesising types. Both types, however, modify the composition and function of the starch, since reducing the substrate supply can affect substrate partitioning between the synthases, branching and debranching enzymes that are temporally regulated.

The presence of mutations at five of the loci, the *rugosus* mutants - *r*, *rb*, *rug3*, *rug4* and *rug5* - result in the dry seed attaining a wrinkled appearance. Mutations at the sixth locus, *low amylose* (*lam)*, do not affect the shape of the dry seed, which resembles the round-seeded wild type in appearance. The enzymes affected by each of the loci influencing starch composition have been characterised and include starch synthases, sucrose synthase, starch branching enzyme and plastidial phosphoglucomutase, mutants in the last having the most severe effect on starch by making the seed virtually starchless. The genes encoding the enzymes have all been cloned and sequenced, including at least one mutant allele at each locus. In the case of alleles at the *r* locus, the severity of effect on starch can be related to the position of a mutation within the gene, those outside of the main active site being less severe than those at or near the active site.

We are currently studying the effects of gene interaction on starch composition by combining mutations from different loci to produce double mutant lines. Except for combinations with *rug3*, in all *rugosus* double mutant combinations the starch content is reduced below that of either single mutant. In any combination containing a *rug3* mutation, the *rug3* phenotype dictates the final starch amount. Double mutants between *r* and *lam* mutants have starch levels much lower than that found in either single mutant, while the *lam/rug5* double mutant contains an amount of starch that is very similar to *rug5*. Starch grain shapes and iodine staining properties are dramatically affected in some combinations. Data will be presented which covers the detail of the phenotypes of the mutants as a prelude to investigations of granule structure and gelatinisation presented elsewhere.

## STRUCTURE AND PROPERTIES OF POTATO STARCH

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Normal (non-waxy) potato starch granules are different from cereal starches in terms of composition (they contain little or no lipid but relatively high levels of amylopectin phosphorylation), fine structure of amylose and amylopectin, crystallinity (B-type as opposed to A-type), relatively large granule size and, large swollen volume when heated in water. Although lipid does not *directly* interact with exterior chains of amylopectin to modify gelatinisation and swelling properties, the formation of amylose-lipid complexes in cereal starches is associated with increased gelatinisation temperatures but decreased swollen volume. Because of this complication, the crystallisation of amylopectin during starch biosynthesis and annealing is much easier to investigate with potato starches grown under the same conditions – particularly when a range of amylopectin phosphorylation is studied.

To facilitate understanding of the starch granule structure, twelve potato varieties were grown under the same conditions in Perthshire during 1997 and harvested at maturity. Starches were extracted from the tubers and subjected to rigorous physico-chemical analysis. The starches were annealed according to a matrix of starch:water ratio, temperature and time to identify how crystallinity could be perfected in these starches and the molecular events underpinning this process.

Annealing conditions were optimised using a commercial potato starch and, using these optimised conditions, the crystallisation of the amylopectin molecules within the different starches was investigated. The optimum condition for annealing potato starch was established to be 55°C, at least 80% moisture (w/w) and for at least 7days. On average, the onset of gelatinisation by DSC ($T_o$) increased from 59.1 to 70.3°C, the peak ($T_p$) from 63.2 to 72.8°C and the conclusion temperature ($T_c$) from 70.3 to 78.4°C. This was associated with a decrease in the gelatinisation range from 11.1°C for the native starches to 8.1°C post annealing. The corresponding average enthalpy of gelatinisation for the native starches was 18.6 J $g^{-1}$, and 20.6 J $g^{-1}$ post annealing.

Whilst we believe that the primary factor which controls the development of starch order is the registration of amylopectin double helices, this process is influenced by the amylopectin phosphorylation. The relative significance of these effects will be discussed in relation to starch functionality.

## ANALYSIS OF LOW LEVELS OF NITROGEN IN STARCH BY DUMAS METHOD USING ELEMENTAR VARIO MAX

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In the production of high quality starch, the nitrogen levels are measured to assess the degree and efficiency of starch extraction. For many years the standard method for nitrogen measurement was the kjeldahl method. This method however, is slow, inefficient and environmentally unfriendly. Here is described a new instrument, the vario MAX that utilises the Dumas combustion method. Samples are analysed in less than 8 minutes by the clean and easy method. The measuring range is from less than 50 ppm Nitrogen upwards.

## INDENTIFICATION OF THERMAL TRANSITIONS IN LOW MOISTURE STARCH SYSTEMS BY PREPARATIVE DSC

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Thermal processing of starch product at low moisture condition is an established technique applied to the manufacture of starch-based bioplastics and expanded food products. DSC can be used to monitor the thermal transitions during processing. However, DSC thermograms of low moisture starch systems are complex, featuring three transitions for cereal starches and four transitions for potato starch. In order to identify those transitions, the concept of 'preparative DSC' was developed and implemented.

Preparative DSC is a method which allows the study of starches which have been subjected to a precisely defined thermal treatment without inadvertent moisture loss. To that aim, the sample is loaded into a hermetically sealable stainless steel DSC cup in which a silicon ring is inserted. This allows the sample to be easily withdrawn after the run. The sample (typical size 20 mg) is then ready for characterisation of its molecular and physical structure and properties by e.g. microscopy, micro-ATR-FTIR, SEC-MALLS-RI, and miniaturised swelling and solubility measurements.

Potato Starch at 16% moisture content features four endotherms at ca. 60, 120, 155 and 190 °C which are denoted as LT, M1, M2, and HT, respectively. The LT endotherm originates in enthalpy relaxation as was elucidated by time and temperature programmed ageing experiments[1]. After heating the sample to a temperature between LT and M1 and cooling, the glass transition is revealed in a second heating scan[1]. Samples with and without silicon ring had similar DSC traces.

For identification of M1, M2, and HT, cups were heated to 142, 178 and 210°C and opened after cooling. Birefringence loss was nearly complete after M1 and complete after M2. The individuality of the starch granules was preserved even after M2. However, the swelling capacity after heating the sample in excess distilled water at 92°C strongly decreased from 82 ml/g for untreated potato starch to 13 ml/g after M1 and 5 ml/g after M2, whereas solubility was 24% for native starch, 9% after M1, and 13 ml/g after M2. This suggests that a severe heat-moisture treatment has occurred in this temperature range. After HT the sample had a glossy appearance and its density was decreased from 1600 to 1400 kg/m3 as judged from micropycnometry. The pellet appeared to contain numerous air bubbles. It showed complete solubility after heating in excess water at 92°C with M = 274 kDa, which indicates that significant molecular degradation has occurred on heating to 210°C. The magnitude of the HT endotherm was strongly dependent on the packing density of the sample which suggests that this endotherm is related to a volume change in the sample rather than to a thermal transition. Our results demonstrate that HT in potato starch is related to the transition of individual gelatinised starch granules into a melt.

Maize starch at 16% moisture showed only three transitions at ca. 60, 155, and 190°C. Traces for samples with and without silicon rings did not coincide. Furthermore it was observed that substantial birefringence was retained at the start of HT. Therefore, preparative DSC cannot be used for maize starch without some adjustments in sample preparation conditions.

1. H.J. Thiewes, P.A.M. Steeneken, *Carbohydr. Polymers*, 1997, **32,** 123-130.

# Subject Index

# Author Index

Starch, in its many forms, provides an essential food energy source for the world's human population. It is therefore vital for manufacturers (and ultimately consumers) to have increased understanding of the granule synthesis and its behaviour in modern food processing.

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