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REVIEW ARTICLE

Starch: as simple as A, B, C?

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Abstract

Starch is the main carbohydrate reserve in plants and an important part of our nutrition. Increasingly, it is being seen by industry as a useful raw material to include in foodstuffs and with which to produce other carbon-based polymers. Our understanding of starch biosynthesis and chemistry has advanced rapidly over the last few years, but our knowledge of how this translates into structure and thence into physico-chemical properties and function is still lacking. Here, we have reviewed this information with an emphasis on genetics and physical properties, especially using data from the model crop, pea (*Pisum sativum* L.).

Key words: Genetics, pea, *Pisum sativum* L., physical properties, starch.

Introduction

Starch is the dominant carbohydrate reserve material of higher plants, being found in leaf chloroplasts and in the amyloplasts of storage organs such as seeds and tubers. At normal temperatures starch granules are extremely insoluble in water and exert a minimal effect on the osmotic pressure of the cell. Granules and their properties, especially the nature of the rings, have exercised scientific curiosity for hundreds of years. Van Leewenhoek used them as one of his subjects in his seminal work on microscopical discoveries (Fig. 1; Van Leewenhoek, 1719). In fact, it could be said that he carried out the first experiments on starch gelatinization:

'I have frequently repeated the experiment of placing a portion of thefe globules of meal, no larger than a grain of fand, upon a clean glafs; and, after pouring a drop of water on them, brought it to the fire. After the water and globules were heated, and the moifture was evaporated, the globules affumed a flat fhape, very like that of cakes ...' (from Hoole, 1800).

Most of the starch utilized world-wide comes from a relatively small number of crops, the most important being maize, potato, wheat, and tapioca with smaller amounts from rice, sorghum, sweet potato, arrowroot, sago, and mung beans. In general, starches from tapioca and sorghum are used only for food, whereas those from maize, potato and wheat are used for food and other, non-food, purposes. Within Europe, the main sources of starch are maize, wheat and potato, although in Sweden and Finland small quantities of oats and barley are used for non-food purposes (Batchelor *et al.*, 1996).

Starch forms the main source of energy in the human diet and comprises on average about 30% of the UK diet by weight, mainly in the form of bread and pasta products produced from milled wheat seeds. In general, native starch is rapidly digested in the gastro-intestinal tract and very little passes into the colon for fermentation by bacteria. Most starch, however, is consumed after various cooking processes, which disrupt the starch granules. The molecular reassociation following these treatments results in the starch becoming more resistant to digestion. Unlike native starch, this resistant starch is not readily digested

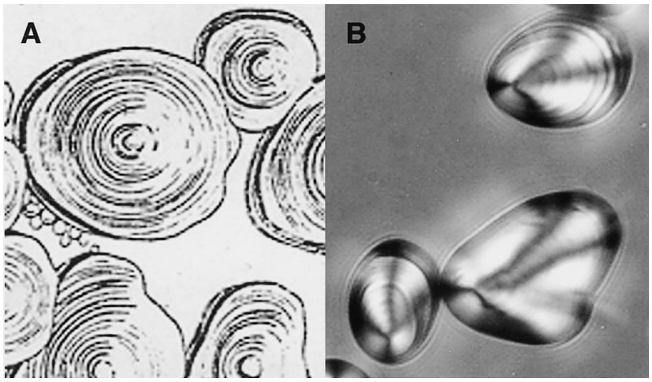

Fig. 1. Starch granules. (A) An image of wheat grains as drawn by van Leewenhoek (1719) observed using the first microscope. (B) A modern image of potato starch granules viewed under polarized light.

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and so much of it becomes available for fermentation in the colon. Fermentation is characterized by the production of small chain fatty acids, especially butyrate, which are believed to affect the metabolism, structure and function of epithelial cells lining the large intestine such that they may prevent several colonic diseases, including colon cancer. The susceptibility of native and cooked starch to digestion and fermentation is determined by the plant source and by genetic variation affecting starch composition (Liljeberg, 1995).

Although the main role of starch is its inclusion in the diet as a high-calory food source, it is also used in food manufacture since it improves the functional properties of foods such as gelling and pasting. Starch pastes and gels are used to control the consistency and texture of sauces, soups, dressings, and spreads. The rheological properties required to provide this texture control have to survive freeze–thaw cycles, conditions of acid pH and heat pasteurization or ultra-high temperature sterilization. They also need to resist the breakdown of the gel or the loss of water (syneresis) during the high shear experienced in processing and during the shelf life of the end product. To achieve these properties most starches are chemically modified by introducing inter-chain cross links or other functional groups (Wurzburg, 1986). Other gelling agents, consisting of plant or microbial non-starch polysaccharides may also need to be used. Much of the starch utilized in the processed food industry is hydrolysed to produce glucose, fructose, maltose, and syrups for drinks and confectionery.

Maize starch is popular amongst processors because there is a guaranteed supply from the USA. There is also a wide range of genetic variation available in maize, producing starches with different chemical and functional properties. The by-products remaining after starch extraction from maize are mainly protein and oil, both of which are commercially valuable. The main disadvantages associated with the use of maize are that the starch is relatively difficult to extract and separate from proteins and it has a relatively high level of associated lipids (*c.* 1%), which affects the starch functional properties (Swinkels, 1992). Also, within Europe there is the additional factor that virtually all maize starch is imported.

The main alternatives to maize starch for processors are wheat and potato. One of the major reasons why wheat is becoming more popular is the demand for the protein co-product, gluten, and the need to develop new uses for grain surpluses within Europe. A major problem with wheat as a source of starch is that there is a lack of genetic variation. Furthermore, wheat starches contain two populations of different-sized starch granules (A and B), making purification of the starches difficult and affecting their functional properties. There is a substantial market for potato starch in Europe, but most of this is taken up by industries in the non-food sector. The functional properties of potato starch are generally regarded to be superior to those of wheat starch, this being attributed to the very low lipid levels in potato starch (Swinkels, 1992). The by-products following starch extraction from potatoes are, however, of little commercial value and, as with wheat, there is little genetic variation available to exploit. There are also major problems with the harvesting, transport and storage of potatoes.

In the future, grain legumes, in particular peas, could become another source of starch for the processor. Up to 50% of the mature pea seed is starch, the remainder being mainly protein and fibre, both of which are commercially valuable. The physical structure of legume starches is different from that of cereal and potato starches and this will be discussed later. Legume starches also have improved functional properties. For example, pea starches have a higher level of solubilization and have restricted starch grain swelling compared with cereal and potato starches. Suspensions of pea starch also maintain a high viscosity when heated and cooled and the viscosity is stable when the suspensions are stored at high temperature (Stute, 1990; Hoover and Sosulski, 1991). In addition, the level of available genetic variation in pea greatly exceeds that of wheat and potato and is comparable to maize. Pea has therefore been established as a model for the study of starch biosynthesis and properties.

Although the greatest proportion of processed starch is used in food, there is also a significant market for starch in non-food industries. In Europe, more than 80% of the starch produced from potatoes is utilized in this sector of industry, although the largest amount of starch for this purpose still comes from maize. The pattern of industrial applications of starch has changed with time, with those uses in older industries, such as textiles, being replaced by new ones in developing industries, for example those producing biodegradable plastics. The new applications have developed as new types of starches have become available. For example, the plastics industry requires starches that have small granules and are high in amylose. This is one area where pea starches are beginning to make an impact through the existence of a high amylose mutant (*r*), which will be discussed in more detail later.

A major challenge will be to predict the effects of genetic changes on the functional properties of starch and hence to produce designer starches to suit specific uses. To achieve this long-term objective will require a multidisciplinary approach involving geneticists to provide genetically-characterized lines, biochemists to determine changes in starch biosynthesis, analytical chemists to analyse changes in the molecular structure of starch, and physical chemists and physicists to understand starch granular structure and the interactions within the granule which contribute to the functional properties of the starch.

Many studies have utilized genetic variation to understand starch biochemistry and the link with the chemical composition of starch. There is a gap, however, in understanding how changes in starch biochemistry and chemistry affect starch granule structure and starch functional properties. In part, this gap in our knowledge can be attributed to a poor understanding of starch granular structure. The main aim of this review, therefore, is to present information on granular structure and where possible to relate structural changes in the granule to genes affecting starch content and composition. Such an understanding would allow the genetic manipulation of starch granular structure directly without a detailed knowledge of the link with biochemistry and chemistry. This approach requires a wide range of well-characterized genetic material. Another major aspect of this review, therefore, is to present an assessment of the genetic variation in pea which could be used for this purpose. The ideas are supported with information on the chemistry, biochemistry and biology of starch, and sufficient information is provided to allow the reader to understand each section in turn, although these sections are not meant to be exhaustive and the reader, therefore, is referred to the reviews of the subjects cited therein.

Chemistry

Starch can be fractionated into two types of macromolecule, amylose and amylopectin. The history and supporting evidence for these two separate structures within the starch granule has been well documented elsewhere (Whistler and Daniel, 1984) and hence will not be covered here. Amylose is an essentially linear molecule of molecular weight between 5×10^5 to 10^6 and is composed of anhydroglucose units connected through $\alpha(1,4)$ linkages. Amylopectin has a molecular weight of several millions and is a much branched polymer formed by anhydroglucose units linked $\alpha(1,4)$, but additionally with 2–4% $\alpha(1,6)$ linked branches (Hizukuri and Takagi, 1984; Takeda *et al*., 1984, 1986). Starches from most species are composed of about 30% amylose and 70% amylopectin, although, as discussed in detail in later sections, mutations affecting starch biosynthesis can dramatically affect the amount of both molecules in the starch granule.

At present, the most widely used techniques for studying the chemical structure of starch are based on analysing the size distribution patterns of the glucose chains comprising the branches. Such studies utilize amylolytic enzymes, such as β-amylase, which degrade glucose chains to maltose, and debranching enzymes, such as pullulanase and isoamylase, which cleave branch points in the molecules. The use of such debranching enzymes, followed by size exclusion chromatography, has shown that amylose does contain some branches and that the frequency and length of these branches differs according to plant species. For example, wheat amylose has an average branch number per molecule of 1.9 with an average chain length (degree of polymerization, DP) of 300, whereas potato amylose has an average of 7.3 chains per molecule and an average chain length of 670 DP (Takeda *et al*., 1984, 1986). This compares to branch points every 20–25 glucose units for amylopectins from most species. Size exclusion chromatography also has been used to separate amylose from amylopectin and to show that amylose from different plant species varies in molecular weight (Hizukuri and Takagi, 1984). For example, amylose from wheat has an average size DP of 570 (i.e. 300×1.9 branch points) compared with potato amylose which has an average size DP of 4920 (i.e. $670 \times c.\ 7.3$).

Hizukuri (1986) used enzymes to debranch amylopectins, followed by size exclusion HPLC to determine the branch size distribution. Using this method it was possible to separate the chains into two main peaks, one with an average DP of 11–16 and the second with an average DP of 40–45 (Hizukuri, 1986). This study, together with others based on similar types of analyses, contributed to the formation of the generally accepted 'cluster' model describing the three dimensional structure of amylopectin (Fig. 2; reviewed in Manners, 1989). This model proposes that the amylopectin molecule is made up of three broad classes of glucose chains, A, B and C, the A chains bind in clusters only to B chains, B chains bind to other B chains or to a C chain which has a reducing end and of which there is one per molecule. Hizukuri's (1986) analysis showed the A chains were the shortest and the B chains, the longest. This analysis also subdivided the B chains into B1–4, the B4 group containing the longest chains.

A second method for analysing the chain length distributions from debranched amylopectin was developed by Ammeraal *et al*. (1991). This method uses anion exchange chromatography together with a pulsed amperometric detector to separate chains with specific DP values. This method, however, can detect chain lengths up to a DP of 30–40 only, because there is a fall off in the sensitivity of the detector with increasing chain length. Size exclusion and anion exchange chromatography have both been used extensively to determine the effects of mutations at different steps in the starch biosynthetic pathway on the chemical composition of starches (see sections on Genetics and on Biochemistry). An example of this type of analysis is given in Fig. 3, which illustrates the effects that the *r* mutation in pea has on amylopectin structure. Although the profiles containing individual polymers (Fig. 3B, D) look very similar there are differences in the polymers around DP15 (Lloyd *et al*., 1996*a*). Size exclusion chromatography revealed a distinct shoulder between DP15 and 22 in the *r* mutants which was much less pronounced in the wild type (Fig. 3A, C). Colonna and Mercier

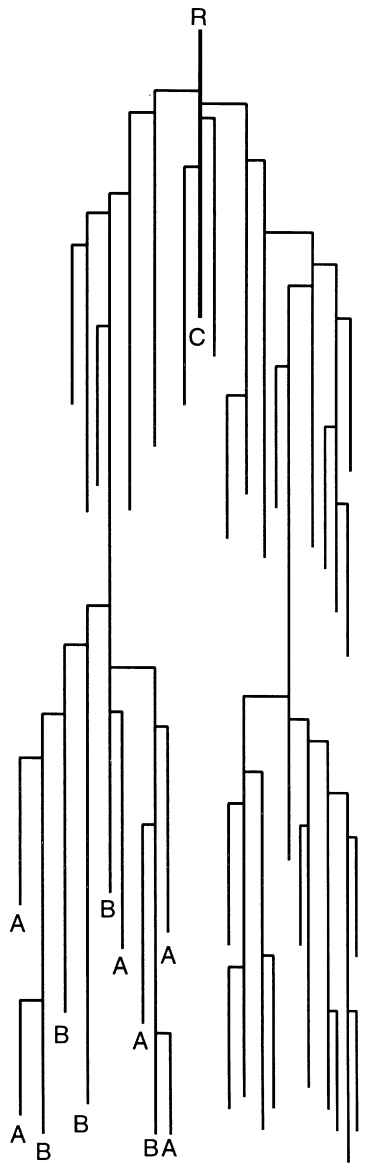

Fig. 2. A cluster model for the arrangement of amylopectin chains: (A, B) indicate the position of A and B chains in one cluster and (C) indicates the position of the C chain in the molecule with its reducing end, R. Based on Manners (1989).

(1984) reported that this mutant produced a starch fraction similar in size to amylose, but branched like amylopectin. This fraction was termed 'intermediate material' (see Genetics section).

The most common chemical characterization of amylose and amylopectin relates to their interaction with iodine. Amylose reacts with iodine to give a bluish colour while the colour given with amylopectin is reddish brown. This difference relates to the affinity that each compound has for iodine, amylose binding on average 20% of its weight of iodine at 20 °C, whereas amylopectin binds less than 0.2% (w/w). The difference in the ability to bind iodine has been used by chemists to define these two compounds (Banks and Greenwood, 1975).

At a molecular level the different reactions to iodine reflect the relative differences in the frequency of branching between amylose and amylopectin, decreased frequency resulting in long chains of glucose moieties or a high DP for each chain. Chains with a DP of 20 or less, commonly found in amylopectin, have a very weak interaction with iodine and those with a DP of 200 or more, usually associated with amylose, having a very strong interaction. When iodine binds to amylose it brings about a conformational change in the molecule, changing it from a flexible coil to a helix. The conformational change observed when iodine binds to the long amylose chains is also important because it forms the basis of another technique for separating amylose from amylopectin, using small hydrophobic molecules, such as butan-1-ol and thymol. These small molecules cause similar comformational changes in the amylose to those brought by iodine and result in the amylose being preferentially precipitated from solution (Ring *et al.*, 1993).

Some species produce amylopectins that have a proportion of constituent chains which are considerably longer than DP 20. For example, some rice amylopectins have chains of 85–180 DP and these are able to bind iodine (Takeda *et al.*, 1987), which results, therefore, in erroneous estimates for amylose/amylopectin ratios based on iodine absorption. Similar anomalies have been noted in some high amylose mutants where it is believed that much of the amylose may be a loosely-branched amylopectin (see Genetics section). Hence iodine binding can be used only as a guide to the nature of the polymer and not a method of classification. The interactions between the polymers and iodine is extremely complex and almost a complete science in itself (Yu *et al.*, 1996).

Biochemistry and molecular biology

Intricate detail of the pathway and the enzymes involved will not be presented here since it has been reviewed many times in recent years (Martin and Smith, 1995; Nelson and Pan, 1995; Preiss and Sivak, 1996; Smith *et al.*, 1997). Indeed in the most recent critique (Smith *et al.*, 1997), it has been stated that our understanding of the enzymes has outstripped the understanding of the nature and regulation of the processes in which they are involved and that predictive models are now required. It could be argued equally that our understanding of the relationship between the enzymes and the function and properties of the starches they create lags even further behind. It is this relationship that is crucial to any rational manipulation of starch. The current understanding of the general pathway and some of the species differences that have been identified from recent

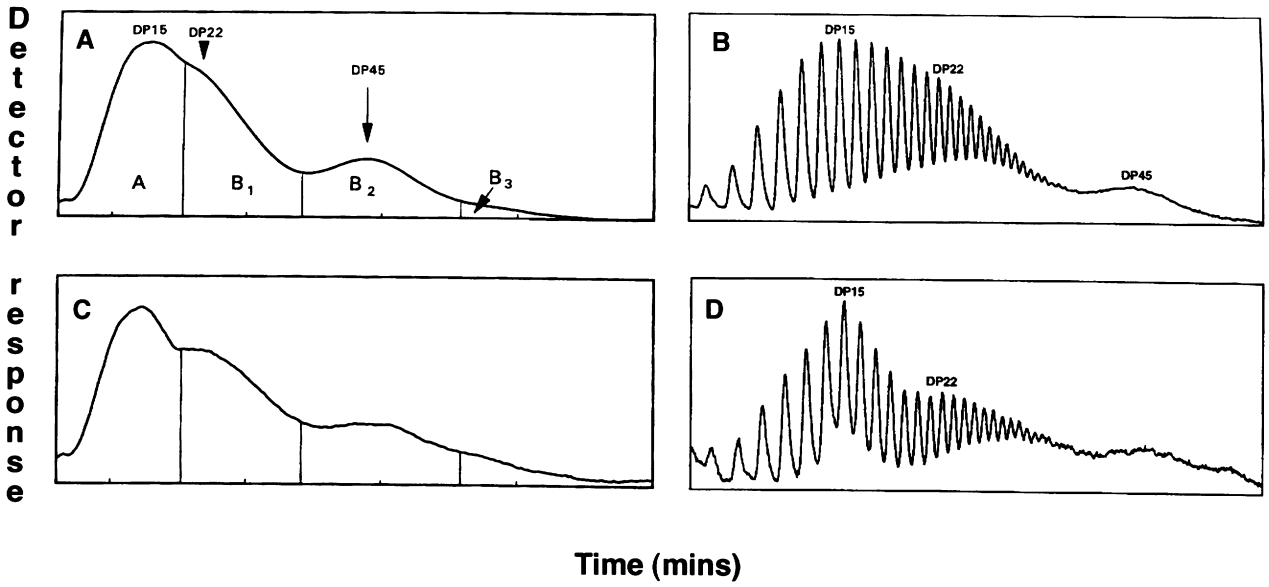

Fig. 3. Chromatographic separations of amylopectin chains in pea starch: (A, C) size exclusion chromatography of wild type and *rr* mutant amylopectin respectively; (B, D) high performance anion exchange chromatographic separation linked to pulsed amperometric detection of constituent chains of wild type and *rr* mutant, respectively. A and B_1–B_3 denote the areas used by Hizukuri (1986) to distinguish differences between starch types. DP (degrees of polymerization) indicate the chain lengths of individual fractions.

biochemical and molecular studies (Martin and Smith, 1995) and our own genetic data is presented here.

There has been widespread acceptance that three enzymes are involved in the synthesis of starch in the amyloplast—ADPglucose pyrophosphorylase, starch synthase and starch-branching enzyme. The pathway for the production of starch is shown in Fig. 4. The first enzyme in the pathway is responsible for the synthesis of the substrate in all plant tissues (Smith *et al.*, 1997) and the other two for the production of the starch polymers. Evidence for an alternative pathway (Okita, 1992) is much weaker and is not supported by current information from mutant analysis. ADPglucose pyrophosphorylase is also considered by many to be a key enzyme in the regulation of the pathway since it is allosterically regulated by both inorganic phosphate (an inhibitor) and 3-phosphoglycerate (an activator) (Preiss and Sivak, 1996). Furthermore, it may be an important enzyme by which one can manipulate crop yields. Modification of the enzyme in different ways can lead to either an increase or a decrease in starch content (Müller-Röber and Kossman, 1994). For example, the maize mutant, Rev6, in which ADPglucose pyrophosphorylase activity is decreased and displays reduced sensitivity to inorganic phosphate, has an increase in seed weight without any decrease in seed number (Giroux *et al.*, 1996). The enzyme consists of large and small subunits encoded by small multigene families, the genes showing both temporal and spatial differences in expression. This variation may mirror the various ways in which starch synthesis may be regulated (Smith *et al.*, 1997). The small subunits are considered to be the site of catalysis, whereas the large subunits modulate the sensitivity of the small ones to allosteric regulation (Preiss and Sivak, 1996).

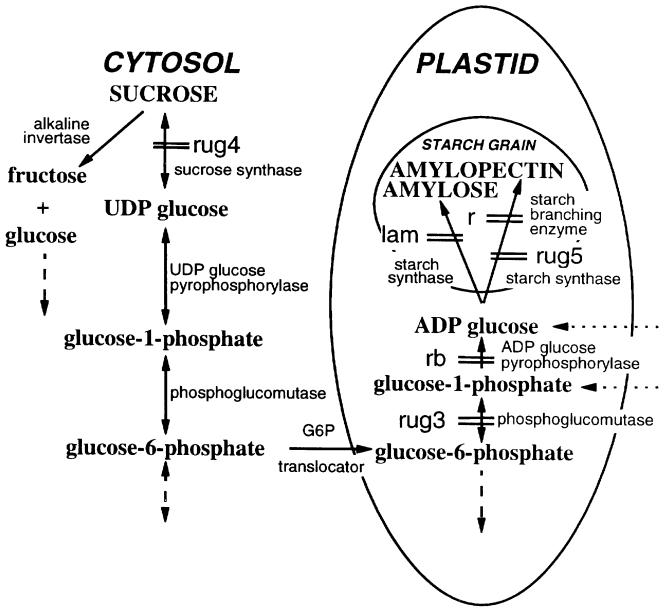

Fig. 4. The pathway of starch biosynthesis in pea. The lesions in the pathway represented by the pea mutants cited in the text are indicated by double lines. The dotted lines indicate substrates that can be imported into the plastid in other species (see text for further details).

Amylopectin and amylose polymers are generated by the action of starch synthases and starch-branching enzymes. There are a number of different isoforms of each enzyme, some of which show differences in organ-

specificity and temporal regulation, but the contribution of the different isoforms to the overall activity in most tissues is not known. Both soluble and granule-activities are present in plant tissues, but whether an enzyme is exclusively or partly associated with the starch granule appears to be an intrinsic property of the protein rather than the starch, as indicated by experiments using transgenics (Edwards *et al*., 1996; Smith *et al*., 1997). The major isoform of the granule-bound synthases, GBSSI, is believed to be responsible for the synthesis of amylose (Smith and Martin, 1993; Preiss and Sivak, 1996), since its elimination in a number of mutants (see below) greatly decreases the amylose content of the starch. There is evidence in pea, however, that there are other isoforms that potentially could take on this role, for example, in pea pods (Denyer *et al*., 1997; see later). Furthermore, there is evidence from mutants that GBSSI is also involved in amylopectin synthesis since the nature of the amylopectin in these mutants differs from that of the wild type (Delrue *et al*., 1992; see also *rug5* below). Starch-branching enzymes are responsible for creating the α-1,6 linkages in amylopectin. The enzymes from a number of different plants fall into two classes based on their primary sequence (Burton *et al*., 1995), yet no clear role for the different isoforms has emerged to date (Smith *et al*., 1997). In pea, the branching enzymes, SBEI and SBEII, are maximally active at different times during development, SBEI being earlier. Deficiencies in SBEI lead to a decrease in starch content whereas those in SBEII are believed not to do so (Burton *et al*., 1995) since no mutants of this type have been isolated (see the *rugosus* loci, below).

Debranching enzymes are also considered to be important in the synthesis of starch (James *et al*., 1995; Ball *et al*., 1996). Debranching enzyme-deficient mutants of maize, rice and *Chlamydomonas* have been isolated and all accumulate phytoglycogen, so-called since it is more similar to the animal carbohydrate reserve, glycogen, than to starch, in that it possesses a greater proportion of α(1,6) linkages and is therefore more highly branched. The action of the debranching enzymes has been suggested to cause 'preamylopectin trimming' to generate an outer layer of short-chained polymers upon which soluble starch synthases can act to create longer chains required as a substrate for starch-branching enzymes (Ball, 1995; Mouille *et al*., 1996). The production of preamylopectin is considered by some (Mouille *et al*., 1996) to be a mandatory step for the production of starch. From the predicted effects in *Chlamydomonas* and maize, i.e. a decrease in starch and its incomplete replacement with phytoglycogen, it might be expected that such mutants would have been isolated in any screen for wrinkled-seeded pea mutants, yet none were found. This indicates that debranching may not be important in the synthesis of pea starch, or it may have a different effect to that in other species, or its loss may be lethal. Activities of two types of debranching enzyme, isoamylase and pullulanase, however, have been found in pea embryos during development (Zhu, Hylton and Smith, personal communication).

The supply of substrate to ADPglucose pyrophosphorylase differs in different tissues and in different organisms (Fig. 4). In is generally accepted that in storage tissues such as the cotyledon or endosperm, hexose phosphates are imported into the amyloplast whereas fructose-6-phosphate from the reductive pentose phosphate pathway is utilized in photosynthetic plastids (Keeling *et al*., 1988; Bowsher *et al*., 1996). In cereals, there have been clear demonstrations that glucose-1-phosphate is the imported substrate (Keeling *et al*., 1988; Bowsher *et al*., 1996) rather than ADPglucose or glucose-6-phosphate. This path is now believed to be of minor importance, however, since there is evidence from barley and maize that most ADPglucose pyrophosphorylase activity is located outside the plastid and that ADPglucose is specifically transported into the plastid by a protein, in the case of maize, encoded at the *brittle1* locus (Kleczkowski, 1996). Nevertheless, in pea, there is both biochemical (Hill and Smith, 1991) and genetic evidence (from *rug3* mutants) that glucose-6-phosphate is the only substrate of importance for starch synthesis. There is also biochemical evidence for this path in *Brassica napus* (Kang and Rawsthorne, 1994). By analogy, a similar situation should hold for *Arabidopsis* and tobacco (see *rug3* below).

Biology

Starch accumulates in seeds such as those of maize and pea, in fleshy fruits such as banana, in tubers (potato), in roots (cassava), and in root nodules. For a detailed survey of the occurrence of starch see Jenner (1982). Accumulation is only transient in photosynthetic organs such as leaves, but it is also transient in some seeds such as soybean and rape, where it accumulates early in development, to be replaced later by oils (da Silva *et al*., 1997). The various organs represent biological differences not only in their ontogeny, but also in their nature, for example, seeds are reproductive tissues, tubers vegetative. Furthermore, reproductive tissues have a maternal barrier to transport, with no symplastic connection for the transfer of the sugar required from the plant for starch synthesis (Gunning and Pate, 1974). Seed storage organs also differ: that in pea, the cotyledon, is part of the embryo and hence diploid (Liu *et al*., 1996); that in maize, is the extra-embryonic endosperm and is triploid (Brown *et al*., 1996). The imported substrate used for starch synthesis (see the Biochemistry and molecular biology section) may differ by virtue of these tissue differences alone.

Martin and Smith (1995) point out a number of variables that can affect enzyme activities and can alter

during the production of a starch granule, namely, isoform differences, flux control, spatial distribution, temporal regulation, and developmental regulation. These differences, which account only for the starch enzymes themselves, may have an impact on the type of starch and its function, and the biochemical route by which it is made. These enzyme variables, however, are merely a reflection of the fact that the biosynthesis of starch is also regulated by the biological environment in which it is carried out; there are many genes, other than those discussed above, that are not involved directly in the pathway which will influence the production of the starch granule, however slight their influence. Too often, the assumption is made that the enzymatic processes of starch synthesis are the same whatever the tissue and that altering these processes alone modifies the function of the starch. It would be foolish to discount other factors in the biological environment that will influence the functionality of the starch, since each organ and species will be subjected to different constraints. It is often accepted that there will be differences in the regulation of starch accumulation in photosynthetic organs versus storage organs (Ball, 1995). Similarly, it would be sensible to believe that there are going to be major differences between species. Furthermore, there will be differences within individual organs since many, for example those in pea (Smith, 1973), possess gradients of starch accumulation. None of these biological differences has been taken into account when analysing extracted starch which consists of a mixture of different granules. Such differences, however, are reflected by the fact that blocking starch synthesis in potato (Müller-Röber and Kossman, 1994) or *Arabidopsis* (Caspar *et al*., 1985) has a much more severe effect on the plant than it does in pea (Harrison *et al*., 1998). Hence, it will be extremely difficult to produce, for example, potato starch granules in the pea merely by expressing the potato starch biosynthetic enzymes in pea (or vice versa), something which should be taken into consideration when genetic manipulation is contemplated. Enzyme activities alone do not make functionality. The enzymes create the chemicals which together with the biology create the functionality.

Genetics

Although detailed biochemical analysis can reveal which enzymes may be involved in a particular pathway, it is the generation of lesions in that pathway (mutants) that defines a physiological role for those enzymes. Maize is by far the most studied seed, though the information gained recently from using model systems such as pea (Casey *et al*., 1993; Wang and Hedley, 1993*b*; Smith *et al*., 1997) and *Chlamydomonas* (Ball *et al*., 1996) has been invaluable. Neither of the last two are recognized as starch crops, but it is interesting to note that 50% of the dry weight of the pea seed is starch and that this starch is very different in physical properties to other starches (see later). C*hlamydomonas* normally makes little starch, but can be forced to accumulate large amounts by nitrogen, phosphate or sulphur starvation (Ball *et al*., 1990). A large number of starch mutants have been recognized in maize for many years (Creech, 1965) and there are many parallels between this species and pea in the type of mutant that has been isolated. Both crops serve as a vegetable and both are available in genotypes that are especially prized for their sweet taste. It is interesting, however, that different mutations have served as the main source of genetic variation for sweet vegetables in these two crops; a debranching mutant, *sugary* (*su*), in corn (Hannah *et al*., 1993) and a branching mutant, *r*, in pea (Wang and Hedley, 1993*b*). This review will concentrate on describing the mutants of pea, especially the newly-isolated ones, since the work on maize has been described many times before and is now well established (Nelson and Pan, 1995, and references therein).

Both naturally-occurring and induced mutants of pea have been characterized that affect the composition of the starch accumulated in the seed, the new ones all being induced quite recently by chemical mutagens (Wang *et al*., 1990; Wang and Hedley, 1991). In pea, chemical mutagenesis has proved extremely useful in providing a large number of mutants (Blixt, 1972). It has both the advantage and disadvantage of being random; advantageous because it can lead to the unpredictable, disadvantageous as it cannot be directed to a particular end. Some of the advantageous effects will come to light as the mutants in pea are described below.

Six loci have now been identified that affect starch (Table 1); in five of them, the *rugosus* loci, the dry seeds of the mutant are wrinkled (*rugosus* meaning wrinkled; Wang and Hedley, 1993*a*), whereas in the other one, *lam*, they are indistinguishable from the round seed of the wild type (Wang *et al*., 1994). Some of the characteristics of the pea loci are given in Table 2 together with some equivalent mutants in other species. The mutants are of both substrate-supplier and starch-synthesizing types. The positions of the lesions represented by the mutants are indicated in Fig. 4. Both types, however, can modify the composition and function of the starch, since reducing substrate supply can affect the partitioning of this supply between the synthases, branching and debranching enzymes that are temporally regulated. Classifications into Class 1 and Class 2 types of mutation that have been applied to maize especially (Boyer, 1996), depending on the extreme nature of the phenotype, are not particularly helpful since they ignore the fact that both types can affect composition and the fact that a wide variation in phenotype can be obtained between alleles at a single locus, e.g. those at the *r* locus (see below). The fact that a number of alleles for each locus (Table 1) were isolated

during the pea mutagenesis programme, indicated that the screen was saturated, i.e. mutants were obtained that represent all the loci which affect the wrinkling of the seed. A corollary to this hypothesis is the fact that mutants representing genes encoding enzymes of the starch pathway other than those so far identified (e.g. SBEII), will not cause the seed to be wrinkled and are not likely, therefore, to cause significant decreases in starch content.

The rugosus *loci*

In 1962 Kooistra (Kooistra, 1962) described two mutant lines that had severely wrinkled seeds. The dry seed of both mutants had a starch content reduced by about 40% (on a dry weight basis). He identified one as being at the original *rugosus* (*r*) locus, described by Gregor Mendel many years earlier (Mendel, 1865) and given the gene symbol by White (1917), whereas the other represented a new locus. Kooistra renamed Mendel's locus (incorrectly) the r_a locus, and he termed the second r_b. Both loci were subsequently renamed correctly as *r* and *rb* by Blixt (1977).

Mutations at the *r* locus exhibit considerable pleiotropy. The locus contains the gene for starch-branching enzyme (SBEI or SBEA; Bhattacharyya *et al*., 1990; Martin and Smith, 1995), the lack of activity in the mutant decreasing the amylopectin content of the starch considerably. Wild-type pea starch usually contains about 30% amylose. In the *r* mutant, this increases to 70%. In this respect, the mutation is equivalent to *amylose-extender* in maize (Shannon and Garwood, 1984). The consequences of the decrease in starch are increases in the lipid content of the embryo, the sugar content, the osmotic pressure and the water uptake. There is also a change in the composition of the storage protein, as the major mRNA for one of the components, legumin, appears to be less stable in such an environment, compared with those for the other component, vicilin (Turner *et al*., 1990).These events have been linked using a 'Jigsaw' model (Wang and Hedley, 1991), which holds that the change in the osmotic environment is the key event in the pleiotropy of the locus. Essentially the model pertains to all loci that bring about a decrease in the starch content of the embryo. Differences in transcript abundances have also been attributed to the accumulation of sugars as well as amino acids in some maize starch mutants (Doehlert and Kuo, 1994).

The mutation analysed by Mendel (1865) is caused by a transposon-like insertion in the gene (Bhattacharyya *et al*., 1990). The alleles produced by chemical mutagenesis all have single base-pair changes, the predicted mode of action of the alkylating agent used (MacLeod, 1994). Together the mutants generate a range of starch compositions from 29% to 41% of the dry embryo weight (the wild type has *c*. 50%), the severity of the decrease corresponding to the relative importance of the position of the mutation in the gene. The lowest value represents a null group since one of its members (*r-f*) produces no transcript, whereas the highest value is produced by *r-g*, an allele with a mutation in the least conserved region of the

Table 1. *Pea loci and alleles affecting starch synthesis*

Loci and alleles are given in italics according to the most recent normal convention for *Pisum*. The original line number is given in brackets.

r	*rb*	*rug3*	*rug4*	*rug5*	*lam*
r (WL200)	*rb* (WL1685)	*rug3-a* (SIM1)	*rug4-a* (SIM11)	*rug5-a* (SIM51)	*lam-a* (SIM501)
r-c (SIM53)	*rb-c* (SIM14)	*rug3-b* (SIM32)	*rug4-b* (SIM91)	*rug5-b* (SIM52)	*lam-b* (SIM502)
r-d (SIM54)	*rb-d* (SIM15)	*rug3-c* (SIM41)	*rug4-c* (SIM201A)	*rug5-c* (SIM81)	*lam-c* (SIM503)
r-e (SIM55)	*rb-e* (SIM16)	*rug3-d* (SIM42)			*lam-d* (SIM504)
r-f (SIM56)	*rb-f* (SIM101)	*rug3-e* (SIM43)			*lam-e* (SIM512)
r-g (SIM57)	*rb-g* (SIM102)				
r-h (SIM58)	*rb-h* (SIM103)				
r-i (SIM59)	*rb-i* (SIM103W)				
r-j (SIM61)					
r-k (SIM71)					

Table 2. *Characteristics of pea starch loci and similar loci in maize and* Chlamydomonas

Loci	Enzyme activity affected	Starch[a]	Amylose[b]	Maize	*Chlamydomonas*
r	starch branching enzyme	27–36	60–75	*ae*	–
rb	ADPG pyrophosphorylase	30–37	23–32	*sh2*	*sta1*
rug3	plastidial phosphoglucomutase	1–12	12	–	*sta5*
rug4	sucrose synthase	38–43	31–33	*sh1*, *sus*	–
rug5	starch synthase	29–35	43–52	–	*sta3*
lam	starch synthase	39–49	4–10	*wx*	*sta2*

[a]Starch is given as a percentage of the dry weight.
[b]Amylose is given as a percentage of the starch on a dry weight basis.

gene (MacLeod, 1994). As mentioned, the starch has a high amylose content due to the decrease in branching activity. By analogy to maize *ae*, this may not be a true amylose (Shannon and Garwood, 1984) although it has absorbance characteristics of amylose (λ_{max} *c.* 630 nm). Colonna and Mercier (1984) reported that an 'intermediate material' of a similar size to amylose, but branched similar to amylopectin was produced in high amylose peas. A detailed study of starch from the *r* mutant indicated a difference in the *amount* of amylose and the *type* of amylopectin only (Lloyd, 1995). Furthermore, using thymol precipitation, Tomlinson *et al.* (1997) showed that the 'intermediate material' was almost certainly a thymol-soluble fraction (and hence branched) with a peak elution time slightly later than amylose and with a λ_{max} higher than amylopectin of the wild type. In these respects, the effects of the *r* mutation may differ from those of maize *ae*.

The *rb* mutants decrease the activity of the enzyme ADPglucose pyrophosphorylase (Hylton and Smith, 1992) and increase its sensitivity to allosteric regulation. Mutants at this locus, therefore, are akin to the maize *shrunken2* mutants and affect the large subunit of the enzyme (Martin and Smith, 1995). The new alleles (Table 1), produced by chemical mutagenesis, include two nulls (*rb-e* and *rb-f*) that possess no large subunit and, consequently, very little activity (C. Hylton and A. Smith, personal communication), and all show a decrease in the starch content to about 30% of the dry weight, a very similar amount to those at the *r* locus. The effect on starch composition is quite different, however, in that the amylose content is lowered to *c.* 20% of the starch.

Mutants at the *rug3* locus have a severe effect on the starch content of the seed, lowering it to such an extent (1–12% of the dry weight, depending on the allele) that the seed is well-nigh starchless (Harrison *et al.*, 1998). The occasional very small granule can be observed in amyloplasts of the cotyledonary parenchyma cells by microscopy, indicating that the small amount of starch detected in chemical assays is real and not an artefact of the assay. Similar mutants have been isolated in *Nicotiana sylvestris* and *Arabidopsis thaliana*. In both these species, mutants have been isolated whose leaves have been shown to lack starch. In *rug3* mutants, however, the starchless phenotype has been demonstrated in leaves, roots and seeds. In *N. sylvestris* the starchless mutants affect plastidial phosphoglucomutase activity (Hanson and McHale, 1988), whereas in *A. thaliana* they affect either this activity (Caspar *et al.*, 1985) or that of ADPglucose pyrophosphorylase (Lin *et al.*, 1988). There is also a preliminary report of plastidial phosphoglucomutase mutants in *Chlamydomonas*, although in this organism the mutants retain 20% of the wild-type activity, due to the presence of a minor cytosolic form of the enzyme, and 4–12% of their starch content (Van den Koornhuyse *et al.*, 1996). The evidence from biochemical assays and linkage studies indicated that the pea mutant alleles decrease the activity of plastidial phosphoglucomutase (Harrison *et al.*, 1998) which, in pea, is the minor form of the activity. This indicates that the hypothesis of Hill and Smith (1991) is correct in that glucose-6-phosphate must be the imported substrate for starch synthesis in peas (Fig. 4). The tiny amount of starch present may indicate that another substrate can enter the amyloplast in very low amounts and may reflect a very low affinity of the glucose-6-phosphate translocator for glucose-1-phosphate or, alternatively, the ability of an ADP/ATP translocator to import small amounts of ADPglucose (Pozueta-Romero *et al.*, 1991), or residual enzyme activity. Although it has been speculated that imported materials may be different in photosynthetic and non-photosynthetic tissues (Ball, 1995), of the same species, the *rug3* mutants provide strong evidence against any such hypothesis since the mutation is expressed in all tissues so far examined. It has also been suggested that the apparent absence of starchless mutants from starch-storing crops is a consequence of a general feature of amyloplasts in that they have the ability to import hexose phosphates in the form of both glucose-6-phosphate and glucose-1-phosphate (Ball, 1995). This hypothesis is clearly abrogated by the *rug3* mutants which demonstrate unequivocally that starchless mutants can exist in starch-accumulating crop plants.

The *rug3* mutants are interesting in that they are completely viable, the lack of starch affecting neither the germination of the seed, nor the establishment and subsequent growth of the plant (Harrison *et al.*, 1997). They raise the obvious question of why the plant should store such huge quantities of starch if it is not really needed. The *rug3* mutants also provide evidence, therefore, to support the view held by Porter (1953) that starch is 'the product of excess assimilation' rather than 'a purposive reserve for future metabolic events'. The notion that the quantity of starch accumulated by an organ is related to its putative function as a purposive reserve for future metabolic events has been challenged on several grounds (Jenner, 1982) and these mutants clearly add weight to such arguments.

The starch content of *rug4* embryos is *c.* 40% of the dry weight, which is only 10% less than the wild type (Table 2). The seed are mildly wrinkled and the phenotype is, consequently, the least different from the wild type of all the *rugosus* mutants. Although the amylose content is similar to the wild type, the physico-chemical properties of the starch are different (see later). Investigations into the *rug4* locus indicate that it affects the activity of sucrose synthase, embryos from the mutants having only 5% the activity of wild type (Craig, 1997). *rug4* mutants are akin, therefore, to those at the maize *sh1* and *sus* loci (Nelson and Pan, 1995). In leaves the reduction is about 50%,

whereas in nodules activity is reduced such that the nodules cannot function correctly and the plants consequently are nitrogen deficient and derive little nitrogen from fixation by *Rhizobium* (Craig, 1997).Three alleles (Table 1) at this locus were isolated using chemical mutagenesis (Wang and Hedley, 1993*a*), two of which were affected in both the synthesis and degradative direction and one was affected in the degradative direction only. This characteristic of a single allele, plus the fact that the degree of effect on enzyme activity differs in different tissues, indicates that *Rug4* gene encodes an isoform of sucrose synthase. Such mutants give a clear indication that the alternative degradation pathway for sucrose via alkaline invertase cannot compensate for a lack of sucrose synthase activity. They further demonstrate that defects in sucrose synthase can affect starch accumulation and that such mutants are not evidence for alternative pathways of starch synthesis via imported ADPglucose (Okita, 1992).

As discussed in an earlier section, there are a number of isoforms of starch synthases. Mutants affecting the major granule bound starch synthase do not affect the amount of starch significantly, as discussed in the next section. Evidence to date from *rug5* mutants, however, indicates that other isoforms can alter both the amount and composition of the starch. In pea, there is an active starch synthase isoform of 77 kDa (SSII; Smith, 1990). Mutants at the *rug5* locus lack this enzyme activity (Craig, 1997). Furthermore, these mutants map to the same locus as SSII, indicating that *Rug5* encodes a starch synthase. The starch content of such mutants is decreased to the same degree as in *r* and *rb* mutants, but the amylose content, by chemical analysis, is increased to *c*. 50% of the starch. Structural studies indicate that the starch from *rug5* is very different from that of the wild type in that it contains more short chains (DP15 and less), more long chains (DP >1000), but fewer intermediate chains (DP15–45) in the amylopectin (Lloyd, 1995). The physicochemical properties of the starch are very different from those of the wild type and any other pea mutant (see below) as are the starch granules (Lloyd, 1995; Hedley *et al*., 1996; Bogracheva *et al*., 1997; Wang *et al*., 1997). A mutant (*sta*3) with similar effects on starch structure has also been isolated from *Chlamydomonas* (Fontaine *et al*., 1993). This mutant affects the activity of one of the soluble starch synthases in this organism.

Uncharacterized wrinkled-seeded mutants

Three additional wrinkled-seeded mutants were isolated in the screen that produced the *rug* mutants. Two were seed lethal, neither being able to germinate, whereas the third was a seedling-lethal chlorophyll-deficient type which died a few weeks after germination (Fig. 5). All three had reduced amounts of starch in their seeds. Such

Fig. 5. SIM31. Young seedlings of wild type (to the left of the pot) and SIM31, a chlorophyll-deficient wrinkled-seeded pea mutant.

mutants demonstrate that while decreasing the starch content can produce wrinkled-seeded mutants, the lesions may not lie directly in the pathway of starch biosynthesis.

Another screen—another gene: the lam *locus*

Since it was likely that all the loci causing the wrinkled-seeded phenotype had been identified in the initial screens, any further mutations would need to be detected by other screens. In maize, mutants that do not affect the amount of starch in the endosperm, but affect its composition, have been established for many years (Sprague *et al*., 1943) and indeed provide a source of starch with novel properties for industrial use (Corn Refiners Association, 1994). Such mutants affect the kernel so that it appears 'waxy' (Shannon and Garwood, 1984). Waxy mutants are devoid of amylose and have been isolated from many different cereal species and other higher plants (Boyer, 1996) and *Chlamydomonas* (*sta2*; Delrue *et al*., 1992). The *wx* locus encodes a major granule-bound starch synthase (GBSSI; see earlier).

In potato, the equivalent mutant to 'waxy' maize, *amf*, was isolated on the basis of iodine staining. The *amf* mutant and other such 'waxy'-like mutants contain essentially amylopectin. As mentioned, this polymer has different iodine affinity and staining properties to amylose, producing a red colour rather than the usual indigo blue of normal starches. The suggestion was made, therefore, that such a screen would permit the isolation of similar mutants in pea. This was indeed the case and a screen of the original mutagenized population based on the coloration of starch grains transferred from dry seeds to filter

paper (Denyer *et al.*, 1995) led to the isolation of five alleles (Table 1) at a locus later named *lam* (*l*ow *am*ylose; Wang *et al.*, 1994). These mutants were similar to others in their class in that they produced no, or very low amounts of, amylose depending on how the measurement was performed. The mutants were shown to lack starch synthase activity and, in all but one case, also lacked the relevent protein (Denyer *et al.*, 1995). Furthermore, the starch grains had a characteristic appearance when stained with iodine in that they possessed a large dark blue core with a very pale, often reddish halo.

Since the *lam* mutants lack the major 59 kDa starch synthase and lack amylose in their starch, they have proved very useful for identifying other proteins with synthase activity of similar molecular weights whose activity would otherwise have been masked by GBSSI. To this end a 60 kDa protein with substantial synthase activity has recently been identified in pea pods. The activity is such that the pods of *lam* mutants contain as much amylose as those of wild-type plants indicating that this isoform makes a major contribution to pod starch synthesis (Denyer *et al.*, 1997).

Double your money— double mutants

The creation of double mutants has an enormous potential to provide a deeper understanding of starch synthesis, as shown in maize (Hannah *et al.*, 1993). In pea, the first *rugosus* double mutants were investigated by Kooistra (1962) when he reported on the discovery of *rb*. Kooistra showed that the *r*/*rb* double mutant had only a slightly reduced amount of starch compared with the parental lines, but a different amylose content from either of these single mutants. Furthermore, the generation of the double mutant demonstrated that the compound starch granule of the *r* mutants was epistatic to the simple one of *rb*. More recent studies using near-isogenic pea lines (Lloyd, 1995; Lloyd *et al.*, 1996*b*; Wang *et al.*, 1997) have shown that the starch content of *r*/*rb* is reduced to about 20% of the dry weight, much lower than either single mutant. The mutants, therefore, have the same effect on the accumulation of starch in pea, whereas the effects in the double mutant were additive to either single mutant, which may reflect the fact that one operates through the supply of substrate and one through the branching of the polymers. In the *r*/*rb* double mutants, the starch granule size is reduced indicating that the reduced starch content is brought about through granule size rather than number. In fact, in most *rugosus* double mutant combinations the starch content is reduced below that of either single mutant (Lloyd, 1995) except for combination with *rug3*. In all hybrids produced to date with this mutant (unpublished data), the *rug3* phenotype usually dictates the final starch content. With regard to granule shape, it is clear that those loci that have a strong influence on shape, such as *r* and *rug5*, have a controlling influence on the final granule shape of the double mutant (Lloyd, 1995; Wang *et al.*, 1997) as illustrated in Fig. 6.

Preliminary data from double mutants produced between mutants at the *rugosus* and *lam* loci provide some interesting information. In maize *ae*/*wx* double mutants the starch content is reduced considerably over that in either of the single mutants, as predicted by their action on the individual pathways to amylose and amylopectin (Hannah *et al.*, 1993). Likewise, *r*/*lam* double mutants contain *c.* 13% starch, far less than either individual mutant. In these double mutants the apparent amylose content is very similar to *r* mutants, but, again, it is unclear what this component is in reality. In *ae*/*wx* mutants, the apparent amylose is an abnormal 'loosely-branched' amylopectin (Boyer *et al.*, 1976) although, in pea, the properties have not yet been fully examined. An attempt to generate an equivalent of this double mutant in potato has also been made by producing a transgenic plant with a decreased SBE activity in the *amf* mutant (Flipse *et al.*, 1996). In this transgenic, the chemistry of the starch was apparently unchanged, but its physicochemical properties were different from those of *amf* mutant.

Double mutants of *lam* and *rug5*, however, contain an amount of starch and amylose very similar to *rug5*. If the Lam protein produces amylose exclusively, neither *lam* combination should show any amylose and it remains to be seen whether these double mutants produce a true amylose fraction or an intermediate fraction as suggested for similar mutants in cereals (Nelson and Pan, 1995; Wang Y.-J. *et al.*, 1993*b*) and for the *r* mutants (see above). In both the single *r* and *rug5* and the double mutants with *lam*, the 'amylose' fraction has a slightly later elution time from a gel filtration column than authentic amylose. It is interesting to note that the apparent amylose content in *r*/*lam* double mutants is far greater than that in the equivalent maize double mutant (Shannon and Garwood, 1984). Combinations of *rb* or *rug4* with *lam* have similar starch contents to the respective *rugosus* locus, but similar amylose contents to the *lam* mutant.

A, B, C

The importance of biology in determining granule structure has been discussed, and this structure will now be examined in more detail. Amylose and amylopectin molecules are arranged in granules, which are complex structures consisting of crystalline and amorphous areas. It is a common point of view that short chains in the amylopectin molecule are organized into double helices, some of which then form crystalline lamellae (or crystallites) (French, 1984; Blanshard, 1987). The remaining double helices and the crystallites form the ordered part of the

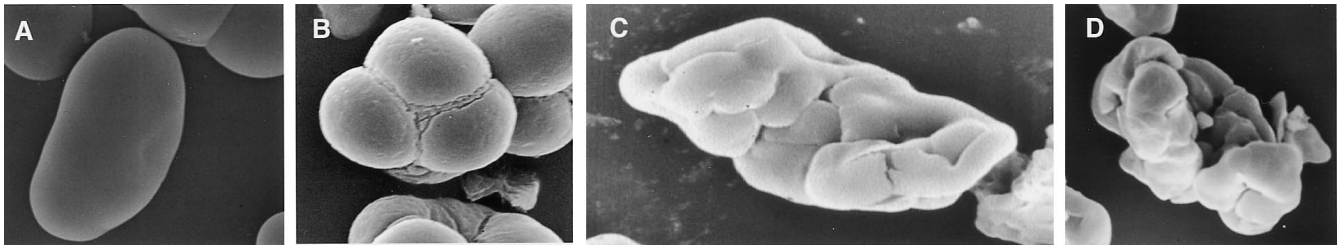

Fig. 6. Scanning electron micrographs of starch grains from the wild type (A), *r* mutant (B), *rug5* (C) and a double *rug4*/*rug5* mutant (D) of pea. Not to scale.

starch granule which is thereby semi-crystalline, the remaining part being called the disordered (or amorphous) part. The amorphous part of the starch granule is believed to consist of amylose and long chains from the amylopectin (French, 1984). There is evidence that there are alternate layers of semi-crystalline and amorphous material in the starch granule (French, 1984; Perez and Imberty, 1996). The presence of crystallites causes the starch granule to be birefringent and this can be studied using light microscopy with cross polarizers. The amorphous region contains no ordered structures by definition and cannot be distinguished from the background. The interference pattern observed takes the form of a Maltese cross as shown in the Introduction (Fig. 1) which indicates that there is an orderly arrangement of the crystalline areas within the granule. The use of a specific plate (the so-called λ-plate or red 1 compensator; Patzelt, 1974; Morris and Miles, 1994) in conjunction with the cross polarizers makes sectors that appear blue or yellow, representing starches as biaxial crystalline polymers (Patzelt, 1974), the background appearing pink (Fig. 7). Crystallites ordered differently with respect to the plane of polarization, either towards or perpendicular to it, give rise to the different colours (French, 1984; Morris and Miles, 1994).

A three-dimensional model has been proposed to describe the arrangement of crystallites in potato starch granules (Fig. 8; Oostergetel and van Bruggen, 1993). In this model it is suggested that short chains in the amylopectin molecule form double helices approximately 5 nm long, which are crystallized into 5 nm thick lamellae. These lamellae alternate with the amorphous layers in which the $\alpha(1,6)$ branch points are located. The crystalline lamellae have cavities each with a diameter of about 8nm and form a more or less continuous super helical structure (Fig. 8). The double helices forming the lamellae are packed in polymorph structures. In the case of potato starch the polymorphs are termed B type. A second type of polymorph structure can be found in starches from other species (e.g. maize) and this is termed A type. These

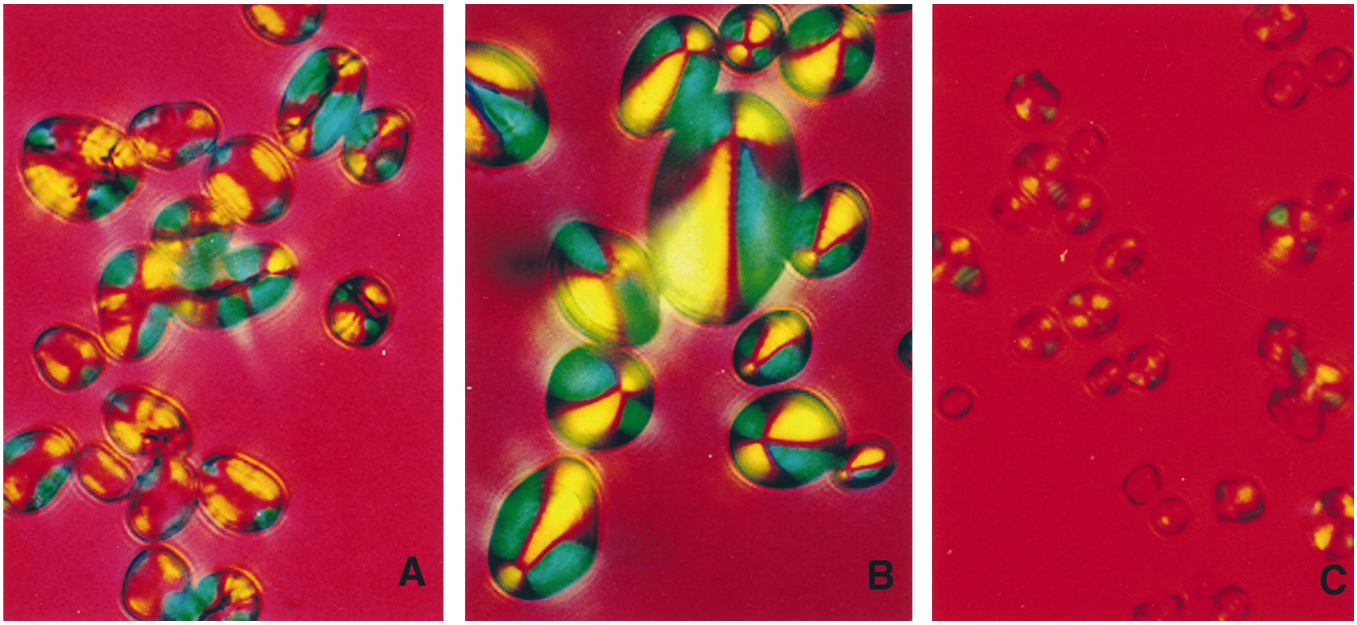

Fig. 7. Starch granules in water viewed using polarized light in conjunction with a λ plate. (A) Wild-type pea starch, (B) potato starch, (C) maize starch.

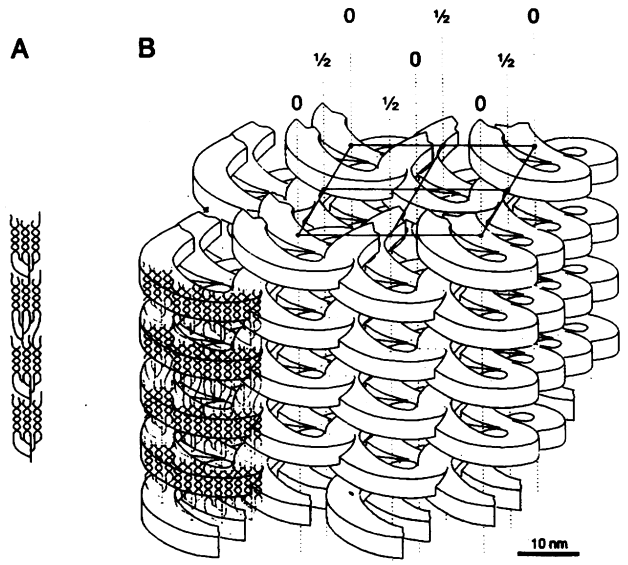

Fig. 8. Schematic model for arrangement of amylopectin in potato starch. (A) model for an amylopectin molecule showing clustering of the α-(1→4), α-(1→6) branch points and the double helical linear α-(1→4) glucan chains. (B) model of the crystalline structure of the granule. 5 nm crystalline lamellae contain double helical linear segments. The crystallites are packed into a continuous network consisting of left-handed helices packed in a tetragonal array. Neighbouring helices are shifted relative to each other by half the helical pitch (indicated by 0 and 1/2). Four amylopectin molecules (A) are projected into one of the helices. Reproduced from *Carbohydrate Polymers* **27,** 7–12, 1993, Oestergetel and van Bruggen, with kind permission of Elsevier Science Ltd, Kidlington, UK.

two types of polymorph differ in the geometry of their single cell units, the packing density of their double helices and in the amount of bound water within the crystal structure, A being more dense and binding less water than B (Fig. 9; Sarko and Wu, 1978; Imberty and Perez, 1988; Imberty *et al.*, 1988). Different starches contain either A, B or both polymorph forms and they are called A-, B- or C-type starches, respectively. C-type starches are found typically in the seeds of grain legumes, for example, peas. A comparison between maize, potato and pea starch granules using polarized light is shown in Fig. 7.

It is possible, using wide-angle X-ray diffraction, to calculate the proportion of the starch which is formed into crystallites. In order to calculate total crystallinity correctly it is necessary to measure a wide range of angles, from at least 4 to 30 degrees 2θ, and take into account the fact that the diffraction pattern also contains a contribution from the amorphous part. In the past, these two criteria have not always been taken into consideration, mainly because it is difficult to measure the amorphous part. Recently, however, Cairns *et al.* (1997), following the work of Chinachoti and Steinberg (1986), have used a method for estimating the amorphous part by recording wide-angle X-ray diffraction patterns of the starches, whose crystalline part had been destroyed (Fig. 10). It has been shown by Hermans and Weidinger (1961) that the total crystallinity of semi-crystalline poly-

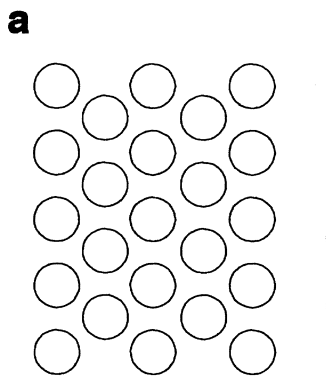

Fig. 9. An illustration of the arrangements of amylopectin double helices. (a) A-type and (b) B-type starch polymorphs. Each circle represents a view down the z-axis of a double helix. Adapted from Sarko and Wu (1978). Note the distances between helices are not to scale, absolute values for the distances can be found in Ring *et al.* (1993).

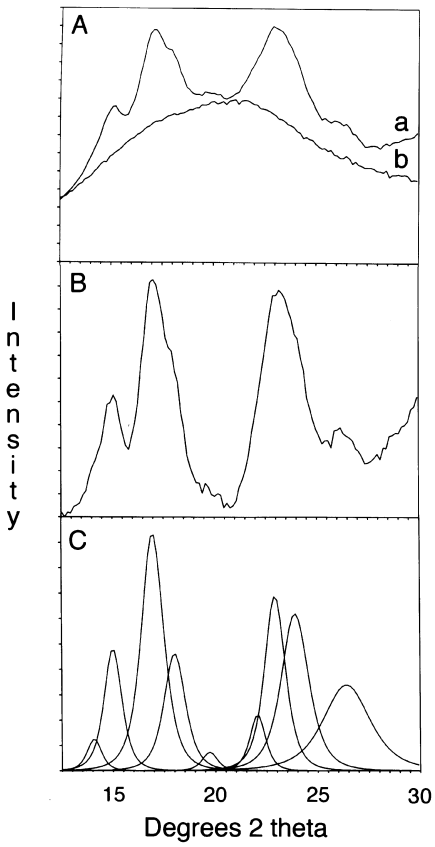

Fig. 10. (A) X-ray diffraction pattern of wild pea starch in native form (a) and of amorphous starch (b) adjusted with reference to (a) according to Cairns *et al.* (1997). (B) X-ray diffraction pattern of the crystalline portion of wild pea starch obtained by the subtraction of pattern b from pattern a in (A). (C) peak profiles for the X-ray pattern of the crystalline portion of wild pea starch in (B), calculated as described in Cairns *et al.* (1997).

mers can be calculated using the formula below, once the contribution from the amorphous part is known.

$$\% \text{ crystallinity} = \frac{Q_{st} - Q_{am}}{Q_{st}} \times 100\% \quad (1)$$

Where Q_{st} and Q_{am} are the measured areas under the wide-angle X-ray patterns from the semi-crystalline and amorphous samples, respectively.

Wide-angle X-ray diffraction can also be used to determine if starches have A-, B- or C-type crystallinity. As with the calculation of total crystallinity, it is necessary to subtract the contribution made by the amorphous part from the overall X-ray pattern. Using this procedure, typical A- and B-type polymorph patterns have been obtained for maize and potato starch, respectively (Cairns *et al.*, 1997). A computer peak fitting program has been used to determine precisely the peak positions which are characteristic of A- and B-type polymorphs (Cairns *et al.*, 1997). The polymorph composition of a given starch can be determined from these characteristic peak positions. For example, this method has been used to obtain the peak profile for the crystalline part of pea starch (C-type; Fig. 10), which has been shown to be composed of peak profiles from A- and B-type polymorphs. Using this information the proportion of A- and B-type polymorphs in the C-type starch derived from pea has been calculated and found to be *c.* 56% and 44%, respectively (Cairns *et al.*, 1997). These values are, by chance, similar to the values obtained previously (61% and 39%, respectively; Gernat *et al.*, 1990) even though no consideration was taken of the amorphous part in this study. This is simply because the amorphous parts of the standards and the samples were similar. If they are not, then ignoring the component due to the amorphous part leads to errors.

Gelatinization/melting–two different mechanisms

Heating starch in the presence of water results in the disruption of ordered structures. This process is often studied by differential scanning calorimetry (DSC; for a review of the subject see Jones, 1979). Using this technique, a starch sample is heated at a defined rate and the changes in heat capacity are measured as a function of temperature. It has been shown that the disruption of ordered structures within starch granules are characterized by an endothermic process which is often called the order–disorder transition (Donovan, 1979; French, 1984; Blanshard, 1987; Cooke and Gidley, 1992; Zobel and Stephen, 1995). It has also been shown that the character of this transition is strongly dependent on the water content (Donovan, 1979; Biliaderis *et al.*, 1980, 1986; Eliasson, 1980; Colonna *et al.*, 1987; Svensson and Eliasson, 1995). At different water contents, the disturbance of ordered structures in most starches may follow two different mechanisms. These two mechanisms are usually called melting and gelatinization. Melting occurs in low moisture conditions (less than 30% of water (w/w) for most starches), when there is no free water in the system, and gelatinization occurs in excess water (usually greater than 70% water), when there is an excess of free water (Evans and Haisman, 1982; Blanshard, 1987). Both of these processes occur when starches are heated in intermediate conditions of moisture (between 30% and 70% water content). The DSC representative curves for the melting and gelatinization of potato starch are shown in Fig. 11. The melting endotherms are on the 0.28 and 0.36 thermograms; gelatinization is on the 0.81 thermogram. The 0.45, 0.51 and 0.64 thermograms represent the occurrence of both processes: the lower temperature peak is characteristic of gelatinization and the higher one of melting. The character of the melting and gelatinization curves is very different: very wide endothermic peaks, with a peak temperature that is strongly dependent on the moisture content of the sample, are characteristic of melting, whereas gelatinization produces large, relatively

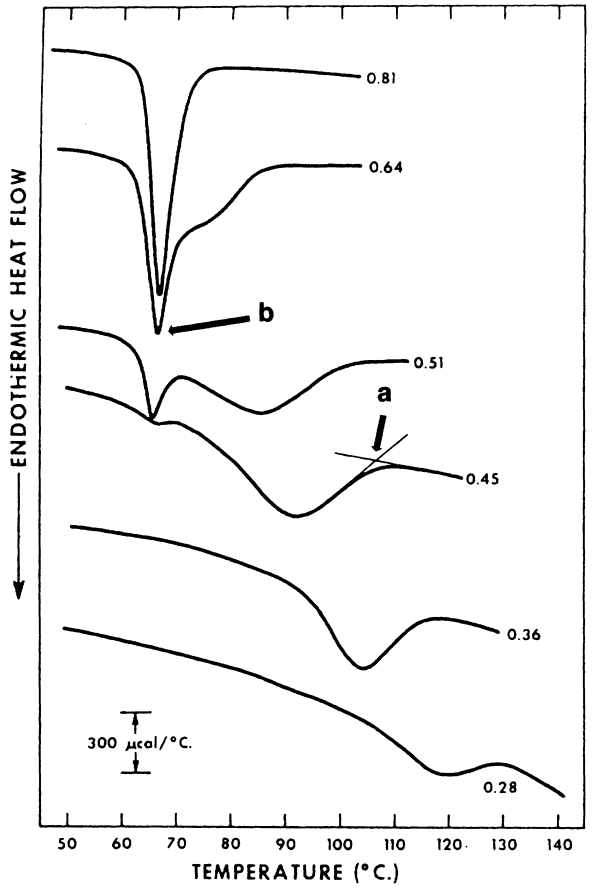

Fig. 11. DSC thermograms of potato starch. Heating rate 10 °C min^{-1}. The volume of the fraction of water is given against each line. The intersection of straight lines (marked as a) drawn for the thermogram with a water fraction of 0.45, shows the method of extrapolation used to determine T_m. The peak (b) is used to determine T^p. Adapted from Donovan (1979).

narrow endothermic peaks that are generally believed to represent co-operative first-order transitions (Donovan, 1979; Zobel and Stephen, 1995). There is no obvious dependence of peak temperature on water content in gelatinization (Colonna *et al*., 1987).

The theory relating the effect of increasing diluent content to melting temperature (T_m) of crystalline polymers was developed by Flory (1953) and was based on equilibrium thermodynamics. The Flory–Huggins equation for the thermodynamic parameters of melting is presented below.

$$\frac{1}{T_m}-\frac{1}{T_m^0}=\frac{R}{\Delta H_u}\times\frac{V_u}{V_1}\left[v_1-\chi_1 v_1^2\right] \qquad (2)$$

Where: T_m^0 is the equilibrium melting point of crystallites without diluent; T_m is the melting point in presence of diluent; R is the gas constant; H_u is the fusion enthalpy per polymer repeating unit; V_u is the molar volume of repeating unit of crystalline polymer; V_1 is the molar volume repeating unit of diluent; v_1 is the volume fraction of diluent; χ_1 is the polymer-diluent interaction parameter.

Although the Flory theory was developed for the melting of hypothetically perfect crystals during heating, reflecting reversible equilibrium conditions in the presence of diluent, it does not distinguish between the concentration of diluent and, therefore, Equation 2 can be and is used commonly for the description of melting *and* gelatinization behaviour of starch crystals (Lelievre, 1976; Evans and Haisman, 1982; Biliaderis *et al*., 1986; Lim and Jane, 1996). Evans and Haisman (1982) showed that, if starch granules are equilibrated in the presence of water, the volume fraction of diluent in Equation 2 should be based on the water content inside the starch granule. Since the disruption of crystallites is essentially an irreversible process, the Flory–Huggins equation can be used only for a description of the initial disruption temperature. It can be predicted from this equation that the order–disorder transition during melting will happen at lower temperatures following an increase in the proportion of water and this can be seen clearly in DSC thermograms of starch in Fig. 11. Gelatinization happens in conditions of excess water, when starch granules are swollen to an equilibrium level, and changes in the quantity of external water will not affect granule composition. Hence, in relation to Equation 2, the initial gelatinization temperature should be independent of the proportion of the water in the starch suspension. It is well known that gelatinization is accompanied by a large swelling of the granule, however, this does not happen during melting. It can be suggested that this swelling behaviour is the main reason for the differences in the progress of the gelatinization and melting processes.

The DSC curve describing the gelatinization process shows that there are sharp changes in the absorption of heat, which are normally described as changes in enthalpy (ΔH). ΔH is related to the changes in free energy (ΔG) of the system. There are reviews discussing the components of this enthalpy change (Gidley, 1992; Zobel and Stephen, 1995) and they conclude that this has only one component which is the disruption of helical order. We propose, however, that there are other components contributing to this process. The change in free energy during gelatinization may be represented by the sum of the energies required for the disruption of the different types of ordered structures and those required for swelling. It can be suggested that, in general, the change in free energy of a population of starch granules during heating will include the sum of: ΔG_1, ΔG_2, ΔG_3, and ΔG_4, where ΔG_1 is the change of free energy of disruption of crystalline order within the granules; ΔG_2, the change of free energy of disruption of double helices within the granules; ΔG_3, the change of free energy following disturbance of the interactions involving the amorphous parts of the granules; ΔG_4, the change of free energy during swelling.

Some of the four components of ΔG may make larger contributions then others and some of them may be influenced by the conditions of the process (e.g. concentration, rate of heating). It is apparent, for example, that ΔG_4 can be strongly influenced by the concentration of starch in suspension and the parameters of heating. The difference between gelatinization of the starches with different crystalline orders (A- and B-type starches) should be related to the value of ΔG_1. In contrast, the value of ΔG_2 should be uniform for every type of starch. It is apparent, therefore, using this approach that ΔH also will be strongly influenced by the structural parameters of granules as well as the parameters of the swelling process.

Use of quasi-equilibrium conditions

It is well known that starch granules swell to a limited degree in excess water and that this swelling is reversible (French, 1984; Blanshard, 1987). No significant change was found in the total water absorption between 20 °C and the initiation of the disruption process (Evans and Haisman, 1982; Blanshard, 1987). There is a common point of view, however, that significant changes happen in the amorphous part at this stage of heating, that destabilize the crystallites (Donovan, 1979; Biliaderis, 1986; Blanshard, 1987; Zobel and Stephen, 1995) until, at a certain temperature, disruption of the crystalline structure occurs followed by a large, irreversible swelling of the granules to complete the gelatinization process (French, 1984; Zobel and Stephen, 1995). The gelatinization is related to the endothermic peak observed in the DSC studies, as mentioned above. It is apparent, therefore, that the initial reversible swelling of the granule, which occurs relatively slowly, is an important part of

the gelatinization process. Also, it is apparent that irreversible swelling is strongly influenced by the conditions of the gelatinization process. The changes in granules that happen before and during gelatinization are related to the thermodynamic parameters of the endothermic transition. It has been shown that the rate of heating and the concentration of starch in suspension influences the initial, peak and final temperatures of gelatinization as well as ΔH (Fig. 12; Evans and Haisman, 1982; Shiotsubo, 1984; Shiotsubo and Takahashi, 1984; Blanshard, 1987). If heating occurs too fast, overheating ensues and the process is condensed so that individual stages cannot be completed. By analogy, if you heat a cake too fast, it will burn rather than bake! It has been shown, however, that the thermodynamic parameters of the transition are not influenced by a heating rate of 1 °C min^{-1} or less and a concentration of starch in suspension of 5% or less (Shiotsubo, 1984; Shiotsubo and Takahashi, 1984; Davydova *et al.*, 1995). Under such conditions, the process becomes quasi-equilibrium. In the literature most of the reports (Biliaderis, 1986; Hoover and Sosulski, 1991; Evans and Haisman, 1992; Gidley, 1992; Zobel and Stephen, 1995) relating to the use of DSC studies of starch have used heating rates of 10–20 °C min^{-1} and concentrations of starch suspensions of 10–30%. This has resulted in problems in interpreting the measured values of ΔH and gelatinization temperatures.

The gelatinization behaviour of A-, B- and C-type starches have been studied and compared using quasi-equilibrium conditions (Shiotsubo and Takahashi, 1984; Bogracheva *et al.*, 1994, 1995, 1997; Hedley *et al.*, 1996, 1997). Under these conditions potato starch (B-type) has a T^p in water which is *c.* 2–4 °C lower than that for maize (A-type; T Bogracheva, unpublished data). This compares with data reported in the literature using non-equilibrium conditions which report differences between these two starches of about 7 °C (Zobel and Stephen, 1995). The higher T^p for A-type starch compared with B-type is in accord with the predictions made using the Flory–Huggins equation (equation 2), which indicated that more dense polymorph structures (such as A type in starch) will have higher temperatures of disruption of their crystallites compared with less dense structures (such as B type in starch).

During the gelatinization process water plays the role of a plasticizer. The use of different additives results in a change in the thermodynamic parameters of gelatinization (Evans and Haisman, 1982; French, 1984; Blanshard, 1987; Jane, 1993). Small additions of neutral structure-making salts, for example, NaCl, increase T^p for both maize and potato starches (Evans and Haisman, 1982; Jane, 1993). It can be suggested that this effect is due to salt solutions being less effective plasticizers than pure water. The effect of KCl on the quasi-equilibrium gelatinization of pea starch is illustrated in Fig. 13. In pure water, pea starch has a single peak of transition (Bogracheva *et al.*, 1994, 1995; Davydova *et al.*, 1995). The addition of KCl increases the temperature of transition and a distinct double peak of transition replaces the single peak. It has been proposed that the double peaks represent a double transition, the first peak corresponding

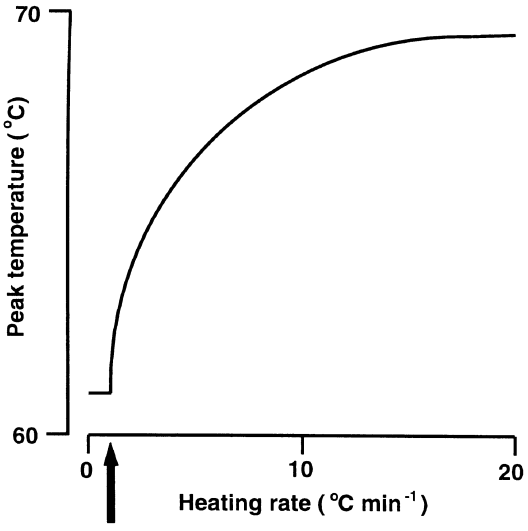

Fig. 12. The influence of the rate of heating on the peak temperature of DSC thermograms. The arrow indicates the temperature below which T^p is independent of the heating rate. Redrawn from Shiotsubo and Takahashi (1984).

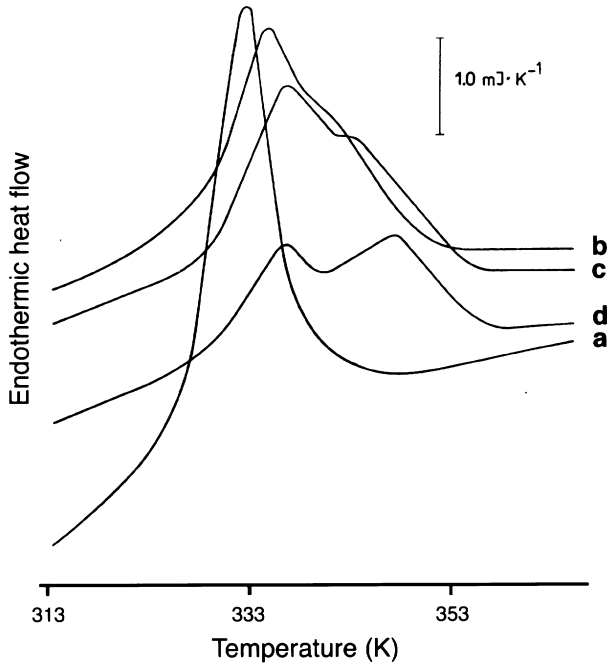

Fig. 13. The DSC thermograms of starch from pea (var. Sproot) in excess water and different concentrations of KCl: distilled water (a), 0.3 M KCl (b); 0.5 M KCl (c); 1.5 M KCl (d). Heating rate 1 K min^{-1}. Concentration of starch in suspension is 0.44–0.5% (w/w). Adapted from Davydova *et al.* (1995).

to the disruption of B polymorphs and the second to A polymorphs (Bogracheva *et al.*, 1994, 1995, 1997; Davydova *et al.*, 1995). It has been possible by using such additives coupled to heating and cooling cycles to completely distinguish the two transitions (Bogracheva *et al.*, unpublished data).

Behaviour of starches from mutants

A wide range of mutants have been described which have lesions affecting the content and composition of starch. Many of these mutants have been characterized with regard to their biochemical effects on starch synthesis and their chemical effects on amylose and amylopectin and these have been discussed earlier in this review. There are few reports, however, of the effects of such mutations on starch granule structure and physico-chemical properties, most of them have been on maize (Wang Y.-J. *et al.*, 1992, 1993*a*; Katz *et al.*, 1993; Campbell *et al.*, 1995; Kasemsuwan *et al.*, 1995; Shi and Seib, 1995) and relatively few on pea (Colonna and Mercier, 1984; Stute, 1990). Recently, an examination has begun of the unique set of pea mutants discussed earlier (Bogracheva *et al.*, 1995, 1997; Hedley *et al.*, 1996, 1997).

The most widely studied maize mutants are those affecting the 'waxy' character. These starches have very low amylose levels, but are still A-type with a slightly higher amount of total crystallinity compared with starch from the wild type (Gidley, 1992). There are no reports describing the structure of any of the starches produced by mutants affected at other loci in maize, although there are such reports using double and triple mutants containing the *wx* gene (Shi and Seib, 1995). When the *wx* gene is in combination with the *ae* mutant allele there is an indication of B polymorphs in the crystalline structure, although it is not clear if this starch still contains A polymorphs, which would make the starch C-type. The X-ray diffraction pattern of starch from the triple mutant, *ae du wx*, in maize, however, appears to be a more typical C-type pattern.

In relation to gelatinization behaviour, 'waxy' maize starches have higher T^p and enthalpy than starch from the wild type (Wang Y.-J. *et al.*, 1992; Katz *et al.*, 1993). Gelatinization studies have been carried out on starches from most of the other maize mutants and double mutants, in particular those combined with the *wx* gene. All of these mutants, with the exception of those containing the *ae* mutant allele, give narrow endothermic peaks of gelatinization with very small differences in T^p and ΔH. With regard to starch from *ae* mutants, there is a very wide transition rather than a definite peak as found with the other mutants (Wang Y.-J. *et al.*, 1992; Katz *et al.*, 1993). It should be borne in mind that all of these studies on maize starches have been carried out using starch concentrations of about 30% and rates of temperature change of $10\,^{\circ}C\,min^{-1}$, which are not quasi-equilibrium conditions and, therefore, must be viewed with caution.

As mentioned earlier, a number of pea mutants have been isolated and characterized with regard to their effects on the biochemistry of starch synthesis. In addition, the effects of the mutations on the chemical composition of the starches are known and have been discussed earlier. With regard to the granular structure and gelatinization properties of the starches, most information is available for the *r* and *rb* mutants, which have been known for many years, and for the double mutant, *r*/*rb* (Bogracheva *et al.*, 1995, 1997; Hedley *et al.*, 1996, 1997). There is less information on the starches from the most recently characterized pea mutants (*rug3*, *rug4*, *rug5* and *lam*; Bogracheva *et al.*, 1997; Hedley *et al.*, 1997).

In relation to granular structure, there is no significant effect of the *rb* mutation, the total crystallinity and the proportions of A and B polymorphs being similar to those found in starch from the wild type. The gelatinization behaviour, however, differs from the wild type in having a much higher T^p and ΔH (Fig. 14; Bogracheva *et al.*, 1995). Significant differences, however, have been found for the granular structure of starch produced by the *r* mutant. The total crystallinity is much lower than starch from the wild type and the proportion of B polymorphs is increased to about 80% of the total. The gelatinization process of this starch shows no obvious peak of transition (Fig.14). The crystalline structure the *r*/*rb* double mutant starch is in many ways similar to that of the *r* starch and very different from *rb*; it has a low level of total crystallinity and a very high proportion of B polymorphs. The gelatinization behaviour of this starch, however, is different from both the *r* and *rb* starches, showing a wide endothermic peak of gelatinization with very high T^p and very low ΔH (Fig. 14).

With regard to starches from the most recently derived pea mutants, their gelatinization behaviour can be divided into two groups, one group having similarities to the behaviour of starch from the *rb* mutant and the other being more similar to starch from the *r* mutant. The first group of starch behaviour, seen in starches from *rug3*, *rug4* and *lam* mutants, shows a narrow endothermic peak of gelatinization, with a $10\,^{\circ}C$ range in T^p. The lowest peak temperature was for starch from the *lam* mutant and the highest for starch from the *rug3* mutant. The second group of behaviour, found in starch from the *rug5* mutant, is similar to *r* in that it shows a very wide transition. In this respect it also behaves in a similar way to starch from the *ae* mutant in maize (Bogracheva *et al.*, 1997). At present the gelatinization properties of starches from the new pea mutants cannot be related to changes in granule structure because there is a lack of structural information.

The gelatinization properties of starches are related to their functional properties which are those that are utilized

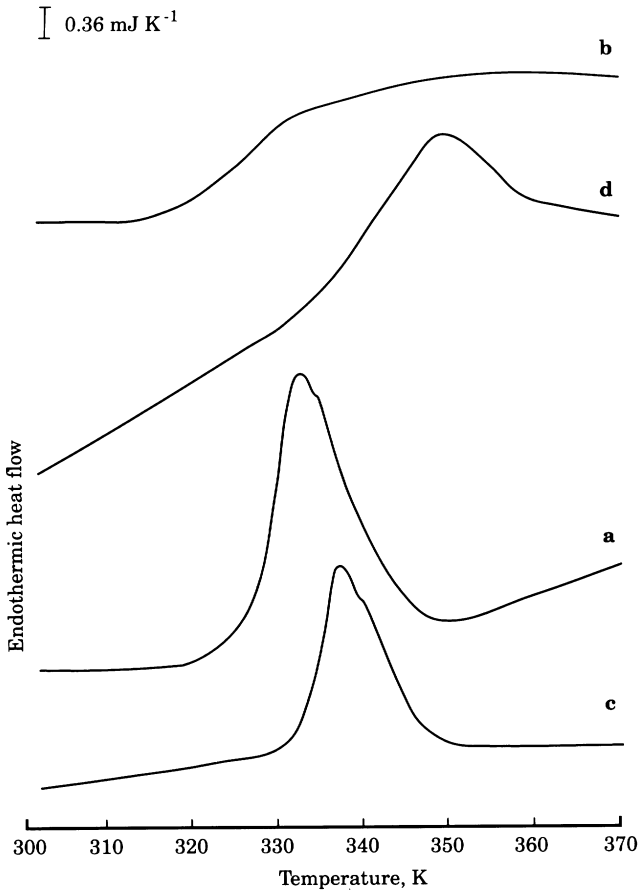

Fig. 14. DSC thermograms for starches of the near-isogenic pea lines in excess water. Genotype and the concentration of starch suspension: a—wild-type starch, 0.54%; b—*r* mutant starch, 0.50%; c—*rb* mutant starch, 0.07%; d—*r*/*rb* double mutant starch, 0.80%. Heating rate 1 K min^{-1}. Reproduced from Bogracheva *et al.* (1995) with permission of the journal.

when starches are processed. It is important, therefore, to understand the basis of gelatinization behaviour. It can be suggested that gelatinization is in some way determined by the amylose content of the starch. It is apparent, however, that when the amylose content is low, as in 'waxy' maize starch and in starch from the *lam* mutant in pea, the changes in gelatinization properties can be either positive or negative compared with starch from their respective wild type. For example, the T^p for 'waxy' maize starch is higher than for normal maize starch, while the T^p for *lam* starch is lower than for normal pea starch. In addition, when the amylose content is high, as in starch from *ae* maize and from *r* mutant and *r*/*rb* double mutant pea, this can result either in an increase in T^p, as in the *r*/*rb* starch, or in the lack of a narrow peak transition, as in the *r* and *ae* starches. It can be concluded from these examples, therefore, that the amylose content in starches is not the direct reason for their gelatinization behaviour.

As discussed earlier, starches which have a similar total and type of crystallinity can have different gelatinization properties. For example, starches from wild type and from *rb* mutant peas have a similar total crystallinity and polymorph composition, but have very different T^p and ΔH parameters of gelatinization. It is apparent in this case that these parameters of starch crystalline structure cannot account for the gelatinization behaviour. It can be proposed that the difference in behaviour may be due to different sizes of crystallites, to defects of the crystallites, or to different properties of the amorphous part of the starches.

For some starches it is possible to relate gelatinization to the biochemical function of the genes. It is apparent, for example, that genes which encode enzymes that directly affect amylopectin structure, such as *r* and *rug5* in pea and *ae* in maize, produce starches which have similar gelatinization behaviour. This gelatinization behaviour contrasts with that of starches affected by genes which encode enzymes that do not directly affect amylopectin structure, such as *rug3*, *rug4* and *lam* in pea and *wx* in maize. At present, the reasons for this apparent relationship are not clear in terms of starch granular structure.

Conclusions

Starch is an extremely important part of our diet. It is becoming increasingly significant as an industrial raw material, yet we are a considerable distance from understanding the link between the functional properties required for starch use and the effects of individual genes, despite rapid advances in our knowledge of its biosynthesis. It is clear, however, that some features of the physical properties that dictate the functionality of starch can be ascribed to specific genetic differences.

Starch is a far more complex structure than its chemistry would imply. There are A, B and C glucan chains in amylopectin, A and B polymorphs in granules, A and B granules in some species and there are A, B and C types of starch. Learning your A, B, C might seem trivial to some of those involved in starch science, but to the child, learning your A, B, C seems extremely difficult. Furthermore, in trying to relate starch structure to gene action, we are all still children. Our inability to escape the first three letters of the alphabet has also not helped. Go learn your A, B, C!

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