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The effect of mutant genes at the *r*, *rb*, *rug3*, *rug4*, *rug5* and *lam* loci on the granular structure and physico-chemical properties of pea seed starch

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Abstract

The granular structure and gelatinisation properties of starches from a range of pea seed mutants were studied. Genes which affect the supply of substrate during starch synthesis (*rb*, *rug3*, *rug4*) affected the total crystallinity and possibly increased the content of A polymorphs in the starch. Conversely, genes directly affecting the synthesis of starch polymers (*r*, *rug5*, *lam*) increased the content of B polymorphs, but had a minimal effect on total crystallinity. During gelatinisation, starches from the *rb*, *rug3*, *rug4* and *lam* mutants had narrow endothermic peaks which were similar to starch from the wild-type, although all the starches had different peak temperatures and enthalpy changes. Starches from *r* and *rug5* mutants were very different to all other starches, having a very wide transition during gelatinisation. In addition, the amylopectin in starch from these mutants had altered chain lengths for those parts of the polymer which form the ordered structures in the granule.

Keywords: Starch; Pea seed mutant; Polymorph

1. Introduction

Starch is the most important carbohydrate reserve in seeds. It can be fractionated into two types of macromolecules, amylose and amylopectin. Amylose is an essentially linear molecule composed of anhydroglucose units connected through $\alpha(1,4)$ linkages. Amylopectin is a much branched polymer which is also formed by $\alpha(1,4)$ linked anhydroglucose units, but additionally with $\alpha(1,6)$ linked branches (Banks & Greenwood, 1975; Manners, 1989). These two types of polymers are arranged in granules, which at room temperature are extremely insoluble in water and exert a minimal osmotic effect on the plant cells in which they are produced.

The structure of starch granules is made up of ordered and disordered, or amorphous, regions. The ordered parts consist of double helices formed from short branches of amylopectin molecules (French, 1984). The majority of these double helices are organised into crystalline lamellae, making the granule into a so-called 'semi-crystalline' structure. Two types of crystalline or polymorph forms, A and B, have been found in starch granules. The two forms differ in the packing density of the double helices, A being more dense than B. The type of polymorph present in the granules characterises the type of starch and, in general, is species dependent. For example, starch from normal (wild type) maize seeds contains A-type polymorphs and is termed A-type starch, while starch from normal (wild type) potato tubers contains B-type polymorphs and is termed B-type starch (Hizukuri & Nikuni, 1957; Sarko & Wu, 1978; Imberty, Buleon, Tran & Perez, 1992; Perez & Imberty, 1996). A third type of starch, typically found in the seeds of grain legumes such as pea (*Pisum sativum*), is called C-type starch. The crystalline structure of C-type starch granules is composed of both A and B polymorphs (Gernat, Radosta, Damaschun & Schierbaum, 1990; Cairns, Bogracheva, Ring, Hedley & Morris, 1997), the B polymorphs being arranged centrally and the A polymorphs peripherally within each of the starch granules (Bogracheva, Morris, Ring & Hedley, 1998).

The structure of granules can be disrupted by heating starch in the presence of water (French, 1984; Blanshard, 1987; Cooke & Gidley, 1992; Zobel & Stephen, 1995; Wang, Bogracheva & Hedley, 1998). In excess water, this disruption is accompanied by high swelling of the granules and an increasing viscosity of starch suspensions (French,

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Table 1
Starch characteristics for pea seed mutants[a]

Genotype	Enzyme activity affected	Starch content (% dry weight)	Amylose content (% starch)
Wild-type		50	30
rug4	Sucrose synthase	38–43	31–33
rb	ADPG pyrophosphorylase	30–37	23–32
rug3	Plastidial phosphoglucomutase	1–12	12
lam	Starch synthase	39–49	4–10
rug5	Starch synthase	29–35	43–52
r	Branching enzyme	27–36	60–75

[a] Data obtained from Wang et al., 1998.

1984; Zobel, 1984; Kokini, Lai, & Chedid, 1992; Rao, Okechukwu, Da Silva & Oliveira, 1997). This process, termed gelatinisation, plays an important role in starch applications. It has been shown that A-, B- and C-type starches differ for the thermodynamic parameters of the gelatinisation process (Donovan, 1979; Biliaderis, Maurice & Vose, 1980; Biliaderis, Page, Maurice & Juliano, 1986; Zobel, 1988; Noel & Ring, 1992; Bogracheva et al., 1998) and that these differences are mainly determined by the crystalline structure of the starch granules (Bogracheva et al., 1998).

Our understanding of starch biosynthesis has been greatly enhanced by the use of genetic variation and, in particular, the identification of specific mutations affecting steps in the starch biosynthetic pathway (Wang et al., 1998). There have been very few studies, however, where genetics has been used as a tool for studying starch granular structure and the link between granular structure and the functional properties of starch. This is partly due to the need to develop physical methods for determining structural parameters in the granule and partly to the need to develop suitable genetic material. A range of techniques have been used for a number of years for studying granular structure, in particular, X-ray diffraction, differential scanning calorimetry and various light and electron microscopy methods (Yamaguchi, Kainuma & French, 1979; Donovan, 1979; French, 1984; Blanshard, 1987; Biliaderis et al., 1986). More recently, progress in quantifying the ordered structures within starch granules has been achieved using X-ray diffraction and NMR methods (Gidley & Bociek, 1985; Gidley & Robinson, 1990; Cairns et al., 1997). The development of suitable genetic material has been less successful. Although numerous mutants have been described, particularly in maize (Wang, White & Pollak, 1992; Katz, Furcsik, Tenbarge, Hauber & Friedman, 1993; Wang, White & Pollak, 1993a,b), they invariably have been produced in different genetic backgrounds, which makes comparative studies of genetic effects very difficult.

A range of mutants have been developed in pea which are known to be affected at specific steps in the starch biosynthetic pathway (Harrison, Hedley & Wang, 1998; Wang et al., 1998; Hylton & Smith, 1992; Wang et al., 1994; Hedley, Bogracheva, Lloyd & Wang, 1996; Denyer et al., 1995). These mutants have been back-crossed into a common genetic background and now can be considered near-isogenic except for genes at the loci where we have identified mutations. All of the mutants produce starch that differs from that of the wild-type in the proportion of amylose and amylopectin (Table 1). Also, with the exception of mutants with lesions at the *lam* locus, the mutants have a reduced starch content in their seeds (Table 1). Genes at three of the loci (*rb*, *rug3* and *rug4*) encode enzymes which affect the supply of substrate during starch biosynthesis; namely, ADPglucose pyrophosphorylase; phosphoglucomutase and sucrose synthase, respectively (Wang et al., 1998). Genes at three other loci (*lam*, *rug5* and *r*) encode enzymes which directly affect the synthesis of the starch polysaccharides; these are respectively starch synthase I, involved in amylose synthesis, and starch synthase II and starch branching enzyme A, both involved in amylopectin synthesis (Wang et al., 1998).

In the present article we present the results of a study of starch granular structure from pea mutants affected at each of the identified loci. The phenotype of each mutant has been characterised in terms of the crystalline structure of starch granules and the thermodynamic parameters of their gelatinisation. This study is the fore-runner of a more detailed genetic analysis of starch granular structure in pea seeds which will utilise not only differences between the six loci known to affect starch synthesis, but also intra-allelic differences at each locus and the interaction between genes at the different loci.

2. Materials and methods

2.1. Materials

The normal and waxy potato starches were obtained from AVEBE PREMIER (UK) and AVEBE (The Netherlands). The normal and waxy maize starches were obtained from ROQUETTE (France) and AMIOCA (USA).

Starch was extracted from the seeds of the wild-type parental line and from a series of mutants that are near-isogenic to this wild-type line except for genes at the *r*, *rb*, *rug3*, *rug4*, *rug5* or *lam* loci. The pea seeds were ground

in a Cyclotec 1093 sample pin-mill (Sweden) and the resulting meal was slurried in water at a solid–liquid ratio of 1 to 12, the pH being adjusted to 8.5 with 0.1 M NaOH. The mixture was centrifuged at 2500 g and the supernatant then removed. The precipitate had a very viscous green top layer and a solid white main part. The green top layer was removed and the rest of the precipitate was resuspended in distilled water. This centrifugation and separation procedure was repeated twice, until the viscous layer was no longer present. The resulting uniform white precipitate was then resuspended in distilled water and screened sequentially through two sieves, 300 and 53 μ. The resulting starch was washed with distilled water and air dried.

Samples of amorphous starch were obtained using a method described previously (Cairns et al., 1997). A 2% suspension of pea starch was gelatinised by heating for 1 h at 100°C with continuous shaking. The samples were placed in sealed Pyrex tubes and transferred to a block heater preheated to 100°C. The temperature of the block was raised to 170°C and maintained at this temperature for 10 min. The samples were then cooled to about 100°C for few minutes, removed from the tubes, rapidly frozen, using a mixture of dry ice and acetone, and freeze-dried. The freeze-dried samples were then placed under vacuum at 70°C for 2 h.

2.2. Wide-angle X-ray diffraction

Measurements were made using two powder diffractometers operating at CuKα. A Philips diffractometer with a PW 1830 generator, operated at 40 kV and 50 mA, with a PW 3710 mpd control and PW 3020 goniometer. For this instrument, the diffractometer had a 1° divergence slit, a 15 mm beam mask, a 0.2 mm receiving slit and a 1° scatter slit. The samples were scanned in the range 4.0–40.0°2θ at a speed of 0.004°2θ/s with a step size of 0.1°2θ. The second diffractometer was a Philips Scientific diffractometer with a PW 1730/10 generator operated at 40 kV and 30 mA. The instrument had a vertical goniometer (PW 1820) with an Anton Paar TTK camera. The incident beam of X-rays was collimated using an automatic divergence slit (Philips PW 1386/55). A 0.3 mm receiving slit with a 1° scatter slit was used. The samples were scanned in the range 4.5–30.0°2θ at a speed of 0.005°2θ/s and a step size of 0.15°2θ. For both instruments, data were collected using a proportional detector and then stored and processed on a PC using Philips PC-ADP (version 3.6b) automated powder diffraction software. Variation due to the instrument background was determined by scanning the empty sample holder under the same conditions used for the samples. Lotus 1-2-3 software was used to subtract the background variation from the sample scans and to subtract the amorphous starch pattern from the pattern given by the native starches. The computation of the peak profiles was carried out using the peak-profile fitting facility of PC-APD, as described previously (Cairns et al., 1997).

2.3. Acid hydrolysis

0.2–0.5 g samples of dry starch were suspended in 6 ml 2.2 M HCl and held at 34°C for 40 days, with occasional shaking by hand. The samples were then neutralised with 1 M NaOH and centrifuged at 3000 g. The supernatant was removed and the remaining acid-resistant starch residues were washed with distilled water and air dried.

2.4. Isoamylase treatment

Using Pyrex tubes, 1 mg samples of the acid-resistant starches were suspended in 1 ml of diluted acetate buffer (1 part acetate buffer (pH 3.6)/15 parts water) and dissolved by heating at 120–130°C for 10 min. After cooling, 2 μl of isoamylase solution (4 344 000 units/ml Sigma Chemical Co., USA) was added to each sample and left overnight to incubate, after which the reaction was stopped by boiling. The samples were dissolved in either water or DMSO/water solution.

2.5. Ion-exchange chromatography

The chain-length distributions of the acid-resistant starches (before and after debranching with isoamylase) were analysed by high-performance anion-exchange chromatography using a Dionex PA-100 column and a Dionex pulsed amperometric detector. The column was eluted with 0.1 M NaOH (A) and 0.1 M NaOH containing 0.6 M sodium acetate (B), using a gradient of 25–60% B from 5–48 min. The column was calibrated using commercially available malto-oligomers (maltose to maltoheptose of $>$ 95% purity (Sigma–Aldrich, Dorset, UK).

2.6. Differential scanning calorimetry (DSC)

A Setaram Micro-DSC (Lyons, France) was used. The concentration of starch in suspension was 1.7–2.0%, for those starches giving a large sharp peak of heat capacity change, and 2.0–4.0% for those starches giving a small wide change in heat capacity during gelatinisation. The heating rate was 1°C/min. The specific enthalpy of gelatinisation (ΔH) was determined by measuring the peak area as described previously (Bogracheva et al., 1995). The peak width (ΔT) was calculated from the difference between the onset (T^o) and conclusion (T^c) temperatures. The peak temperature (T^p), T^o and T^c were determined as described in Bogracheva et al. (1995).

3. Results

3.1. Comparison between wide-angle X-ray diffraction instruments with either fixed or divergence slits

Wide-angle X-ray diffraction is commonly used for studying starch crystalline structures. Two types of instrument were used in the present study, one with an automatic

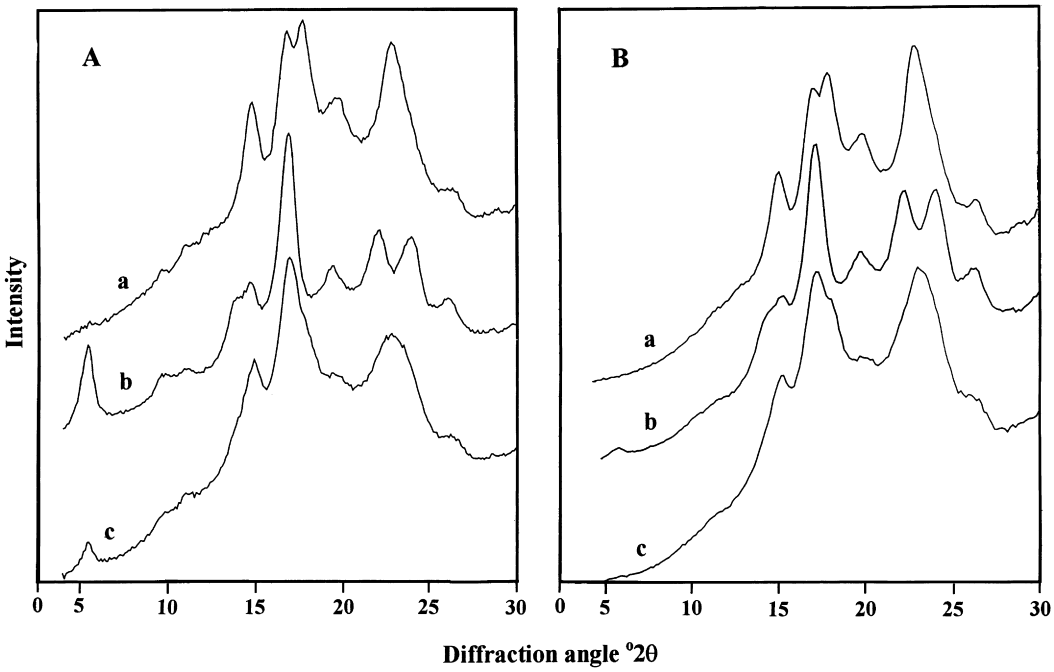

Fig. 1. X-ray diffraction patterns for (a) A-type (wild type maize), (b) B-type (wild type potato) and (c) C-type (wild type pea) starches, using a fixed slit (A), and an automatic divergence slit (B), diffractometer. In each case, the instrument background was subtracted.

divergence slit and the other with a fixed slit. The X-ray beam focus of the instrument with the automatic divergence slit is constantly varied, so that at any angle of the goniometer the irradiated area of the sample remains constant. On the contrary, in the instrument with the fixed slit there is an increase in the irradiated area of the sample when the angle of the goniometer is decreased. Using this fixed-slit device, the peaks at small angles appear to be relatively larger than when the automatic divergence slit device is used.

A comparison between the X-ray diffraction patterns of the two instruments using A-, B- and C-type starches (represented by maize, potato and wild type pea) is shown in Fig. 1A and B. Using the instrument with the automatic divergence slit, it is difficult to identify the peaks at angles 4.5–12.5°2θ, while these peaks can be identified using the fixed slit instrument. It is possible to identify peaks at the larger angles equally well with both the instruments (Fig. 1A and B). In order to study the polymorph composition of the starches it is necessary to identify the peak positions for the crystalline parts of the X-ray patterns at both small and large angles (Hizukuri & Nikuni, 1957; Sarko & Wu, 1978; Cairns et al., 1997). Therefore the device with the fixed slit was preferred for this purpose.

The proportion of A and B polymorphs in C-type starches can be calculated from the area of the peaks at the different angles (Davydova et al., 1995; Bogracheva et al., 1995; Cairns et al., 1997). This determination requires the irradiated area of the sample to remain constant at any goniometer angle. The method previously developed for this calculation depends on the measurement of peak areas at the range of angles from 12.5 to 30.0°2θ (Cairns et al., 1997). Therefore in this case, the automatic divergence-slit instrument was preferred and was used to obtain the data presented in the present article.

3.2. Polymorph composition of starches

The X-ray patterns obtained with the fixed-slit instrument for the seed starches from the wild-type and the different mutant peas are shown in Fig. 2. It can be seen from this figure, that the patterns for the different starches were not identical.

The description of the polymorphic composition of the starches is related to the position of the peaks for the crystalline parts of the granules and was carried out as described previously (Cairns et al., 1997). The steps required for this determination are illustrated in Fig. 3, using starch from the wild-type pea as an example. The X-ray pattern for the crystalline part of the starch granules (Fig. 3B) was obtained by subtracting the pattern for the amorphous starch from the pattern for the native starch. The X-ray pattern of the amorphous part was determined by fitting the pattern of the amorphous starch (Fig. 3A(b)) to the pattern of the native starch (Fig. 3A(a)), as described in Cairns et al. (1997). The peak profile (Fig. 3C) was obtained by applying the peak profile fitting facility of PC-APD to the pattern of the crystalline part (Cairns et al., 1997). As the angles were related to the maximum of each peak, the positions of the diffraction peaks could be determined using the peak profile programme (Fig. 3C). This procedure was applied to the patterns from the mutant pea starches and to the patterns

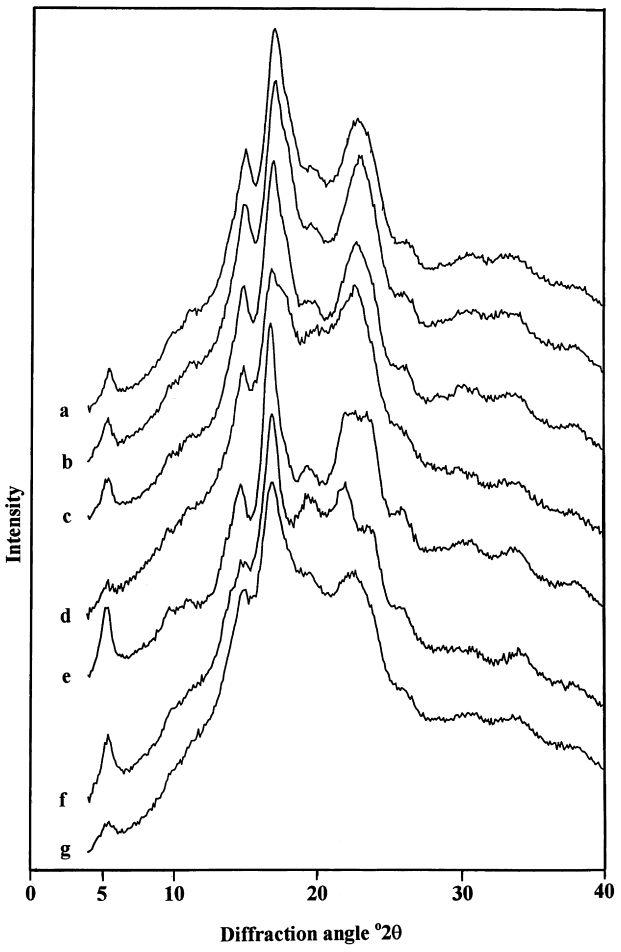

Fig. 2. X-Ray diffraction patterns for (a) the wild-type, (b) *rug4*, (c) *rb*, (d) *rug3*, (e) *lam*, (f) *r*, and (g) *rug5* pea starches, using a diffractometer with a fixed slit. The instrument background was subtracted.

from the A-type (waxy and normal maize) and B-type (waxy and normal potato) starches, for comparison. As shown previously (Cairns et al., 1997), the X-ray diffraction patterns of the amorphous starches from A-, B- and C-type starches (maize, potato and wild-type pea starches) were identical. For this reason the subtraction of the amorphous part from the X-ray patterns of all the native starches was performed using the X-ray pattern for the amorphous starch obtained from wild type peas.

A comparison of the peak positions for the A-, B- and C-type starches is shown in Table 2. The two A-type starches had identical peak positions and we considered these positions, therefore, as characteristic for A polymorphs. Likewise, the peak positions for the two potato starches were considered to be characteristic for B polymorphs. The peak positions for wild type pea starch were characterised by the sum of those for the A and B polymorphs, the deviations never being greater than 0.2°2θ, demonstrating that this starch can be considered as C-type. The data obtained correlated well with that reported by Cairns et al. (1997). The positions of the peaks at the small angles (4.5–12.5°2θ), however, were obtained more accurately in the present study. In addition, the positions of the peaks at the larger angles (30–40°2θ) were not presented in previous publications.

The peak positions for the starches from the mutant peas are presented in Table 3. A comparison between these peak positions and those for A and B polymorphs indicated that starches from the *rug4*, *rb*, *rug3*, *lam* and *rug5* mutants contained both A and B polymorphs and are, therefore, of the C-type. The peak positions for the crystalline part of the starch from the *r* mutant, however, were characteristic for B polymorphs and this starch is, therefore, of the B-type.

3.3. The proportion of A and B polymorphs in starches

The proportion of A and B polymorphs in the crystalline regions of the starches were obtained using the X-ray patterns from the instrument with the automatic divergence slit. The peak profiles of the crystalline parts of the starches from the wild-type and from the mutant peas are shown in Fig. 4, together with the patterns for potato and maize starches for comparison. It has been shown earlier (Sarko & Wu, 1978; Cairns et al., 1997), and it can be seen from Table 2, that peak numbers 7 and 10 are characteristic only for A polymorphs and that peak numbers 1, 4 and 9 are characteristic only for B polymorphs. A comparison between the heights of these peaks and the heights of the other peaks within each pattern indicated that the proportion of A and B polymorphs in the starches from the different mutant peas was different. These proportions were calculated according to the method of Cairns et al. (1997). Using this method, the amount of A polymorphs is determined from the proportion of the total area of peaks represented by the area of peak 7. The amount of B polymorphs is determined from the proportion of the total area represented by the area of peak 4. Starches from the *rug4*, *rb* and *rug3* mutants had an increased proportion of A polymorphs (Table 4) and a consequent decrease in the proportion of B polymorphs compared with starch from the wild-type. In the case of the *rug4* and *rb* mutants, however, the changes are similar to those attributed to the error associated with polymorph determination.

In contrast, starches from the *lam*, *rug5* and *r* mutants had a higher proportion of B polymorphs and a reduced content of A polymorphs (Table 4).

3.4. Total crystallinity of starches

The total crystallinity in the starches was determined according to the method described in Cairns et al. (1997). It was calculated as a proportion of the crystalline area to the total area at the angles between 4.5 and 27.5°2θ using starch samples with 12–15% moisture. For this analysis, the X-ray instrument with the automatic divergence slit was used. The total crystallinity for starch from wild type peas was 20%, while starches from the mutant peas ranged from 17–27% (Table 4).

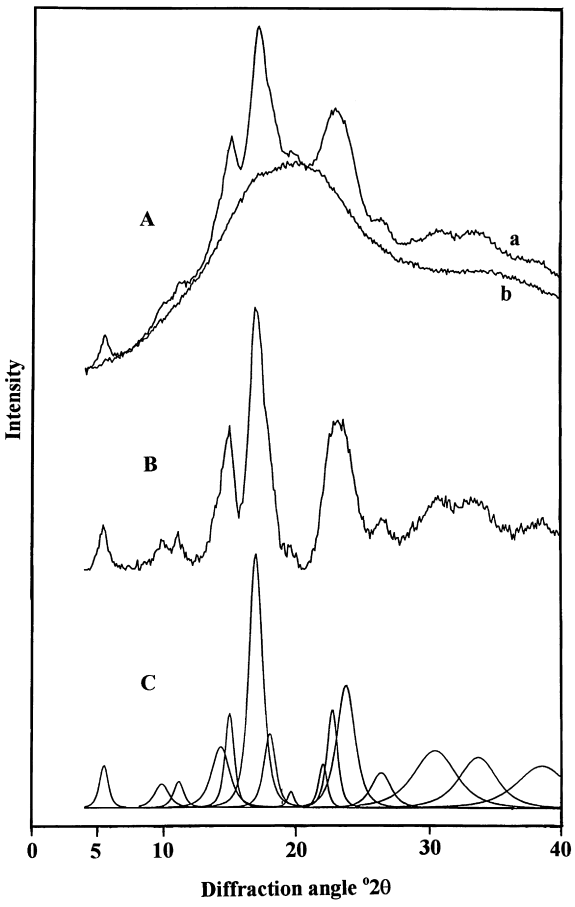

Fig. 3. A: X-ray diffraction patterns for starch from wild-type pea (a) and for the corresponding amorphous starch (b), using the fixed-slit diffractometer. In both cases, the instrument background was subtracted. B: X-ray diffraction pattern for the crystalline portion of starch from wild-type pea obtained by subtracting pattern (b) from pattern (a). C: computer derived peak profiles for the X-ray pattern of the crystalline portion of starch from wild-type pea shown in Fig. 3B.

Table 3
Peak positions for starches from pea mutants

Peak no.	WT	*r*	*rb*	*rug3*	*rug4*	*rug5*	*lam*
1	5.5	5.3	5.3	5.2	5.3	5.3	5.2
2	9.8	9.9	9.7	9.7	9.8	9.9	9.7
3	11.1	11.1	11.0	11.1	11.1	11.2	11.0
4	14.3	14.0	14.0	13.3	14.2	14.1	13.7
5	15.0	14.8	14.8	14.7	14.9	14.9	14.6
6	17.0	16.9	16.8	16.7	16.9	16.9	16.7
7	18.0	—	17.8	17.7	17.8	18.0	17.6
8	19.6	19.5	19.5	19.7	19.6	19.5	19.4
9	22.0	22.0	21.9	21.5	22.0	22.1	21.8
10	22.7	—	22.7	22.7	22.8	22.9	22.6
11	23.7	23.8	23.7	23.8	23.8	24.0	23.7
12	26.3	26.1	26.0	26.0	26.2	26.2	26.0
13	30.3	30.3	30.3	30.1	30.3	30.6	30.2
14	33.6	34.0	33.7	33.4	33.6	33.6	33.9
15	38.5	38.4	38.2	—	38.2	38.2	38.2

3.5. Chain lengths of ordered structures in starches

It has been shown previously, that during acid hydrolysis the amorphous parts of starches are preferentially hydrolysed and dissolved, while the ordered areas remain insoluble and keep their crystalline structure (French, 1984; Komiya & Nara, 1986; Buleon, Bizot, Delage & Pontoire, 1987; Jane, Wang & McPherson, 1997). Ion-exchange chromatography of the acid-resistant part of starch from the wild type and mutant peas, as well as from potato and maize (normal and waxy), are shown in Figs. 5 and 6. The acid-resistant starch from the wild-type and from *rug4*, *rb*, *rug3* and *lam* pea mutants showed a bimodal distribution for the chain lengths (degree of polymerisation, DP). This type of DP distribution was similar to that found for potato, maize, waxy maize, tapioca and banana acid-resistant starches (Fig. 5; Ring, Noel & Bull, 1993; Jane et al., 1997). Following the debranching treatment with isoamylase the position of the first peak remained essentially unchanged, whereas the

Table 2
Peak positions for A-, B- and C-type starches

Peak no.	WT potato	Waxy potato	B-polymorphs[a]	WT maize	Waxy maize	A-polymorphs[a]	WT pea
1	5.4	5.3	5.4	—	—	—	5.5
2	9.8	9.8	9.8	9.8	9.6	9.8	9.8
3	11.1	11.0	11.1	11.1	11.1	11.1	11.1
4	13.9	13.8	13.9	—	—	—	14.3
5	14.8	14.7	14.8	14.8	14.8	14.8	15.0
6	16.8	16.8	16.8	16.9	16.7	16.8	17.0
7	—	—	—	17.9	17.8	17.9	18.0
8	19.4	19.3	19.4	19.7	19.8	19.8	19.6
9	22.0	22.0	22.0	—	—	—	22.0
10	—	—	—	22.8	22.7	22.7	22.7
11	23.8	23.7	23.8	23.8	23.8	23.8	23.7
12	26.1	26.0	26.1	26.3	26.1	26.2	26.3
13	30.2	30.2	30.2	30.3	30.1	30.2	30.3
14	34.2	34.1	34.2	33.4	33.3	33.4	33.6
15	38.4	38.5	38.5	38.3	38.3	38.3	38.5

[a] Peak positions for A- and B-polymorphs were determined from the peak positions of wild-type (WT) and waxy maize and potato starches, respectively.

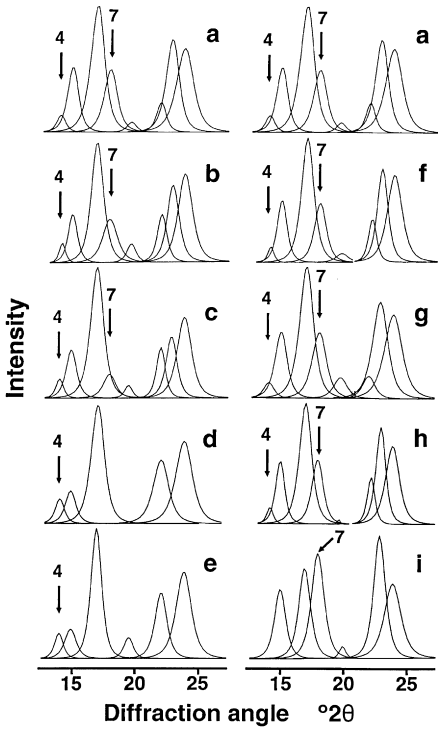

Fig. 4. Computer derived peak profile X-ray patterns for the crystalline portions of starches from maize, potato and peas. a, wild-type pea; b, *rug5* pea mutant; c, *lam* pea mutant; d, *r* pea mutant; e, wild-type potato; f, *rug4* pea mutant; g, *rb* pea mutant; h, *rug3* pea mutant; i, waxy maize. The original X-ray diffraction patterns for the native and amorphous starches were obtained using the diffractometer with the automatic divergence slit. The numbers correspond to the peak numbers given in Tables 2 and 3.

peaks with the higher DP disappeared (Fig. 7). This behaviour was similar to that found for potato, maize and waxy maize starches (Fig. 7; Ring et al., 1993; Jane et al., 1997). In addition, all starches from this group, with the exception of waxy maize starch, had a sharp peak with a DP^{max} of 12 that appeared after isoamylase treatment (Fig. 7). The DP^{max} for the main peaks of acid-resistant starches before and after treatment with isoamylase are given in Table 5. The DP^{max} for potato starch was slightly higher than for maize starches, which agreed with the previous reports (Jane et al., 1997). The DP^{max} for starches from the pea mutants in this group was intermediate between that of the maize and potato starches.

Starches from the second group of mutant peas (*rug5* and *r*) showed a multi-modal distribution of chain lengths both before and after isoamylase treatment (Figs. 6 and 8). The DP^{max} for the first peaks of these starches were slightly higher than for potato starch (Table 5). A large proportion of the chains from these starches had a high DP.

3.6. Thermodynamic parameters of gelatinisation

Gelatinisation is essentially an irreversible process and its thermodynamic parameters are influenced by different experimental conditions, for example, the concentration of starch in suspension and the heating rate (Evans & Haisman, 1982; Shiotsubo & Takahashi, 1984; Blanshard, 1987; Wang et al., 1998). It has been shown that when low starch concentrations and low heating rates are used, the gelatinisation process may be considered to consist of a large number of equilibrium states, i.e. it may be considered to be a so-called quasi-equilibrium process (Shiotsubo & Takahashi, 1984; Blanshard, 1987; Wang et al., 1998). Under these conditions the thermodynamic parameters are not influenced by the experimental conditions and these parameters can be used, therefore, to characterise the starch. In the present study, all of the DSC experiments were carried out using starch concentrations not greater than 4% and a heating rate of 1°C/min. These conditions satisfy the requirements of a quasi-equilibrium process (Shiotsubo & Takahashi, 1984; Davydova et al., 1995; Bogracheva et al., 1995).

The DSC thermograms for starch from the wild-type and from the mutant peas are shown in Figs. 9 and 10. Fig. 9 illustrates the gelatinisation process for starch from the wild-type, *rug4*, *rb*, *rug3* and *lam* mutant peas. All of these starches gave high, narrow peaks of changes in heat capacity (C_p), with peak temperatures which were characteristic for each of these starches. This type of change in C_p indicates that the gelatinisation process for these starches is a first order transition, which is characteristic for starches

Table 4
Parameters relating to the chemical and granular structure and gelatinisation behavior of starches from wild type and mutant pea seeds

Genotype	Amylose content[a] (% starch)	Crystallinity				T^p (°C)	ΔT (°C)	ΔH (J/g)
		Total(%)	%B	%A	B/A			
WT	35	20	45	59	0.8	61.8	13.4	10.8
rug4	33	23	39	57	0.7	65.4	12.5	9.8
rb	23	27	43	58	0.7	66.1	9.0	12.6
rug3	12	17	37	63	0.6	70.0	9.4	7.5
lam	8	22	69	29	2.4	58.6	8.4	6.8
rug5	43	20	52	45	1.2	49.0–57.0	30.0	5.1
r	65	19	73	0[b]	∞	52.5–60.0	34.0	2.4

[a] Data reproduced from Bogracheva et al. (1997).
[b] A-polymorphs not detected.

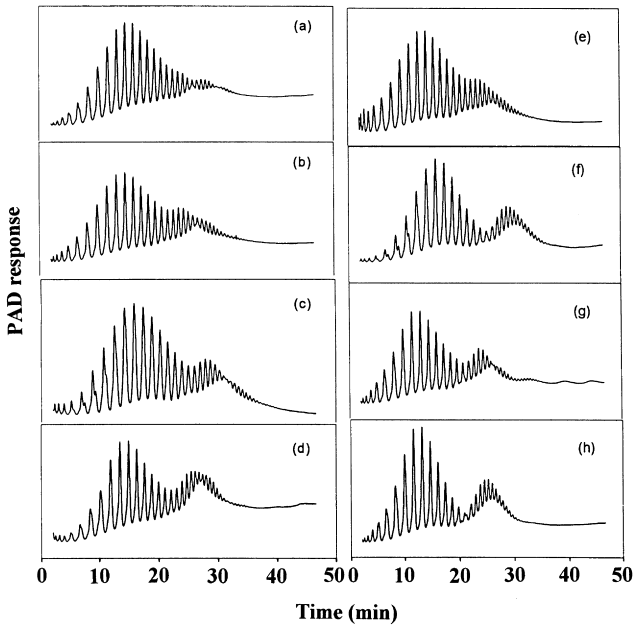

Fig. 5. Chromatograms of the acid-resistant parts of starches. (a) wild type potato, (b) wild type pea, (c) *rb* pea mutant, (d) *rug3* pea mutant, (e) *rug4* pea mutant, (f) *lam* pea mutant, (g) wild type maize and (h) waxy maize.

from other sources (Donovan, 1979; Biliaderis et al., 1980, 1986). The gelatinisation behaviour of starches from the *rug5* and *r* mutants was unusual. The change in C_p of these starches during gelatinisation was very slow and cannot be referred to as a first order transition (Fig. 10). These slow changes are reflected in large values of ΔT (Table 4). The absence of sharp changes in C_p during gelatinisation (Fig. 10), did not allow us to define a peak temperature for these starches and therefore T^p was determined as a temperature range (Table 4). In addition, the changes in C_p were relatively low and were reflected in low values of ΔH (Table 4).

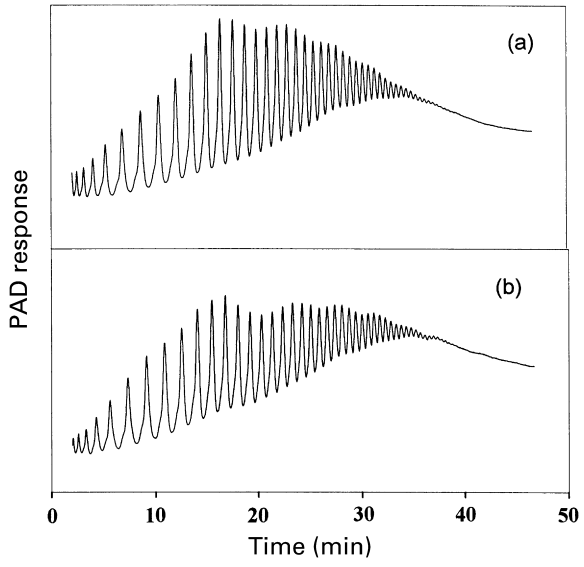

Fig. 6. Chromatograms of acid-resistant starches from (a) *r* pea mutant and (b) *rug5* pea mutant.

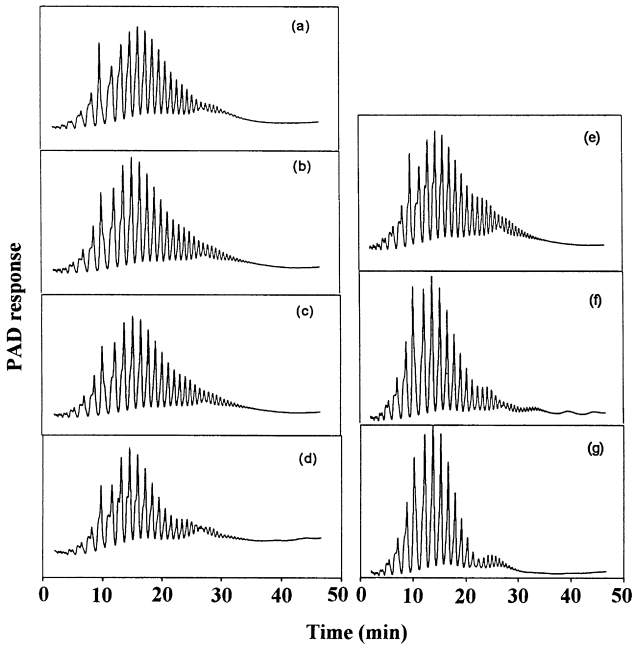

Fig. 7. Chromatograms of acid-resistant parts of starches following debranching with isoamylase (a) wild type potato, (b) wild type pea, (c) *rb* pea mutant, (d) *rug3* pea mutant, (e) *rug4* pea mutant, (f) wild type maize and (g) waxy maize.

4. Discussion

The present studies confirmed previous reports (Gernat et al., 1990; Davydova et al., 1995; Bogracheva et al., 1997; Cairns et al., 1997) that the crystalline parts of starch from wild type pea seeds have both A and B polymorphs. In addition, they have confirmed the previously reported data (Cairns et al., 1997) that at 13% moisture this starch has 20% total crystallinity with a ratio of B- to A-type polymorphs of 0.8 (Table 4). This information has been used as

Table 5
DP^{max} of acid-resistant starches before and after debranching with isoamylase

Source of starch	DP^{max} acid-resistant starch	DP^{max} debranched acid-resistant starch
Wild-type pea	15	15
rb pea mutant	15	15
rug3 pea mutant	14–15	15
rug4 pea mutant	14–15	15
lam pea mutant	15	no data
r pea mutant	16[a]	17[a]
rug5 pea mutant	15[a]	16[a]
Wild-type potato	15	16
Wild-type maize	13–14	14
Waxy maize	13–14	14

[a] The DP^{max} for the first peak.

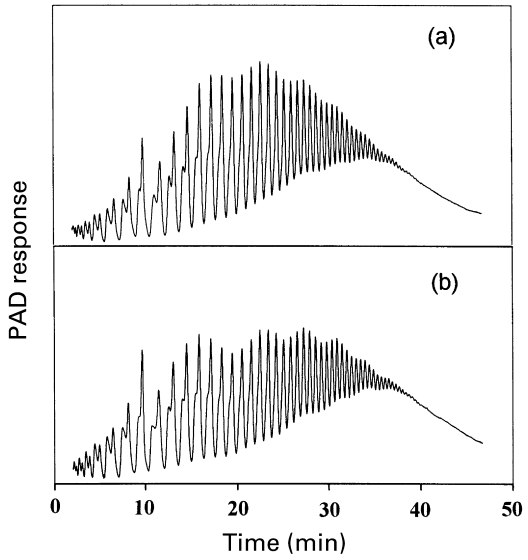

Fig. 8. Chromatograms of acid-resistant parts of starch following debranching with isoamylase (a) *r* pea mutant and (b) *rug5* pea mutant.

the basis for studying the effects of mutations at six loci, known to affect starch biosynthesis in pea seeds, on the crystalline structure and physico-chemical properties of pea starch. Genes at three of these loci (*rb*, *rug3* and *rug4*) affect the substrate supply during starch biosynthesis and genes at the other three loci (*r*, *rug5* and *lam*) are directly involved in the synthesis of the starch polysaccharides (Wang et al., 1998).

The starches from the mutants affecting substrate supply were similar in that they were all C-type (Table 4). The proportion of A and B polymorphs in these starches was affected, however, A being increased relative to B. This change in polymorph composition was more pronounced for starch from the *rug3* mutant than for starch from the *rb* and *rug4* mutants (Table 4). The *rug4* and *rb* starches had an increase in total crystallinity, which correlated with an increase in the proportion of amylopectin in these starches. These effects were more pronounced in starch from the *rb* mutant, in which the proportion of amylopectin was increased by 12% and the total crystallinity by 7%. A link between the total crystallinity and the proportion of amylopectin in the starch is not unexpected as the crystalline areas in starch granules are formed by short chains of amylopectin (French, 1984). The relationship between crystallinity and amylopectin content was less clear, however, for starch from the *rug3* mutant. In this case there was a negative correlation between the change in total crystallinity and the proportion of amylopectin; the amylopectin content increasing by 23% and the total crystallinity decreasing by 3%, compared with starch from the wild-type. It can be suggested that in this starch a smaller proportion of the amylopectin participates in the formation of the crystallites.

The changes in crystalline structure of starches from the mutants directly affecting polysaccharide synthesis (*lam*, *rug5* and *r*) differed from those attributed to mutants

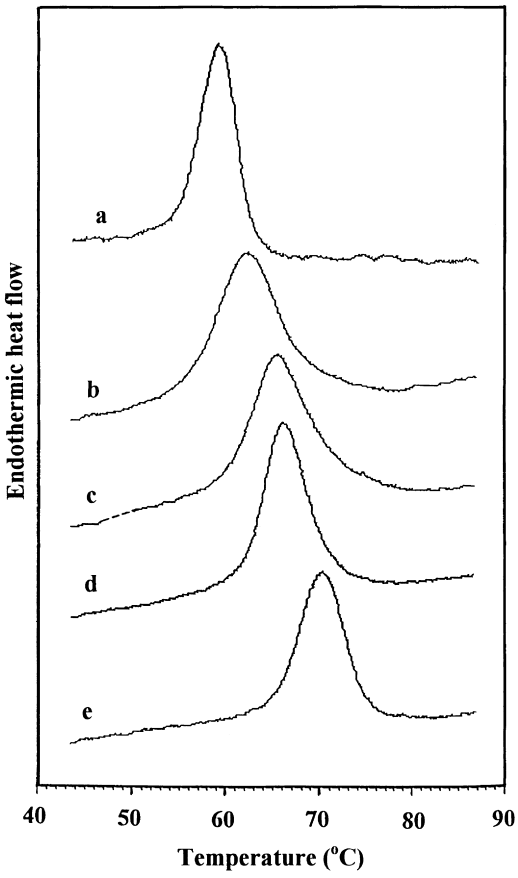

Fig. 9. Differential scanning calorimetry thermograms of starches from wild-type and mutant pea seeds. (a) *lam*, (b) wild-type, (c) *rug4*, (d) *rb* and (e) *rug3*. The instrument background was subtracted. The rate of heating was 1°C/min and the concentration of starches in suspensions was 1.7–2.0%.

affecting the substrate supply. In particular, for this group the proportion of B polymorphs was increased and the proportion of A polymorphs was decreased, the mutants differing for the extent of these changes. For example, the ratio of B to A polymorphs in starch from the *rug5* and *lam* mutants was increased to 1.2 and 2.4, respectively, compared with starch from the wild-type which had a ratio of 0.8. With regard to the increase in the proportion of B polymorphs, the most extreme starch was from the *r* mutant. Examination of the peak positions for starch from this mutant showed that there were no A polymorphs and only peaks associated with B polymorphs were present. When the B polymorph content was calculated using the peak at 14.0°2θ as a reference, a B polymorph value of 73% was obtained (Table 4). This showed that the proportions between the crystalline peaks in this starch and in potato starch, which was used as a reference for B-type crystallinity, were different. From this observation, it can be suggested that the B polymorphs from the *r* mutant starch are arranged in crystallites differing in shape to those in potato and to those in the other pea starches. A similar increase in the proportion of B-type crystallinity has been

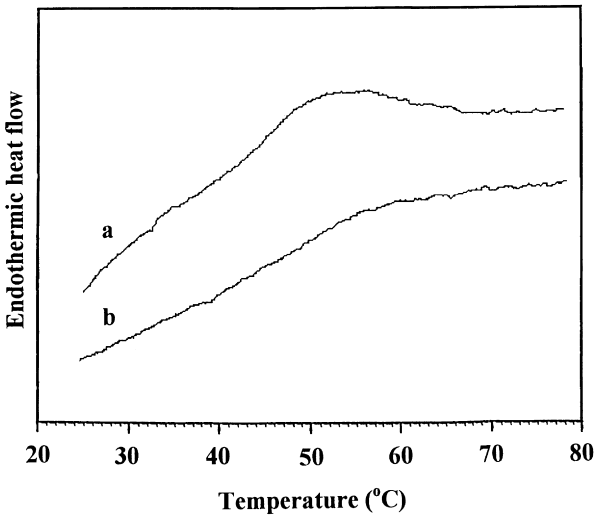

Fig. 10. Differential scanning calorimetry thermograms of starches from (a) *rug5* pea mutant and (b) *r* pea mutant. The instrument background was subtracted. The rate of heating was 1°C/min and the concentration of starches in suspension was 2.0–4.0%.

found in maize starch when the *ae* mutation, which is analogous to *r* in pea, is present (Zobel, 1988; Shi & Seib, 1995; Jane et al., 1997).

The large change in amylopectin content observed in starches from the *rug5*, *r* and *lam* mutants was not reflected in corresponding changes in crystallinity. In particular, the very large increase in the proportion of amylopectin in starch from the *lam* mutant resulted in total crystallinity of 22%, compared with 20% for starch from the wild type. This indicates that the amylopectin in starch from the *lam* mutant has a lower proportion of chains involved in crystalline structures than starch from the wild-type. Conversely, starches from the *r* and *rug5* mutants had a much lower proportion of amylopectin than starch from the wild-type (up to 30% and 52%, respectively compared with 65% for the wild-type). The total crystallinity of these starches, however, was very similar to that found in starch from the wild-type. This indicates that in these starches the proportion of amylopectin chains involved in the formation of crystalline structures is much higher than for starch from the wild type.

The crystalline structures in starch granules are built from the short chains of amylopectin. It is therefore reasonable, to compare changes in the crystalline structure of starches from the different mutants with their amylopectin structure. It has been shown previously, that for starches from two mutants affected in substrate supply (*rb* and *rug4*) the distribution of DP for amylopectin chains is similar to that for starch from wild type peas (Lloyd, 1995; Lloyd, Hedley, Bull & Ring, 1996). In the present article we analysed the chain length distribution of the crystalline areas of starches from this group of mutants, following acid hydrolysis. We assumed that this treatment extracts the amorphous parts of the starch while leaving the crystalline parts intact. Taking this assumption into account, we were able to show that the distribution of amylopectin chains in the crystalline parts of this group of starches was similar to that found for starch from the wild-type. It is evident, therefore, that the changes in crystalline structure observed for this group of starches were not related to changes in the structure of amylopectin.

It has been shown previously that the amylopectin structure of starches from mutants known to be affected in amylopectin synthesis (*r* and *rug5*), differs from that of the starch from the wild-type (Lloyd, 1995; Craig et al., 1998). In these earlier studies, starch from the *r* mutant had a reduction in the proportion of chains with DP of about 22 and 45, while starch from the *rug5* mutant had an increased proportion of chains with DP 15. In addition, starch from the *rug5* mutant had a proportion of unusually long chains (DP more then 1000) not found in starch from the wild-type. The amylopectin chain distributions for the crystalline parts of starch from the *r* and *rug5* mutants differed from the wild-type and from all other known starches (Figs. 5–8; Table 5; Ring et al., 1993; Jane et al., 1997). In particular, the crystalline parts of these starches had a high proportion of unusually long chains, which may be the result of changes in the process of amylopectin synthesis. There is a correlation between the presence of the long chains and an increase in the proportion of B polymorphs in the starch of these two mutants. This may be related to the suggestion that the average DP for the short chains of B-type starches is higher than for A-type starches (Hizukuri, 1985).

Two types of gelatinisation behaviour were found for the different starches. One type of behaviour was found in starches from the wild-type pea and from those pea mutants where the substrate supply (*rb*, *rug3* and *rug4*), or amylose synthesis (*lam*), was affected. All of these starches showed a sharp peak of heat capacity change during gelatinisation accompanying the disruption of crystallinity. These starches had different T^p values, which decreased in the order—*rug3* > *rb* > *rug4* > wild-type > *lam*. These relative changes in T^p correlated with changes in the ratio of B/A polymorphs, the greater the proportion of B polymorphs the smaller the T^p. This observation supports the hypothesis, that A polymorphs have a higher gelatinisation temperature than B polymorphs (Bogracheva et al., 1998). In addition to the differences in the polymorph composition, these pea starches also had different amylose contents, which decreased in the order—wild-type > *rug4* > *rb* > *rug3* > *lam*. With the exception of starch from the *lam* mutant, this decrease in amylose content correlated with an increase in the T^p. It can be concluded, therefore, that the progressive increase in T^p for starches from the wild-type, *rug4*, *rb* and *rug3* is determined by relatively small increases in the proportion of A polymorphs and decrease in the proportion of amylose. With regard to starch from the *lam* mutant, however, the proportion of amylose was very small, whereas the T^p for this starch was lower than for starch from the wild-type. In this case it appears that the relatively

low gelatinisation temperature for this starch was due to the large increase in the proportion of B polymorphs.

Starches from this group of mutants also showed differences in ΔH, ranging from 6.8 to 12.9 J/g. The basis for these differences was not consistent. The changes in ΔH for starches from the *rb* and *rug3* mutants correlated with a corresponding increase or decrease, in the total crystallinity of these starches. The increased crystallinity of starch from the *rug4* mutant however, was not reflected in an increase in the ΔH. In the case of starch from the *lam* mutant, a significant decrease in ΔH was found, although the total crystallinity was slightly increased. It has been shown that the process of crystallinity disruption spreads along the granule because the disrupted parts swell (Bogracheva et al., 1998). It has been suggested (Wang et al., 1998), that the ΔH of gelatinisation is related not only to the disruption of ordered structures during gelatinisation, but also to other changes in the interactions between starch polysaccharides, for example to their interactions with water molecules.

The second type of gelatinisation behaviour was found in starch from the two mutants affected in amylopectin synthesis (*r* and *rug5*). These starches did not show first order transition during gelatinisation. In each case the gelatinisation was accompanied by relatively small and slow changes in C_p (Fig. 10). This is a similar type of behaviour to that found in starch from the *ae* mutant in maize, which is analogous to the *r* mutation in pea (Wang et al., 1992, 1993b; Katz et al., 1993). In addition to their very wide gelatinisation transition, the two mutant pea starches also had very low ΔH. This type of gelatinisation behaviour reflects the differences in granular structure between these two starches and the other pea starches studied. The dramatic changes in the structure of those parts of amylopectin involved in the ordered parts of the granule may result in differences in the sizes of the crystallites, or to some defects in the crystallites, which in turn may result in differences in gelatinisation behaviour. Starch granules also contain amorphous parts. Although the properties of these parts were not studied in the present article, they may greatly influence the physico-chemical properties of starches and, in particular, their gelatinisation behaviour.

5. Conclusion

It is apparent that the presence of mutations known to affect starch biosynthesis in pea seeds results in changes in the crystalline structure and gelatinisation behaviour of the starch, and that different mutations bring about different changes. Mutations in genes that influence the flow of substrate in the starch biosynthetic pathway result in starches with changes in total crystallinity and, possibly, increases in the proportion of A polymorphs. On the contrary, the presence of mutations in genes which directly influence the synthesis of amylopectin and amylose result in very small or no changes in the total crystallinity. The presence of these mutations, however, does bring about changes in the proportion of A and B polymorphs in starch granules, although these changes are different to those found when mutations affecting substrate flow are present. In particular, the proportion of B polymorphs is increased. This effect is dramatic when the *r* mutation is present and results in the starch becoming B-type.

Changes in starch crystalline structure were reflected in gelatinisation behaviour. Starches produced by mutants with lesions affecting substrate supply all showed first order transition of C_p during gelatinisation, which was similar to that shown by starches from wild type peas and from the *lam* mutant. In all of these starches correlations were found between the proportions of A and B polymorphs and the T^p. Variation was found between these starches for ΔH, but the nature of these differences is not completely understood. Mutations affecting amylopectin synthesis resulted in starches which were changed dramatically in their gelatinisation behaviour. The changes in C_p of these starches during gelatinisation could not be referred to as first order transitions. In addition, their ΔH value was significantly lower than for all other pea starches studied. The changes in gelatinisation behaviour for these starches correlated with equally dramatic changes in the structure of the ordered parts of the amylopectin.

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