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Major differences in isoforms of starch-branching enzyme between developing embryos of round- and wrinkled-seeded peas (*Pisum sativum* L.)

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Abstract. In order to determine whether round- and wrinkled-seeded peas (*Pisum sativum* L.) differ in the activity and properties of starch-branching enzyme (1,4-α-D-glucan, 1,4-α-D-glucan-6-glycosyl transferase; EC 2.4.1.18) in their developing embryos, essentially isogenic lines of peas, differing only at the *r* (*rugosus*) locus that confers the round (RR, Rr) or wrinkled (rr) phenotype, were studied. Activity of the enzyme rises rapidly from an early stage of development in embryos of round peas, but only at later stages in embryos of wrinkled peas. The purified enzyme from mature embryos of round peas can be resolved into two isoforms that differ in molecular weight and in their ability to branch amylose. The purified enzyme from mature embryos of wrinkled peas is a single protein with the same molecular weight and branching properties as one of the isoforms from embryos of round peas. The difference in activity of starch-branching enzyme between embryos of round and wrinkled peas is likely to be due to the absence from embryos of wrinkled peas of one of the isoforms occurring in embryos of round peas.

Key words: Embryo development – 1,4-α-D-glucan, 1,4-α-D-glucan-6-glycosyl transferase – *Pisum* (starch-branching enzyme) – Starch-branching enzyme.

Introduction

The aim of this work was to discover whether there is a major difference between lines of peas differing at the *r* (*rugosus*) locus in the activity and properties of starch-branching enzyme (1,4-α-D-glucan, 1,4-α-D-glucan-6-glycosyl transferase; EC 2.4.1.18).

Abbreviations: DEAE = diethylaminoethyl; FW = fresh weight; kDa = kilodalton; SDS = sodium dodecyl sulfate

The *r* locus has profound effects on the morphology and storage-product composition of pea seeds. Pea seeds that are homozygous recessive at the *r* locus (rr) are wrinkled when mature, whereas pea seeds that are heterozygous or homozygous dominant at this locus (Rr, RR) are round when mature (Mendel 1865; Hedley et al. 1986). Mature round seeds contain more starch with a higher proportion of amylopectin than mature wrinkled seeds. Starch is typically 60–70% amylopectin and 50% of the final dry weight in round seeds, but only 30% amylopectin and 30% of the final dry weight in wrinkled seeds (Kellenbarger et al. 1951; Schneider 1951; Greenwood and Thomson 1962; Kooistra 1962). Mature wrinkled seeds contain more sucrose and lipid than mature round seeds. Sucrose and lipid are typically 10% and 5%, respectively, of the dry weight of wrinkled seeds, but only 5–6% and 2–3%, respectively, of the dry weight of round seeds (Kooistra 1962; Coxon and Davies 1982).

A major cause of the differences between round and wrinkled seeds may be a lesion late in the pathway of starch synthesis. The relatively low starch content of wrinkled seeds indicates that the capacity for starch synthesis during development may be lower than that of round seeds. Measurements of amounts of metabolites of the putative pathway of starch synthesis show that the levels (including the level of ADP-glucose, the substrate of starch synthase) are higher in developing embryos of wrinkled-seeded peas than in those of round-seeded peas (Edwards 1985; Edwards and ap Rees 1986a). This indicates that there may be a block very late in the pathway of starch synthesis in embryos of wrinkled peas, leading to an accumulation of metabolites of the pathway.

The most likely cause of a block in the pathway of starch synthesis in embryos of wrinkled peas

is a reduced activity of starch-branching enzyme, the enzyme that catalyses the conversion of amylose to amylopectin. First, the low amylopectin content of starch from wrinkled seeds indicates that branching of amylose is very restricted during embryo development. Second, the maximum catalytic activities of key enzymes of the pathway of starch synthesis up to and including starch synthase are similar in developing embryos of round and wrinkled-seeded peas (Edwards 1985; Edwards and ap Rees 1986a, b). This indicates that the block in the pathway of starch synthesis is not the consequence of a reduced activity of any enzyme of the pathway between sucrose and amylose. Third, activity of starch-branching enzyme in developing embryos of the wrinkled-seeded cultivar Progress 9 was claimed to be up to 12-fold lower than in the round-seeded cultivar Alaska (Matters and Boyer 1982). However, no evidence was provided that the activities reported were accurate reflections of the maximum catalytic activities of the enzyme in the embryos. In addition, the genetic backgrounds of these cultivars are different, so differences between their developing embryos are not necessarily an effect of the *r* locus.

To discover whether or not the *r* locus has a major effect on starch-branching enzyme in developing embryos, lines of round and wrinkled peas that are essentially isogenic except at the *r* locus (Hedley et al. 1986) have been used. The maximum catalytic activity of starch-branching enzyme during embryo development has been estimated and the properties of the purified enzyme from mature embryos have been studied for both of these lines.

Material and methods

Plant material

All experiments were done on round and wrinkled-seeded genotypes of *Pisum sativum* L. derived from JI 430 (John Innes germplasm collection) as described by Hedley et al. (1986). Plants were grown in a greenhouse at a minimum temperature of 12° C and fed twice weekly with a low-nitrogen fertilizer (Solinure; Fisons, Ipswich, UK). For measurements of starch and starch-branching enzyme during embryo development, side shoots and the main apex of plants were removed to leave only one main stem with three flowering nodes per plant. For measurements of starch-branching enzyme during embryo development and preparation of amyloplasts, pods were removed from the plants onto ice and the seeds were used within 30 min. The seed at either end of the pod was rejected. For asssay of starch and purification of starch-branching enzyme, seeds with their testas removed were frozen at −80° C for up to two months prior to use. Seeds were kept on ice between excision of the pod from the plant and freezing, which was usually less than 2 h. In all cases, the testa of the seed was removed and the embryo (cotyledons + axis) was used in experiments.

Assay of starch branching enzyme

1. *Phosphorylase-stimulation.* The assay contained 0.5 ml 200 mM 2-(N-morpholino)ethanesulphonic acid (Mes) pH 6.6, 50 mM [U−^{14}C]glucose 1-phosphate (296 MBq mol^{-1}; Amersham plc, Amersham, Bucks, UK), 0.3 units phosphorylase a (rabbit muscle; Boehringer, Lewes, Sussex, UK) and 2–25 µl of extract at 25° C. Aliquots of 0.1 ml were removed at 30- or 60-min intervals and boiled for 2 min. Glucose polymer was precipitated and washed according to Hawker et al. (1974), and radioactivity in it was measured by liquid scintillation spectroscopy. Activity was expressed as micromoles glucose incorporated into a methanol-insoluble polymer per unit time during the phase of the assay in which the rate of incorporation was linear with respect to time.

Measurements of turbidity in phosphorylase-stimulation assays were carried out in microtiter plates. Wells contained 0.2 ml 200 mM Mes (pH 6.6), 50 mM glucose 1-phosphate, 0.2 units phosphorylase a and 2–20 µl of extract. Plates were incubated at 25° C and the optical density of assays was monitored at 500 nm in a microtiter-plate reader at 15-min intervals.

2. *Amylose-branching.* The assay contained 1 ml 200 mM sodium citrate (pH 7.0), 0.5 mg amylose and 5–50 µl of extract. Tubes were incubated at 25° C, and 0.1-ml samples were removed at 15-min intervals and added to iodine solution in saturated, aqueous $CaCl_2$ solution according to Hawker et al. (1974). Optical density was monitored at 680 nm. Results are expressed as decreases in optical density per unit time under these conditions.

Extraction of developing embryos for enzyme assays

Embryos were extracted first with a pestle and mortar and then with an all-glass homogeniser in approx. 5 vol. of 50 mM Tris-acetate (pH 7.5), 5% (w/v) sucrose, 2 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid (EDTA) at 4° C. The weight of tissue extracted ranged from approx. 0.05 g for embryos of less than 10 mg fresh weight (FW) to approx. 1.8 g for embryos of around 0.6 g FW. Extracts were centrifuged at 10000 · *g* for 10 min at 4° C and supernatants were removed and kept on ice. The time between extraction and assay was less than 30 min.

Preparation of plastids

Plastids were prepared from embryos of approx. 0.1 mg FW of the round-seeded genotype. Tissue was chopped finely into a medium containing sorbitol, filtered through Miracloth, and the crude homogenate centrifuged at a very low *g*-force precisely according to Denyer and Smith (1988). Pellet and supernatant fractions were mechanically disrupted, then assayed for starch-branching enzyme, and for ADP-glucose pyrophosphorylase and alcohol dehydrogenase according to Denyer and Smith (1988). It was not possible to measure the percentage recovery of activity from the homogenate in the pellet and supernatant fractions because of difficulty in obtaining a representative sample of homogenate. As an alternative check that neither plastid nor supernatant fraction contained inhibitory substances, equal volumes of plastid and supernatant fractions were mixed prior to mechanical disruption. Recovery of activity in this mixed fraction was expressed as a percentage of that predicted from activities in separate aliquots of plastid and supernatant fractions. These values were: ADP-glucose pyrophosphorylase 111%, alcohol dehydrogenase 83%, starch-branching enzyme 92%.

Extraction and assay of starch

Frozen embryos were crushed in a mortar then incubated in 10 ml 80% (v/v) aqueous ethanol at 90° C for 10 min. Samples were centrifuged to pellet the tissue, the supernatant discarded and the tissue extracted three times more with 80% ethanol in the same way. Sample sizes ranged from approx. 0.1 g for embryos of less than 25 mg FW to approx. 2.5 g for embryos of up to 0.7 g FW. Ethanol was allowed to evaporate from the final pellets, which were then ground to a fine slurry in water and made up to a known volume. Aliquots of 3 ml were autoclaved for 45 min at 121° C and 0.2 MPa then samples of 0.05–0.2 ml of the autoclaved material were incubated with α-amylase and amyloglucosidase according to Stitt et al. (1978) and assayed enzymatically for glucose according to Lowry and Passoneau (1972).

Purification of starch-branching enzyme

1. *First ammonium-sulfate precipitation.* Samples of about 140 g of mature (greater than 0.45 g FW) embryos were crushed while still frozen in a mortar in 300 ml 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) (pH 7.8), 0.5 mM EDTA, 2 mM dithiothreitol. The homogenate was squeezed through four layers of muslin and the residue re-extracted with a further 150 ml of extraction medium and squeezed through muslin again. The residue was discarded and the combined filtrates centrifuged at 12000·*g* for 15 min at 4° C. The supernatant was brought to 40% saturation with powdered $(NH_4)_2SO_4$ at 4° C and the precipitate collected by centrifugation.

2. *Diethylaminoethyl (DEAE) Sepharose chromatography and second ammonium-sulfate precipitation.* The $(NH_4)_2SO_4$ precipitate was resuspended in 5 ml 20 mM Tris (pH 7.2), 1 mM mercaptoethanol and dialysed for 15 h against 4 l of this buffer at 4° C. The dialysate was centrifuged for 15 min at 28000·*g* and 4° C and the supernatant (about 40 ml) applied to a column (2.6 cm long, 20 cm i.d.) of DEAE Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) equilibrated with 20 mM Tris (pH 7.2). The column was washed with this buffer followed by a gradient of 170 ml from 0 to 1 M NaCl in 20 mM Tris (pH 7.2), 1 mM mercaptoethanol at a flow rate of 2 ml·min^{-1}. Fractions of 3 ml were collected and assayed for starch-branching enzyme. Twelve to 14 peak fractions were pooled and dialysed for at least 3 h at 4° C against 4 l 20 mM Tris (pH 7.2), 1 mM mercaptoethanol. The dialysate was brought to 30% saturation with powdered $(NH_4)_2SO_4$ at 4° C, the precipitate removed by centrifugation and the supernatant brought to 40% saturation. The precipitate was collected by centrifugation.

3. *Mono Q anion-exchange chromatography.* The precipitate collected at 30–40% $(NH_4)_2SO_4$ was dissolved in 6 ml 20 mM 1,3-bis[tris(hydroxymethyl)-methylamino] propane (Bis-tris propane), pH 7.0, 1 mM mercaptoethanol and dialysed 15 h against 4 l of this buffer at 4° C. The dialysate was centrifuged for 10 min at 10000·*g* and 4° C and the supernatant filtered through a 0.2 μm cellulose-acetate filter. Aliquots of 1.5 ml of the filtrate were loaded onto a Mono Q anion-exchange column (Pharmacia; HR5/5) equilibrated with 20 mM Bis-tris propane (pH 7.0), 1 mM mercaptoethanol on a Fast Protein Liquid Chromatograph (Pharmacia). The column was washed with 4 ml of this buffer then eluted with a 24-ml gradient of 0 to 0.42 M NaCl in this buffer at 1 ml·min^{-1}. Fractions of 1 ml were collected and assayed for starch-branching enzyme. The peak two fractions from all four Mono Q runs were pooled together, dialysed for 2 h against 4 l 20 mM Bis-tris propane (pH 7.0), 1 mM mercaptoethanol at 4° C, filtered, and subjected to Mono Q chromatography exactly as previously except that 0.5-ml fractions were collected. The peak 4–6 fractions were pooled and dialysed for 2 h against 4 l 20 mM bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (Bis-tris), pH 6.0, 1 mM mercaptoethanol at 4° C. The dialysate was filtered and subjected to Mono Q chromatography exactly as previously except that the buffer was 20 mM Bis-tris (pH 6.0), 1 mM mercaptoethanol. Fractions of 0.5 ml were collected and assayed and the peak 3–5 fractions pooled and dialysed 15 h against 4 l 50 mM imidazole (pH 7.0), 1 mM mercaptoethanol at 4° C.

4. *Polyanion anion-exchange chromatography.* The dialysate was centrifuged for 10 min at 10000·*g* and 4° C, then loaded onto a Polyanion SI anion-exchange column (Pharmacia; HR5/5) equilibrated with 50 mM imidazole (pH 7.0), 1 mM mercaptoethanol on a Fast Protein Liquid Chromatograph. The column was washed with 4 ml of this buffer, then with a gradient of NaCl in this buffer. From 0 to 0.5 M NaCl the gradient was 9 ml at a flow rate of 0.5 ml·min^{-1}. From 0.5 to 1 M NaCl the gradient was 17 ml at a flow rate of 0.25 ml·min^{-1}. Fractions of 0.5 ml were collected between 0.5 and 1 M NaCl and assayed for starch-branching enzyme. Peak fractions were dialysed for at least 3 h against 4 l 20 mM Tris (pH 7.2), 1 mM mercaptoethanol at 4° C before further experiments were carried out.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Samples to be analysed were mixed with a sample buffer containing 2% sodium dodecyl sulfate (SDS) (Laemmli 1970) to a final protein concentration of 1 mg·ml^{-1}. Where samples were too dilute initially they were incubated for 24 h with 9 vol. acetone at −20° C and the precipitated protein collected by centrifugation and dissolved in an appropriate volume of sample buffer. Samples were boiled for 2 min immediately prior to application to gels. Molecular-weight-marker proteins (Sigma, Poole, Dorset, UK) were treated in the same way as samples. Gels (100·60·1 mm^3) were 7.5% acrylamide and 0.1% SDS with a stacking gel of 3% acrylamide, and were run in a Tris-glycine buffer according to Laemmli (1970). Proteins were stained with Coomassie Brilliant Blue R.

Measurement of protein

Protein was assayed using the Biorad Protein Assay Dye Reagent (Biorad, München, FRG) with a standard curve of bovine serum albumin.

Results

Assay of starch-branching enzyme. Starch-branching enzyme can be assayed either by its ability to branch amylose (amylose-branching assay) or by its ability to stimulate the rate of synthesis of glucose polymer by phosphorylase a (phosphorylase-stimulation assay). These assays are liable to interference by substrates and other enzymes, particularly starch and starch-hydrolysing enzymes, present in crude extracts of plant tissues. To assess the suitability of the assays for crude extracts of

pea embryos, the following checks were carried out on extracts of mature (over 0.45 g FW) and young (around 0.15 g FW) embryos of both round- and wrinkled-seeded genotypes (referred to as round and wrinkled embryos). First, the effect of addition of a range of activities of purified starch-hydrolysing enzymes to the assays was checked. Levels of activity that interfered with the assay were compared with endogenous levels of activity of these enzymes in extracts of pea embryos. This showed that endogenous α- and β-amylase would not interfere with the phosphorylase-stimulation assay, but that endogenous β-amylase was likely to interfere with the amylose-branching assay. The phosphorylase-stimulation assay alone was used in subsequent experiments. Activity in this assay was dependent upon the presence of phosphorylase a, and was negligible in the absence of extract or with boiled extract. Second, the effect of soluble starch in extracts of embryos on the assay was investigated. Additions of either purified soluble starch or aliquots of boiled extract, giving concentrations of starch in the assay five times greater than those normally present due to the addition of extract, had no effect on the activity.

Third, the intracellular location of the putative starch-branching-enzyme activity was investigated. Starch-branching enzyme would be expected to be associated exclusively with plastids (Echeverria et al. 1985). Plastids were prepared from round embryos of about 0.1 g FW by a mechanical method (Denyer and Smith 1988). Older embryos give unacceptably low yields of plastids. Preparations contained 20–30% of the total activity of a plastidial marker enzyme, ADP-glucose pyrophosphorylase, less than 4% of a cytosolic marker enzyme, alcohol dehydrogenase (Table 1) and less than 10% of a mitochondrial marker enzyme, cytochrome oxidase (data not shown). The percentage of the total extracted activity of putative starch-branching enzyme recovered in plastids was very similar to that of the plastidial marker enzyme in three separate experiments (Table 1). Furthermore, when plastids were partly ruptured during preparation the reductions in the percentages of ADP-glucose pyrophosphorylase and putative starch-branching enzyme recovered in the pellet were very similar (Table 1).

These checks show that the activity measured by the phosphorylase-stimulation assay in crude extracts of pea embryos is not an artefact caused by starch hydrolytic enzymes or soluble starch in the extracts. The association of the activity with plastids from young embryos provides further evidence that it is caused by starch-branching enzyme.

Table 1. Localisation of starch-branching-enzyme activity. Cotyledons (about 7 g) of embryos (about 100 mg FW) from round-seeded peas were chopped into a medium containing sorbitol. For intact plastids, an aliquot of the homogenate was filtered and centrifuged at 100·g for 10 min to produce a plastid pellet and a supernatant. For broken plastids the procedure was the same except that the homogenate was subjected to vortex mixing prior to centrifugation. Fractions were mechanically disrupted to rupture organelles prior to assay. Activity in the plastid fraction is expressed as a percentage of the sum of the activities in the plastid and supernatant fractions (see *Material and methods*). Values are means ±SE of three measurements, each made on a separate preparation (intact plastids), or two individual measurements, each made on a separate preparation (broken plastids)

	Percentage of activity recovered in	
	intact plastids	broken plastids
ADP-glucose pyrophosphorylase	22.3±1.7	12.5, 12.2
Alcohol dehydrogenase	2.5±0.6	3.3, 1.9
Starch-branching enzyme	24.6±1.8	16.0, 14.9

Activity of starch-branching enzyme during embryo development. To check whether loss of enzyme activity caused by inhibition or degradation was occurring during extraction of a particular age or genotype of embryo, recovery experiments were carried out. Two replicate samples of each of two sorts of embryo were prepared. One sample of each sort was extracted separately and the remaining two samples were mixed and extracted together. Activity in the mixed extract was compared with that predicted from the activities in separate extractions of the two sorts of embryo. This was done for the following pairs of embryos: mature round + young round, mature round + young wrinkled, mature round + mature wrinkled, mature wrinkled + young round, mature wrinkled + young wrinkled. Activity in mixed extracts was always within 17% of the predicted value. It is thus unlikely that there is any major inhibition or degradation of starch-branching enzyme during extraction of any of these sorts of embryo. For each of these sorts of embryo the concentration of components and the pH of the assay were checked to ensure that the rate was maximum.

Activity of starch-branching enzyme was first detectable in round embryos at a fresh weight of about 0.01 g, and it rose very rapidly as embryo weight increased to 0.1–0.15 g (Fig. 1). Between 0.15 g and 0.35 g there was relatively little increase in activity of starch-branching enzyme. This plateau was observed in two, independent experiments on two, separately-grown batches of peas (Fig. 1

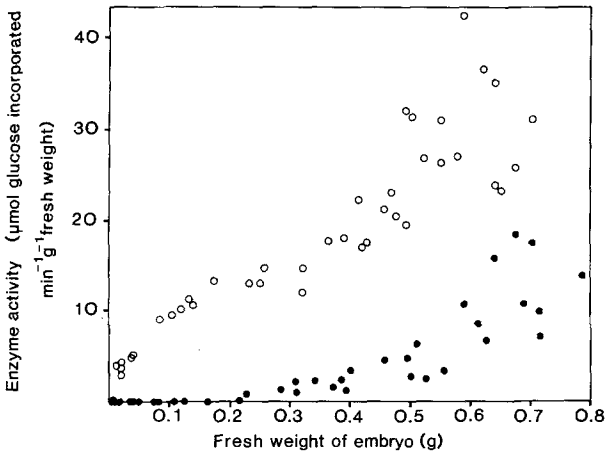

Fig. 1. Activity of starch-branching enzyme in developing embryos of round- and wrinkled-seeded peas. Embryos were harvested at intervals from a single batch of round- and wrinkled-seeded pea plants, and immediately extracted and assayed for starch-branching enzyme. ●, Wrinkled-seeded plants; o, round-seeded plants

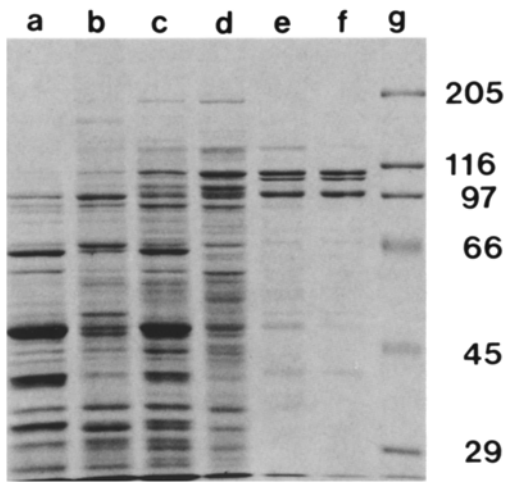

Fig. 2. Separation of proteins from fractions during the purification of starch-branching enzyme from embryos of round-seeded peas. Protein was made up to a concentration of 1 mg·ml^{-1} in sample buffer (Laemmli 1970) and subjected to SDS-polyacrylamide gel electrophoresis in a 7.5% gel. Fractions are as described in Table 2. Lanes contain 5–10 μg protein (*lanes a–d*) or approx. 3 μg protein (*lanes e, f*). *Lane a*, initial supernatant; *lane b*, 0–40% ammonium-sulfate precipitate; *lane c*, DEAE Sepharose chromatography; *lane d*, 30–40% ammonium-sulfate precipitate; *lane e*, Mono Q chromatography pH 7.0; *lane f*, Mono Q chromatography pH 6.0; *lane g*, marker proteins of known molecular weight (kDa)

and data not shown). Between 0.35 g and 0.6 g, activity again rose rapidly. In contrast, activity of starch-branching enzyme was not detectable in wrinkled embryos until they reached 0.2 g FW. Between 0.35 g and 0.7 g activity rose rapidly, at a rate comparable with that in round embryos over the same range of weights.

Purification of starch-branching enzyme. To discover whether the properties of starch-branching enzyme in round and wrinkled embryos are different, the enzyme was purified from mature (greater than 0.45 g FW) embryos of both genotypes. Previously published methods using affinity chromatography on amino-alkyl agarose (Boyer and Preiss 1978a, b) were not effective with the enzyme from pea embryos. The enzyme was purified by ammonium-sulfate precipitation and anion-exchange chromatography on columns of DEAE Sepharose and Mono Q (see *Material and methods*, Table 2). Under the conditions used, the enzyme eluted from these columns as a single peak. Fractions of low activity were rejected at each stage of the purification. A typical purification of the enzyme from the embryos of round seeds is shown in Table 2, and the appearance of stages of the purification on an SDS-polyacrylamide gel is shown in Fig. 2.

Table 2. Purification of starch-branching enzyme from embryos of round peas. A sample of 280 g of embryos was crushed, filtered and centrifuged. The supernatant was brought to 40% saturation with ammonium sulfate and the precipitate was subjected to chromatography on a DEAE Sepharose Fast Flow column. Fractions of highest activity were pooled and brought to 30–40% saturation with ammonium sulfate. The precipitate was subjected to chromatography on a Mono Q column twice at pH 7.0 and then once at pH 6.0. For all fractions but the first supernatant, activity was measured after the dialysis that followed each step

Fraction	Total protein (mg)	Total activity (μmol glucose incorporated·min^{-1})	Specific activity (μmol glucose incorporated·min^{-1}·mg^{-1} protein)
Initial supernatant	14740	7650	0.52
0–40% Ammonium-sulfate precipitation	2460	2830	1.15
DEAE Sepharose chromatography	344	2085	6.1
30–40% Ammonium-sulfate precipitation	42	750	17.9
Mono Q chromatography pH 7.0	1.08	120	111
Mono Q chromatography pH 6.0	0.30	84	280

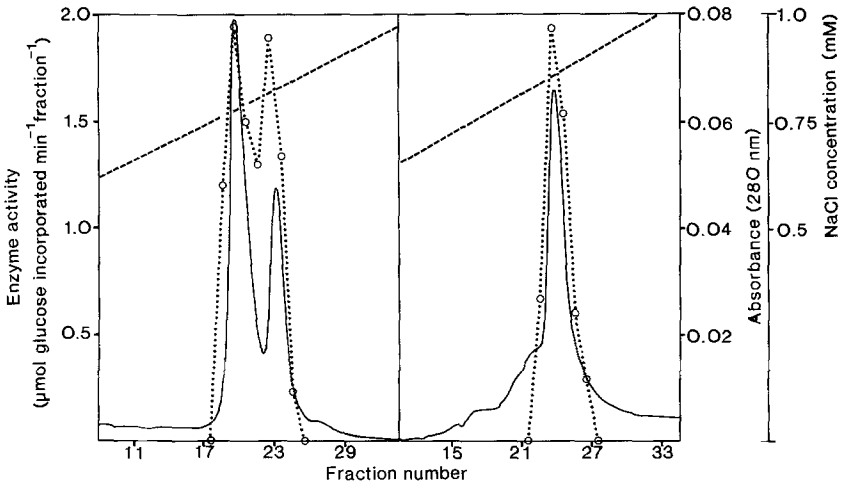

Fig. 3. Chromatography of purified starch-branching enzyme on a Polyanion anion-exchange column. The enzyme was applied to a Polyanion column in 50 mM imidazole (pH 7.0) and eluted with a gradient of NaCl. Fractions of 0.5 ml were collected and assayed for starch-branching enzyme. Activity recovered from the column was 90% of that applied for the enzyme from wrinkled embryos, and 98% of that applied for the enzyme from round embryos. *Left panel*, enzyme from round embryos; *right panel*, enzyme from wrinkled embryos. -----, Salt gradient; —, absorbance (280 nm); ·····, enzyme activity

The purified enzyme from the embryos of round seeds had a specific activity 500- to 1000-fold greater than that of the enzyme in the initial supernatant derived from a crude homogenate of embryos (range of ten preparations). Electrophoresis using SDS-polyacrylamide gels showed that the purified preparation contained three major proteins (Fig. 2, lane f) of approximate molecular weights 114, 108, and 100 kilodaltons (kDa). These proteins could not be separated by Mono Q chromatography. However, chromatography of this preparation on a Polyanion SI anion-exchange column resolved the proteins and the activity of starch-branching enzyme into two peaks (Fig. 3) that eluted at NaCl concentrations of 0.78 M and 0.85 M. The first peak to elute (peak 1) contained only the 114- and 108-kDa proteins, and the second peak to elute (peak 2) contained only the 100-kDa protein (Fig. 4, lanes d, e). When peaks 1 and 2 were re-chromatographed separately on the Polyanion column, all of the activity of starch-branching enzyme recovered from the column co-eluted with the peak of protein in both cases (data not shown). This indicates that the activity of starch-branching enzyme is associated with one or both of the 114- and 108-kDa proteins and with the 100-kDa protein.

The purified enzyme from wrinkled embryos had a specific activity 1000- to 1400-fold greater than that of the enzyme in the initial supernatant derived from a crude homogenate of embryos (range of three preparations). Electrophoresis on SDS-polyacrylamide gels showed that purified preparations contained only one major protein of molecular weight 100 kDa (Fig. 4, lane a). When chromatographed on a Polyanion column this protein co-eluted with starch-branching-enzyme activity at a NaCl concentration of 0.85 M (Fig. 3).

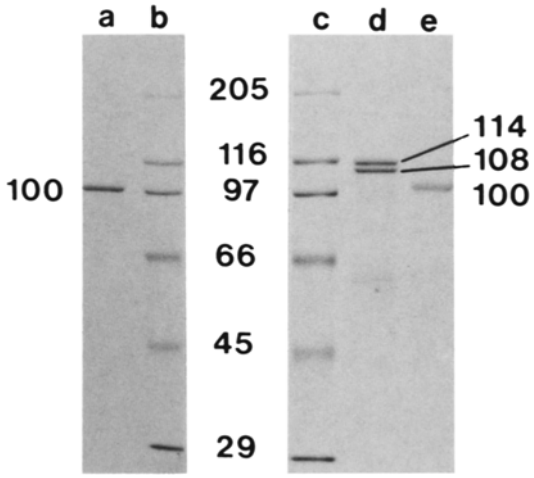

Fig. 4. Separation of proteins from fractions of purified starch-branching enzyme. Protein in peaks of starch-branching-enzyme activity eluted from a Polyanion column (*lanes a, d, e*) was subjected to SDS-polyacrylamide gel electrophoresis in 7.5% gels. *Lane a*, approx. 2 μg protein from wrinkled embryos; *lanes b, c*, marker proteins of known molecular weight (kDa); *lanes d, e*; 1–2 μg protein from peaks 1 and 2, respectively, from round embryos

To check whether the absence of the 114- and 108-kDa proteins and their associated starch-branching-enzyme activity from wrinkled embryos was the result of their loss during extraction and purification, starch-branching enzyme was purified from a mixture of equal weights of round and wrinkled embryos. The purified preparation contained major proteins of molecular weights 114, 108, and 100 kDa (Fig. 5). Thus the presence of wrinkled embryos in the extraction did not prevent the recovery of the 114- and 108-kDa proteins in the final, purified preparation.

Properties of isoforms of starch branching enzyme. To determine whether the putative isoforms of

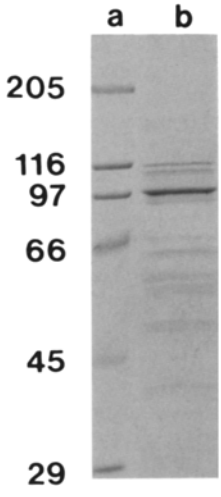

Fig. 5. Separation of proteins from purified starch-branching enzyme from a mixture of round and wrinkled embryos. Protein in the peak of starch-branching-enzyme activity eluted from a Mono Q column (pH 6.0) was subjected to SDS-polyacrylamide gel electrophoresis in a 7.5% gel. *Lane a*, marker proteins of known molecular weight (kDa); *lane b*, 2 μg protein from purified starch-branching enzyme

starch-branching enzyme separated by Polyanion chromatography have different properties, their activities were measured in three types of assay. The specific activity of the enzyme in peaks 1 and 2 from round embryos and the single peak from wrinkled embryos was measured using both the phosphorylase-stimulation and the amylose-branching assays. In addition, the rate of increase in turbidity of the phosphorylase-stimulation assay for these three fractions was measured. The appearance of turbidity in this assay is caused by precipitation of the glucose polymer formed by the joint action of phosphorylase and starch-branching enzyme.

The specific activities of starch-branching enzyme in peaks 1 and 2 from round embryos and the single peak from wrinkled embryos were very similar when measured by the phosphorylase-stimulation assay (Table 3). When measured by the amylose-branching assay, peak 2 from round embryos and the peak from wrinkled embryos had similar specific activities, but the specific activity of peak 1 from round embryos was about 13-fold lower (Table 3). The rate of increase of turbidity in the phosphorylase-stimulation assay of peak 1 from round embryos was proportional to the amount of enzyme protein in the assay (Fig. 6a). However, the rate of increase of turbidity and the final turbidity of the assay were inversely related to the amount of enzyme protein in the assay for both peak 2 from round embryos and the peak from wrinkled embryos. At low protein concentrations turbidity rose rapidly to a final value of about 0.8 A (measured at 500 nm). At higher protein concentrations, turbidity rose more slowly to a final

Table 3. Properties of purified starch-branching enzyme. Peak fractions of starch-branching-enzyme activity eluted from a Polyanion column were assayed by the amylose-branching and phosphorylase-stimulation assays. For explanation of units, see *Material and methods*. Values are means ± SE of three measurements, each made on a separate preparation of purified enzyme

Fraction	Assay	
	Amylose-branching (OD·min^{-1}·mg^{-1} protein)	Phosphorylase-stimulation (μmol glucose incorporated·min^{-1}·mg^{-1} protein)
Round embryo peak 1	0.48 ± 0.06	253 ± 43
Round embryo peak 2	6.72 ± 0.93	317 ± 57
Wrinkled embryo	6.54 ± 1.04	287 ± 34

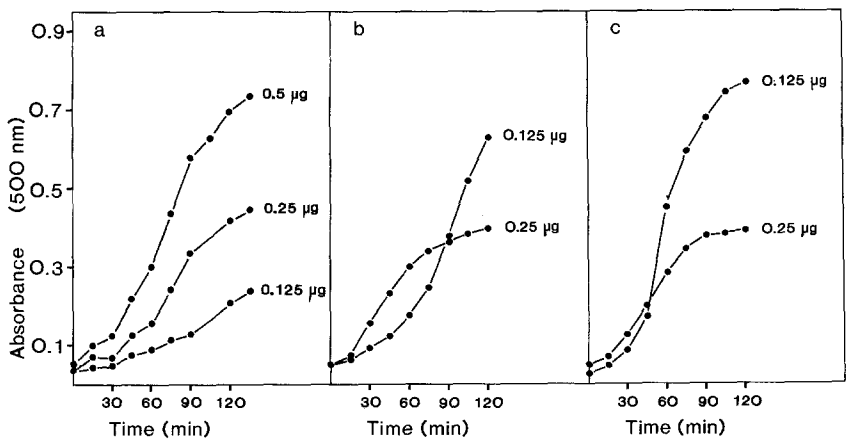

Fig. 6a–c. Precipitation of polymer in phosphorylase-stimulation assays. Peak fractions of starch-branching enzyme activity eluted from a Polyanion column were assayed by the phosphorylase-stimulation assay in a microtiter plate. Precipitation in the assay was monitored at 500 nm. Amounts of starch-branching-enzyme protein used in the assays are indicated.
a, **b** Peaks 1 and 2, respectively, from embryos of round-seeded peas;
c peak from embryos of wrinkled-seeded peas

value of only 0.4 A (Fig. 6b, c). This indicates that the polymer made by the enzymes in peak 2 and the peak from wrinkled embryos at high concentration is more soluble than the polymer made by the enzyme in peak 1.

Discussion

The data presented in this paper show that the *r* locus has a profound effect on the activity of starch-branching enzyme in developing pea embryos. Activity in embryos that are homozygous dominant at this locus rises rapidly from an early stage of development, whereas activity in embryos that are homozygous recessive at this locus does not rise until around the mid-point of development. These changes indicate that the *r* locus may affect one of two forms of the enzyme. Thus there may be a form present in both round and wrinkled embryos which increases in activity only after the mid point of development, and a form present in round embryos only which increases in activity at a much earlier stage of development.

Attempts to obtain reproducible separations of isoforms of starch-branching enzyme from crude extracts of round embryos by ion-exchange chromatography were not successful (data not shown). However, the purified enzyme from round embryos could be separated into two forms by chromatography on a Polyanion anion-exchange column. The properties of the two forms differ considerably. Peak 2 branches amylose more effectively and makes a more soluble, hence probably a more branched, polymer in the phosphorylase-stimulation assay than peak 1. The two major proteins in peak 1, one or both of which may be starch-branching enzyme, differ in molecular weight from the protein in peak 2. It is likely, therefore, that peaks 1 and 2 represent two different isoforms (referred to as isoforms I and II, respectively) of starch-branching enzyme.

The single form of starch-branching enzyme found in purified preparations of the enzyme from wrinkled embryos appears to be the same as isoform II from round embryos. It elutes from the Polyanion column at the same salt concentration and has the same specific activity in the amylose-branching assay as isoform II, and it produces a polymer in the phosphorylase-stimulation assay of similar solubility to that produced by isoform II. The protein has the same apparent molecular weight as isoform II from round embryos. The reduction in the activity of starch-branching enzyme in wrinkled relative to round embryos may therefore be the consequence of the absence of one isoform, isoform I, from wrinkled embryos. It is therefore possible that isoform I is responsible for the rise in the activity of starch-branching enzyme early in the development of round embryos, whereas isoform II is responsible for the rise in activity later in the development of both round and wrinkled embryos.

The effects of the *r* locus on starch-branching enzyme in pea embryos may be analogous to the effects of the locus *amylose extender* (*ae*) on this enzyme in the endosperm of developing maize (*Zea mays* L.) kernels. There are two fractions of starch-branching enzyme, I and II, in maize endosperm. Like the isoforms from pea, they differ in the ratio of their activities in the amylose-branching and the phosphorylase-stimulation assays (Boyer and Preiss 1978a; Hedman and Boyer 1982). Endosperm that is recessive at *ae* resembles wrinkled-pea embryos in that it has reduced activity of starch-branching enzyme and a very high ratio of amylose to amylopectin in its starch grains relative to dominant endosperm (Boyer and Preiss 1978b; Mercier 1973). The reduction in starch-branching-enzyme activity appears to be due specifically to the loss of one of the two fraction-II isoforms, isoform IIb. Isoforms I and IIa are not affected by *ae*. It has been suggested that the gene coding for isoform IIb may be at the *ae* locus (Hedman and Boyer 1982, 1983). However, recent work that indicates that isoforms IIa and IIb are identical proteins calls this into question (Singh and Preiss 1985). Two or more isoforms of starch-branching enzyme with different capacities to branch amylose have been reported from other plant organs (spinach leaf: Hawker et al. 1974; *Sorghum* seed: Boyer 1985). In the few tissues from which the enzyme has been purified it is reported to be a monomer of molecular weight 80–90 kDa (potato tubers: Borovsky et al. 1975; endosperm of maize kernels: Boyer and Preiss 1978b; Singh and Preiss 1985). The enzyme from pea embryos has an apparent molecular weight on SDS-polyacrylamide gels that is considerably above this range (100–114 kDa, Fig. 4).

Some of the effects of the *r* locus on the storage-product composition of pea seeds may be consequences of its effect on isoform I of starch-branching enzyme. First, the reduced amount of starch may be a result of the reduced activity of starch-branching enzyme in wrinkled embryos. The pattern of synthesis of starch (assayed as ethanol-insoluble material digested to glucose by α-amylase and amyloglucosidase) during embryo development is closely correlated with the changes in activity of starch-branching enzyme (Fig. 7). In round

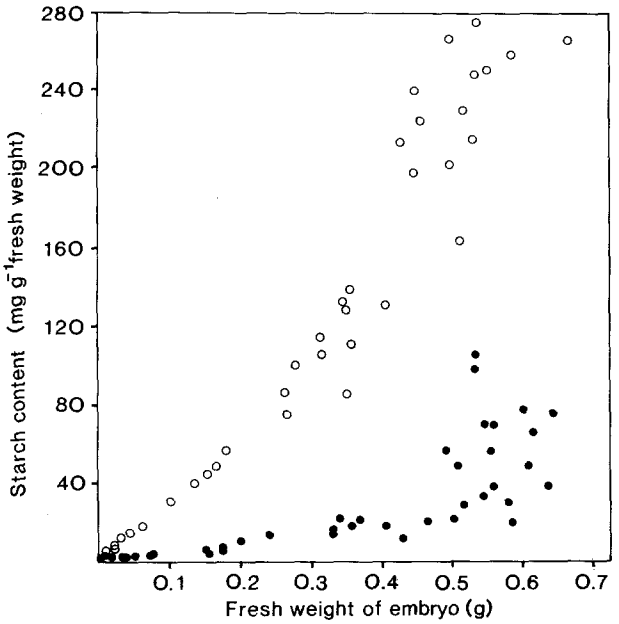

Fig. 7. Starch content of developing embryos of round and wrinkled-seeded pea plants. Embryos were harvested at intervals from a single batch of round and wrinkled-seeded plants, and frozen at −80° C. Frozen samples were crushed and extracted in boiling ethanol, then the insoluble residue was rehydrated, homogenised, autoclaved and digested with α-amylase and amyloglucosidase. Glucose was determined enzymatically on the digestion products. •, Wrinkled-seeded plants; o, round-seeded plants

embryos, in which starch-branching-enzyme activity rises from an early stage of development, starch accumulation also occurs throughout development. In wrinkled embryos, in which starch-branching-enzyme activity rises only late in development, starch accumulates only later in development (Fig. 7). A mechanism by which the activity of starch-branching enzyme could determine the overall rate of starch synthesis is indicated by the observation that the activity of purified starch synthase is considerably stimulated by starch-branching enzyme (Boyer and Preiss 1979; Pollock and Preiss 1980). This is thought to be due to the provision of increased numbers of non-reducing ends of glucose chains as the substrate for starch synthase by the action of starch-branching enzyme. Thus low activity of starch-branching enzyme may reduce the activity of starch synthase in vivo, and hence reduce the overall rate of starch synthesis (Boyer and Preiss 1979; Pollock and Preiss 1980). Second, the increased amount of sucrose in wrinkled embryos could be a consequence of a lower rate of starch synthesis in these embryos. However, synthesis of ADP-glucose via ADP-glucose pyrophosphorylase may be irreversible in vivo since plastids contain an alkaline pyrophosphatase (Gross and ap Rees 1986). If this is the case, it is not obvious how a lower rate of starch synthesis caused by a reduced activity of starch-branching enzyme can affect the rate of synthesis of ADP-glucose from sucrose and hence be responsible for the increased amounts of sucrose in wrinkled embryos.

The rate of starch synthesis in wrinkled embryos is unlikely to be determined solely by the total activity of starch-branching enzyme. During the later stages of embryo development, the relationship between the rate of starch synthesis and the rate of increase of starch-branching-enzyme activity is very similar in round and wrinkled embryos, but the final amylose content of starch in wrinkled seeds is much greater than in round seeds. The isoform of starch-branching enzyme present in both round and wrinkled embryos makes polymers in vitro that are more branched than those made by the isoform present only in round embryos. Thus although in-vitro assays indicate that starch-branching enzyme in wrinkled embryos may have a higher capacity to branch amylose than the enzyme in round embryos, the starch made in wrinkled embryos actually has a higher amylose content than that in round embryos. I suggest that the roles of the two isoforms of starch-branching enzyme in the synthesis of starch grains in vivo are likely to be different.

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