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).

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 amyllopectin than mature wrinkled seeds. Starch is typically 60–70% amyllopectin and 50% of the final dry weight in round seeds, but only 30% amyllopectin 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 amyllopectin. First, the low amyllopectin 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 (Solunure; 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 amyllopectins, 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 assay 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 [\(^{14}C\)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. 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 spectrophotometry. 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 amyllose 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 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. 1.5 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%.