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Determination of the effect of *r* and *rb* mutations on the structure of amylose and amylopectin in pea (*Pisum sativum* L.)

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The structure of starch from pea lines near-isogenic except for mutation at the *r* and *rb* loci was compared with that of the wild-type. The isolated starch polysaccharides were fractionated by size exclusion chromatography, and the constituent chain profile of the branched amylopectin components examined. A mutation at the *rb* locus had little effect on starch structure and composition. A mutation at the *r* locus (a genetic lesion affecting the activity of an isoform of starch branching enzyme) resulted in an increase in the amylose content of the starch and a change in the structure of amylopectin. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Starch is the main storage polysaccharide of higher plants and consists of amylose and amylopectin. Amylose is an essentially linear molecule linked by α-(1–4) glycosidic linkages with an average molecular weight as high as 2×10^6 g mol^{-1}. Natural amylose fractions can contain branched molecules, with recent evidence (Takeda *et al.*, 1993) suggesting that the branches are predominately short chains linked to a longer linear chain. In contrast, amylopectin is a highly branched macromolecule consisting of linear (1–4)-α-D-glucose chains linked through α-(1–6) branch points on average every 20–25 residues. Examination of the constituent chain profile of amylopectin shows a polymodal distribution with two main fractions of short and long chains with a degree of polymerization of ~15 and 45, respectively (Hizukuri, 1986); some amylopectins contain a fraction of much longer chains (Takeda *et al.*, 1987). The distribution is characteristic of the botanical origin of the starch (Hizukuri, 1985). An accepted model of the structure of amylopectin is the 'cluster' model (French, 1984; Robin *et al.*, 1974; Manners & Matheson, 1981) in which 'short' chains are arranged in clusters, which are linked to each other by 'long' chains that are themselves linked together. Studies on starch structure have examined differences between species (Hizukuri, 1986; Takeda *et al.*, 1987), and between cultivars of the same species (Colonna & Mercier, 1984; Takeda *et al.*, 1987).

In peas (*Pisum sativum* L.), there are two distinct genetic loci, *r* and *rb*, mutations at which cause the mature, dry embryo to be wrinkled in appearance (Kooistra, 1962). Mutations at both these loci are known to affect enzymes within the starch biosynthetic pathway (Smith & Denyer, 1992). A mutation affecting the *R* gene decreases the activity of the starch branching enzyme (SBE) during embryo development (Smith, 1988). There are two isoforms of SBE in pea embryos (SBEI and SBEII; Smith, 1988) and embryos which are homozygous recessive at the *r* locus lack the SBEI isoform. Effects of the *r* mutation include a decrease in the rate of starch synthesis (Smith, 1988), an increase in the proportion of amylose in the starch from ~35 to 65–70% (Greenwood & Thomson, 1962; Colonna & Mercier, 1984), a change in granule crystallinity from a C polymorph with a tendency to B, to the B polymorph (Buléon *et al.*, 1987) and a change in the morphology of the starch granules which appear 'compound' in shape compared with the wild-type 'simple' grains (Boyer, 1981). Mutant alleles at the *rb* locus reduce the activity of ADPG pyrophosphorylase (Smith *et al.*, 1989) and reduce the embryo starch content (Kooistra, 1962). A mutation in the *rb* gene has no effect on starch granule morphology (Boyer, 1981). Pea lines have been bred that contain little genetic variation except for alleles at the *r* locus (*rrRbRb*; Hedley *et al.*, 1986), the *rb* locus or

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both loci (*RRrbrb*, *rrrbrb*; Hedley *et al.*, 1994) when compared to the wild-type (*RRRbRb*). These lines are known as near-isogenic and are used in this study as they are particularly useful for determining the effects of specific genes.

Although there is variation in results of studies of the structure of round (*RR*) and wrinkled (*rr*) pea starch (Bertoft *et al.*, 1993), it has been established that in addition to a high molecular weight amylopectin, there is also a much smaller branched component with structural similarities to amylopectin (Colonna & Mercier, 1984). In this paper we examine the effects of mutations at both the *r* and *rb* loci on the fine structure of pea starch.

MATERIALS AND METHODS

All work was performed on starch extracted from the BC3/*RRRbRb*, *rrRbRb*, *RRrbrb* and *rrrbrb* near-isogenic pea lines (Hedley *et al.*, 1986, 1994) by an aqueous extraction procedure.

All reagents used were of analytical reagent grade. Isoamylolysis of amylopectins and amyloses was performed by incubating 5mg of polysaccharide in 0.375 ml deionized water and 0.025 ml of 1 M acetate buffer, pH 3.6 with 5000U *Pseudomonas* isoamylase (ICN Biochemicals) at 37°C for a period of 3 h. The incubation mixture was boiled to destroy any remaining enzyme and immediately analysed to avoid precipitation of the constituent chains.

Chromatography

A non-granular preparation of starch was prepared by dissolution in dimethyl sulphoxide/water (90:10) at room temperature followed by alcohol precipitation. The polysaccharide product was dissolved in 0.1 M NaOH (5 mg/ml) and fractionated on a column (790 × 15 mm) of Sepharose CL-2B (Pharmacia Inc.) by elution with 0.01 M NaOH at a flow rate of 0.5 ml/min at room temperature. Carbohydrate in the eluate was continuously monitored using a index detector (Gilson 132) and fractions (2.5 ml) were collected. After

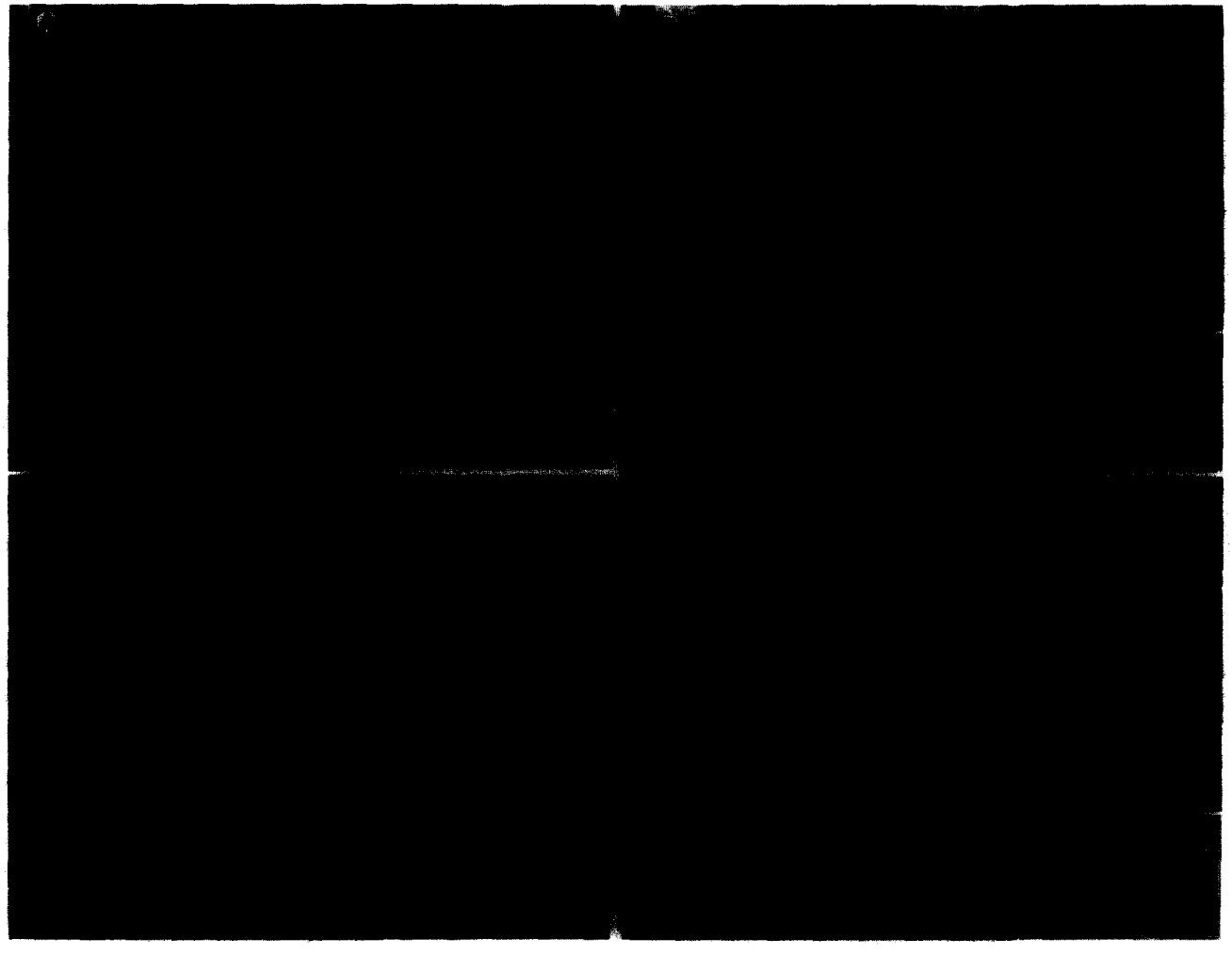

Fig. 1. Scanning electron micrographs of pea starch granules (space bar represents 10 μm) A, *RRRbRb* line; B, *rrRbRb* line; C, *RRrbrb* line; D, *rrrbrb* line.

neutralization with acetic acid, the iodine binding behaviour of the starch fractions was assessed in the usual way.

Debranched amylopectins (~100 μg) were further characterized by gel-permeation chromatography on TSK-gel columns (300 × 7.5 mm) — $G3000SW_{xl}$, $G2000SW_{xl}$ and $G2000SW_{xl}$ (Tosoh) connected in series, eluted with 10 mM acetate buffer pH 5.0 at a flow rate of 1.5 ml/min. The column was calibrated with linear dextrins of known DP and pullulan standards of narrow molecular weight distribution (Polymer Laboratories). The constituent chains of amylopectin were also separated by ion exchange chromatography on a Dionex Carbopac PA1 (250 × 4 mm) column with a PA1 guard column (50 × 4mm). The carbohydrate was eluted with a gradient from 100 mM NaOH, 120 mM Na acetate to 100 mM NaOH, 540 mM Na acetate over 50 min at a flow rate of 1 ml/min. Carbohydrate in the eluate was detected using a pulsed amperometric detector (Dionex).

RESULTS AND DISCUSSION

When viewed using scanning electron microscopy it was confirmed that a mutation in the *r* gene affected starch granule morphology through the appearance of 'compound' granules (Fig. 1B and D), while a mutation at *rb* had no effect (Fig. 1C) compared with the wild-type (Fig. 1A). The amylose content of the starches was determined using a calorimetric assay of iodine binding behaviour. If it is assumed that only the amylose fraction binds the iodine–polyiodide complex, then the amylose contents of the starches were 38 and 31% for the *RRRbRb* and *RRrbrb* isolines, and 72 and 55% for the *rrRbRb* and *rrrbrb* isolines. To obtain more detailed information on the composition of the different starches, they were fractionated by size exclusion chromatography on Sepharose CL-2B. The elution profiles of the starches are shown in Fig. 2A–D and all show the presence of two main components, one eluting close to the void volume and the other, of a lower molecular size, eluting as a broad peak. As amylopectin is a larger molecule than amylose, the peak eluting close to the void volume is likely to consist largely of amylopectin with the other component being amylose. If this is assumed then the amylose content of the starches would be 29% for the *RRRbRb* and *RRrbrb* isolines and 64% for the *rrRbRb* and *rrrbrb* isolines. The λ_{max} of the iodine–polyiodide ion complex of purified amylopectins from pea starch was at 560 nm (Colonna & Mercier, 1984). In the present study, the peak eluting at the void volume had a λ_{max} of 570–580 nm, suggesting a limited contamination with amylose. To obtain an estimate of the magnitude of the contamination, the fraction was debranched with isoamylase and fractionated by size exclusion chromatography. A peak which gave a blue

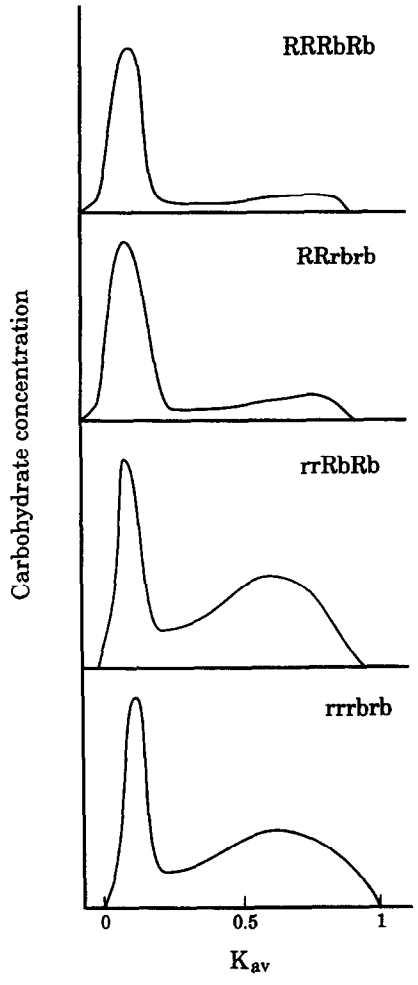

Fig. 2. Elution prophiles of pea starches separated on a sepharose CL-2B column A, *RRRbRb* line; B, *RRrbrb* line; C, *rrRbRb* line and D, *rrrbrb* line.

colour with iodine appeared at the void volume, but accounted for less than 10% of the carbohydrate present.

To examine the structure of the amylopectins in greater detail, debranched products were separated using high-performance anion-exchange chromatography (Fig. 3A–D). Individual DPs were resolved up to about DP 35. The main effect of the mutation at the *r* locus on the chain profile was a more dramatic discrimination of fractions with a DP of 15 and 22.

The debranched product was further examined by size exclusion chromatography (Fig. 4a–d). Peas dominant at the *r* locus (*RRRbRb* and *RRrbrb*) showed similar profiles, with four distinct populations of chains. The average DPs of the populations were 15, 22, 45 and 60, respectively. Similarly, pea starch amylopectin affected by a mutation at the *r* locus (*rrRbRb* and *rrrbrb*) showed characteristic chain profiles which although distinguishable from those dominant at the locus were very similar with small observed differences in the relative amounts of the various fractions

(Table 1). Mutation at the *rb* locus had no obvious effect on amylopectin structure.

The amylose fraction of the starches was examined in more detail. The fractions from the size exclusion chromatography were pooled, and the amylose purified as its 1-butanol complex. The amylose obtained from the complex gave a blue colour with iodine with a λ_{max} of 635–640 nm. The supernatant fractions obtained after 1-butanol complexation from *RRRbRb* and *RRrbrb* starches contained little carbohydrate, <5% w/w of the total present. The supernatant fractions from the *rrRbRb* and *rrrbrb* starches contained larger amounts of carbohydrate (15–20% w/w). When this material was debranched with isoamylase, the consituent chain profile was similar to that of the comparable amylopectins (data not shown).

The present study indicates that mutation at the *rb* locus had a relatively small effect on starch composition and structure. A mutation at the *r* locus resulted in the synthesis of starches with a higher amylose content, and a branched component with a wider range of molecular sizes than for the wild-type. These observations are comparable with those of a previous study on pea starch, which distinguished between a high molecular weight amylopectin and a lower molecular weight, branched, intermediate material (Colonna & Mercier, 1984), and are consistent with the observation that the mutation results in a decreased activity of SBE. Although *in vitro*, the isoforms of SBE have character-

Table 1. Percent mass fractions of the constituent chains of pea amylopectins. These masses were obtained from Fig. 4. Perpendiculars were dropped from the inflection points and troughs of the four peaks. The resulting areas were then integrated (see Fig. 4a).

	Carbohydrate amounts in each fraction (wt%)			
Average DP	DP 15	DP 22	DP 45	DP 60
RRRbRb	41	35	20	4
rrRbRb	39	38	17	6
RRrbrb	41	37	18	4
rrrbrb	40	38	16	6

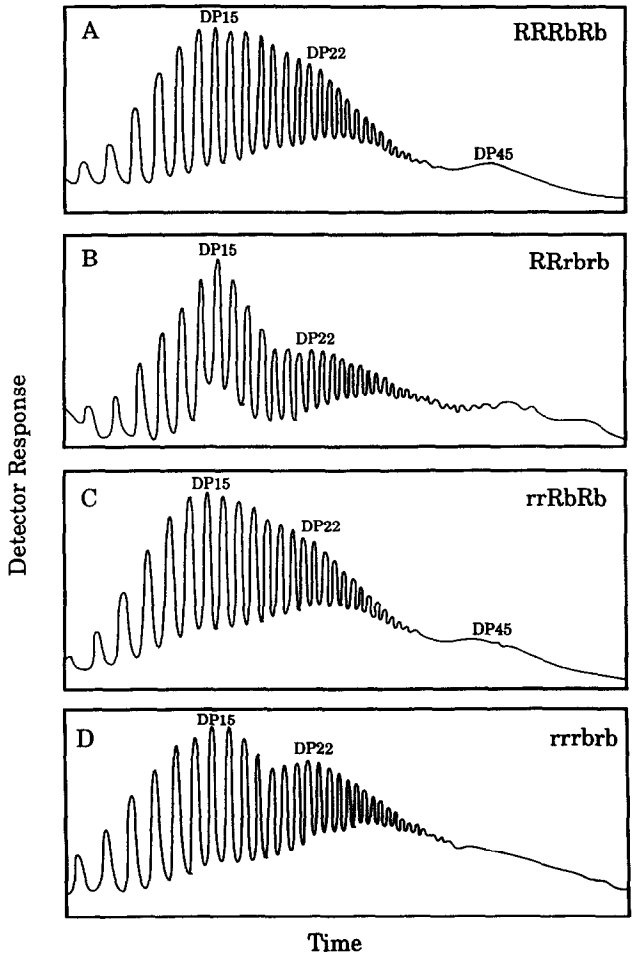

Fig. 3. Separation of the constituent chains of debranched pea amylopectins by HPAEC-PAD A, *RRRbRb* line; B, *RRrbrb* line; C, *rrRbRb* line and D, *rrrbrb* line.

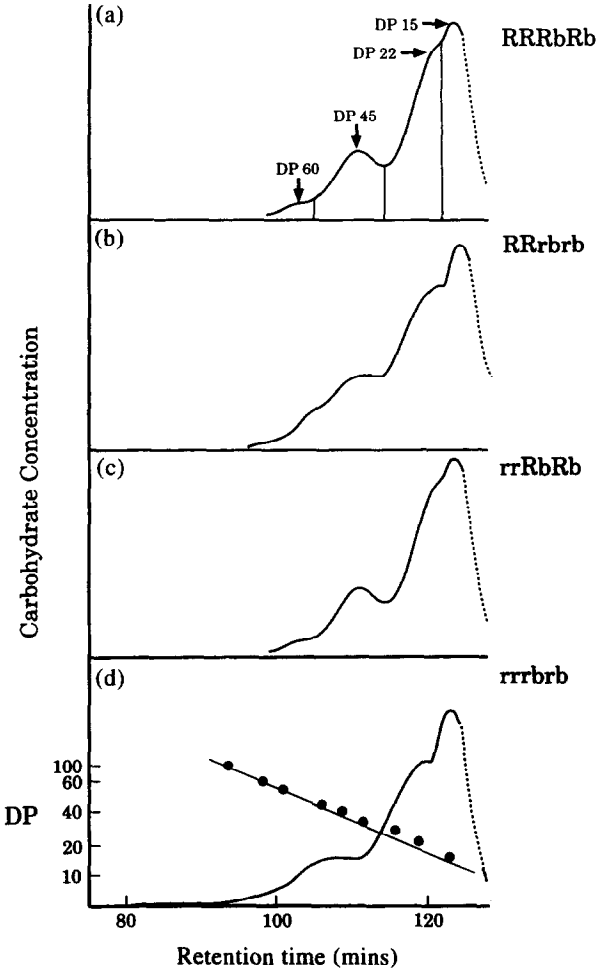

Fig. 4. Separation of chains from debranched amylopectins by size exclusion HPLC a, *RRRbRb* line; b, *RRrbrb* line; c, *rrRbRb* line and d, *rrrbrb* line. Calibration line in d obtained from pullulan standards and linear dextrins.

istic activities (Smith, 1988) *in vivo*, the lack of one isoform in the *r* mutation only has a small effect on the branched structure of amylopectin.

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