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# Characterisation of the (1→4)-α-D-glucan-branching 6-glycosyltransferase by *in vitro* synthesis of branched starch polysaccharides*

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ABSTRACT

Starch branching enzyme (Q-enzyme; EC 2.4.1.18), isolated from young, mature potato tubers (*Solanum tuberosum* L.) and purified by ammonium sulfate precipitations, hydrophobic-interaction chromatography, and size-exclusion chromatography, was completely free of phosphorylase (EC 2.4.1.1) and alpha-amylase (EC 3.2.1.1) activity, had a molecular mass of 64 kDa, was homogeneous in SDS-PAGE, was inhibited by $4 \times 10^{-5}$M oxidised gluthathione, and could be stored at −80° in the presence of SH-reducing agents. The actions of Q-enzyme alone and in combination with potato phosphorylase on amylose, pea starch, potato amylose, potato amylopectin, and waxy maize was investigated. The combination gave high molecular weight polysaccharides, debranching of which yielded patterns of short and long chains similar to those of debranched amylopectin. Treatment of amylose with Q-enzyme resulted in a decrease in the molecular weight averages and broadening of the molecular weight distribution, and debranching of the product yielded a short-chain distribution pattern.

## INTRODUCTION

Branching enzyme [Q-enzyme, (1→4)-α-D-glucan:(1→4)-α-D-glucan 6-glucosyltransferase, EC 2.4.1.18] has been isolated from various sources, including potato tubers[1–5], maize[6–8], teosinte[9], sorghum[10], rice[11], *Pisum sativum*[12], spinach[13,14], cotton leaves[15], mammalian muscle[16], *Escherichia coli*[17,18], and rabbit liver[19], since its activity was first detected in potato juice[20]. The enzyme hydrolyses (1→4) linkages with inter- or intra-chain transfer, including the formation of (1→6) linkages[10]. The wide variety of structures of such glucans depends on the combined action of synthesising and branching enzymes, each of which occurs in multiple forms[4,6,9,12,14,21–23]. The combined actions of branching enzyme severally with phosphorylase[16,17,24], starch synthase[25,26], and glycogen synthase[18,27,28] have been investigated. We now report on the purification of branching (Q) enzyme, and on its action alone and in combination with potato α-glucan phosphorylase [(1→4)-α-D-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1].

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EXPERIMENTAL

*Materials.* — Young mature potatoes ("red eyed" Nr. 2848) were a gift from the Institut für Pflanzenbau (Universität für Bodenkultur).

D-Glucose 1-phosphate, 1,4-dithio-DL-threitol, SDS markers (SDS-70), beta-amylase (sweet potato, EC 3.2.1.2), and isoamylase (*Pseudomonas*, EC 3.2.1.68) were purchased from Sigma. Amylose (Ex-1, d.p. 17) was obtained from Hayashibara Biochemical Laboratories; malto-oligosaccharides from Boehringer Mannheim; pullulan (Shodex standard P-82) from Showa Denko; Fractogels TSK Butyl-650 (M), HW-50 (S), and HW-40 (F) from Merck; and Superose 12, Superdex™ 75 HR, and the gel-filtration calibration kit (17-0442-01) from Pharmacia. Phosphorylase (EC 2.4.1.1) and synthetic amyloses were isolated and prepared as described[29,30].

The following buffers were used: *A*, 50mM Tris/citrate (pH 7.5) with 2.5mM dithiothreitol and 10mM EDTA; *B*, 50mM Tris/HCl (pH 6.6) containing mM dithiothreitol and 5mM EDTA: and *C*, 100mM acetic acid/acetate (pH 4.5).

*Isolation of the enzyme.* — Young, mature potato tubers (2 kg, peeled and stored in $H_2O$ containing 0.1% of $Na_2S_2O_4$) were homogenised and immediately blended with 100 mL of 0.1M Tris/citrate buffer (pH 7.5) containing 10mM EDTA, and 1 g of sodium dithionite as protective agent. The homogenate was filtered through several layers of nylon mesh and centrifuged for 20 min at 30000*g* in a Sorvall RC-5B refrigerated centrifuge. The supernatant solution was treated with ammonium sulfate to 20%, and the protein precipitate was centrifuged and discarded. The enzyme was then precipitated by the addition of ammonium sulfate to 50% saturation, collected by centrifugation, dissolved in 20 mL of buffer *A*, and stored at −80°.

Protein was determined according to Lowry *et al.*[31]. Alpha-amylase activity was determined with the Amylochrome® reagent (Roche); 1 unit (U) corresponds to the formation of 1 μequiv. of reducing groups per min per litre. Carbohydrate was determined by the anthrone–sulfuric acid method[32].

Evidence for the absence of starch-hydrolysing enzymes (alpha-amylase, beta-amylase, R-enzyme, D-enzyme) was obtained by t.l.c. (data not shown) and size-exclusion chromatography (s.e.c.).

The activity of the Q-enzyme was determined by the decrease of absorbance at 620 nm of the iodine–amylose complex, by a modification of the assay described by Borovsky *et al.*[24]. Amylose (1 mg in 3 mL of buffer *A*) was treated with Q-enzyme at 22°. Aliquots (500 μL) were taken at intervals, 0.5 mL of water and 1 mL of iodine reagent (4 mg of KI and 1.25 mg of $I_2$ in 10 mL of $H_2O$) were added, and the absorbance at 620 nm was determined: 1 U is defined as the decrease of 1% of the iodine–amylose complex at 620 nm per min.

The activity of α-glucan phosphorylase was determined from the amount of inorganic phosphate liberated from D-glucose 1-phosphate[33,34].

*Hydrophobic interaction chromatography* (h.i.c.). — The stored potato extract (5 mL) was applied to a column (150 × 25 mm i.d.) of Fractogel TSK Butyl-650 (M) equilibrated with buffer *A* containing 20% of ammonium sulfate. The column was

washed with 600 mL of the equilibrating buffer, and the protein was eluted with a gradient in 2 steps (15 and 10%) of $(NH_4)_2SO_4$ in buffer *A*. The Q enzyme was desorbed with 1800 mL of a linear gradient of 10→0% of $(NH_4)_2SO_4$ in buffer *A* at 90 mL/h (18-mL fractions). Fractions 64–77 contained Q-enzyme activity, and were combined and treated with $(NH_4)_2SO_4$ to 70% saturation. The precipitate was collected, redissolved in 5 mL of buffer *A*, and dialysed overnight against 2 L of buffer *A*.

*Size-exclusion chromatography (s.e.c.).* — The foregoing enzyme (5 mg) was eluted from a column of Fractogel TSK HW-50 (S) (600 × 15 mm i.d.) with 50mM KCl + 0.01% of $NaN_3$ at 0.6 mL/min. The appropriate fractions were combined and concentrated at 20° on a Rotavapor.

The molecular weight distribution of Q-enzyme (500 mg) was determined using the Pharmacia Superose s.e.c. system: columns of Superose 12 (300 × 10 mm i.d.) and Superdex™ 75HR (300 × 10 mm i.d.), with 50mM KCl + 0.01% of $NaN_3$ at 1 mL/min, using the Pharmacia s.e.c. calibration kit (17-0442-01).

The molecular weights and chain-length distributions of branched synthetic glucans were determined using columns of Superose 12 (300 × 10 mm i.d.) + Fractogel HW-40 (F) (450 × 10 mm i.d.) and the above eluent at 0.6 mL/min. Each synthesised α-glucan (5 mg) and digest with isoamylase was desalted by adding 100 mg of mixed-bed resin (Biorad) and then subjected to s.e.c. with 50mM KCl and 0.01% of $NaN_3$. Pullulans and malto-oligosaccharides were used for the calibration.

The chain-length distribution of the isoamylase digests of the synthetic products were determined by reverse-phase h.p.l.c. (Spherisorb ODS-II S3, 125 × 4 mm i.d. column). α-Glucans (600 μg/20 μL) were desorbed with a linear gradient from 0→5% methanol–$H_2O$ within 30 min at 0.8 mL/min. Carbohydrates were detected with the evaporation light-scattering detector Sedex 45 from S.e.d.e.r.e. (France) with $N_2$ as the nebuliser gas at 43°.

*S.e.c.–l.a.l.l.s. technique.* — Double detection of refractive index (d.r.i.) and low-angle laser-light scattering (l.a.l.l.s.) of samples of polysaccharide separated by s.e.c. allows determination of molecular weight distributions without the need for calibration standards[35–37]. TSK-system: TSK PW5000 (600 × 0.8 mm i.d.) and TSK PW4000 (600 × 0.8 mm i.d.) columns (Toyo Soda, Japan), Refracto Monitor III (d.r.i.), KMX-6 (l.a.l.l.s.) and p.c.l.a.l.l.s. software package for data acquisition and data processing from LDC-Analytical (U.S.A.); the eluent was 50mM NaCl at 0.8 mL/min.

*Electrophoresis.* — Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed[38,39] on 12.5% polyacrylamide. Proteins in the polyacrylamide gel were detected by silver stain[40].

*Debranching with isoamylase.* — Digests of the branched glucans (2 mg/mL in buffer *C*) were incubated with isoamylase (15 U) at 37° for 15 h.

De novo *synthesis.* — *De novo* synthesis was carried out in digests containing maltoheptaose (5 mg), D-glucose 1-phosphate (1.2 g), potato phosphorylase (10 U), and branching enzyme (0.5 U) in a total volume of 30 mL of buffer *B* at 37° for 24 h. The chain-distribution patterns of the products were determined by analytical s.e.c. and h.p.l.c.

*Beta-amylolysis.* — For determination of the beta-amylolysis limit of the branched glucans, the branching enzyme was inactivated (100°, 1 min), and 1 mL of buffer *C* and 12 U of beta-amylase were added. After incubation at 37° for 24 h, the mixture was assayed for reducing sugars[41].

TABLE I

Purification of Q-enzyme[a]

| *Step* | *Vol. (mL)* | *Protein (mg)* | *Activity[b] (U)* | *Specific activity (U/mg)* | *Yield (%)* |
|---|---|---|---|---|---|
| Potato juice | 320 | 1200 | 41 | 0.034 | 100 |
| 50% $(NH_4)_2SO_4$ (supernatant) | 260 | 280 | 2 | 0.007 | 5 |
| H.i.c. (TSK Butyl-650) | 16 | 331 | 33 | 0.099 | 80 |
| G.p.c. [HW-50 (S)] | 8 | 4.7 | 14 | 2.980 | 34 |
| G.p.c. (Superose 12 + Superdex™ 75 HR) | 2.8 | 1.3 | 6 | 4.620 | 15 |

[a] The enzyme was purified 132-fold and was homogeneous in electrophoresis. [b] Measured by the decrease of absorption of the complex with iodine at 620 nm.

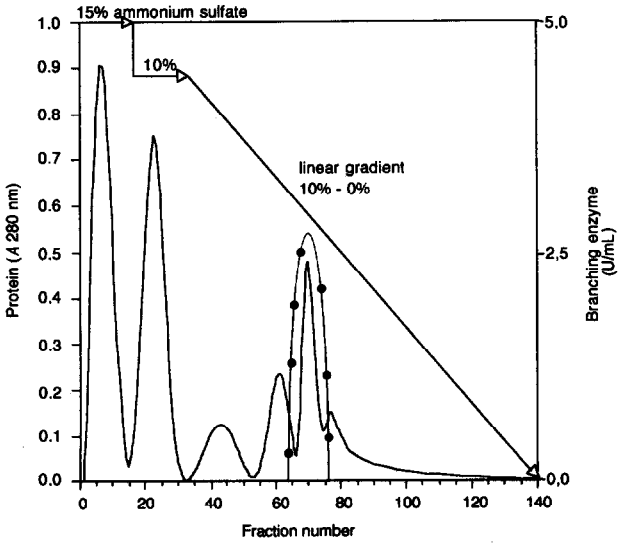


Fig. 1. Elution profile of Q-enzyme activity (●) from Fractogel TSK Butyl-650 (see Experimental). Fractions 64–77 were combined and precipitated with 70% ammonium sulfate for further chromatography.

RESULTS AND DISCUSSION

*Purification of the branching enzyme.* — Starch branching enzyme (Q-enzyme) was prepared from young, mature potato tubers (see Experimental) and the various steps are shown in Table I. The crude extract was concentrated by fractional precipitation with ammonium sulfate (20% / 50%) and by h.i.c. on Fractogel TSK Butyl-650 (Fig. 1). An advantage of this technique is the shorter time of preparation because no

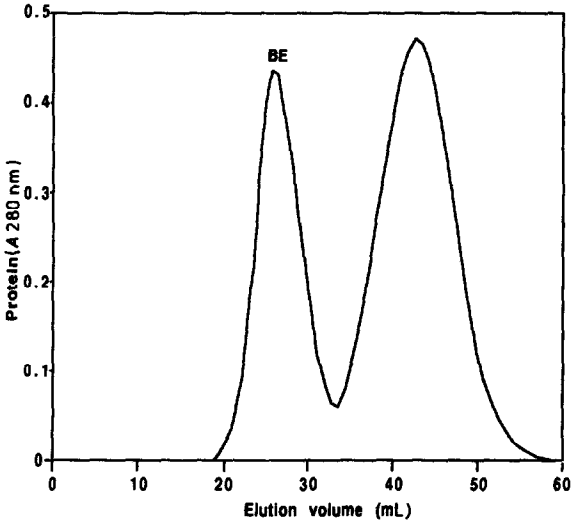


Fig. 2. Gel-permeation chromatographic profile of Q-enzyme (BE) in fractions 64–77 in Fig. 1 on Fractogel HW-50 (S). The enzyme was eluted with 50mM KCl + 0.01% of $NaN_3$.

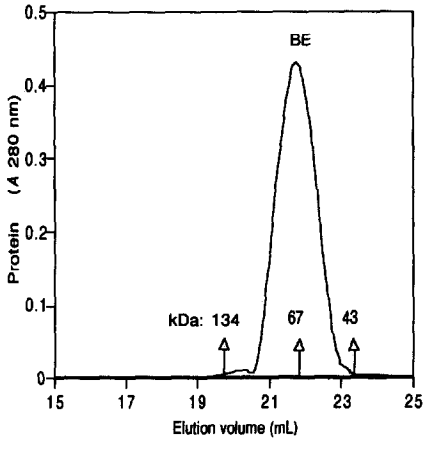


Fig. 3. Gel filtration of the combined fractions from Fig. 2 containing Q-enzyme (BE) from Fractogel HW 50 (S) on Superose 12 and Superdex™ 75 HR. Molecular masses (kDa): BE, 64; bovine serum albumin dimer, 134; and monomer, 67; and ovalbumin, 43.

dialysis is required. The fractions containing Q-enzyme were combined, and the product was purified further on Fractogel HW-50 (S) (Fig. 2) and then on a Superose 12 + Superdex™ 75 HR column system in which Q-enzyme was eluted as a sharp and single peak (Fig. 3) with a molecular mass of 64 kDa. The purified Q-enzyme was free of amylolytic and phosphorylase activities (see Experimental).

A silver stain of Q-enzyme separated by SDS-PAGE is shown in Fig. 4. The electropherogram contains a single band with a relative mobility of 0.30 which corresponds to a molecular mass of 62 kDa [*cf.* 85 (ref. 2), 79 (ref. 24), 103 (ref. 42), and 70 +

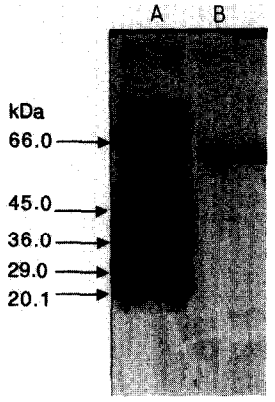


Fig. 4. Silver-stain SDS-electrophoresis of Q-enzyme: lane B contains BE (15 $\mu$g) after gel filtration on Superose 12 and Superdex™ 75 HR; lane A contains 15 $\mu$g each of bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), and trypsin inhibitor (20.1 kDa).

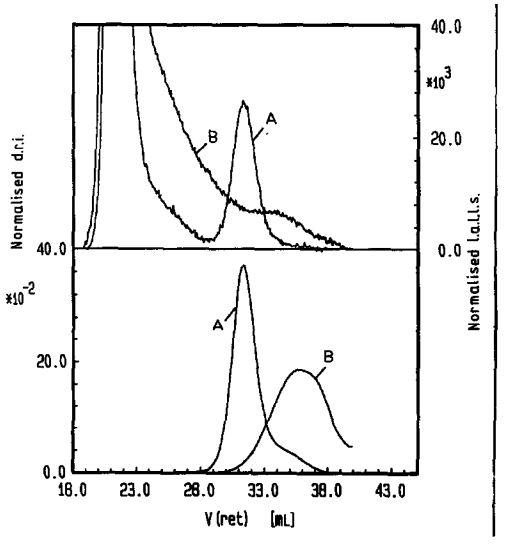


Fig. 5. Normalised d.r.i.- and l.a.l.l.s.-chromatograms (see Experimental): A, synthetic amylose; B, product due to the action of Q-enzyme on A.

20 kDa (ref. 3)]. During the preparation of Q-enzyme with DEAE-Sepharose gels, phosphorylase was always present and, thus, the molecular weight could not be determined. No smaller proteins could be detected by SDS-PAGE, indicating that the enzyme does not consist of subunits as has been suggested[3].

*Action of Q-enzyme.* — Molecular weight distributions for amylose and for the products obtained from amylose + Q-enzyme were determined by means of the s.e.c.–l.a.l.l.s. technique. Fig. 5 illustrates the chromatograms obtained with the nor-

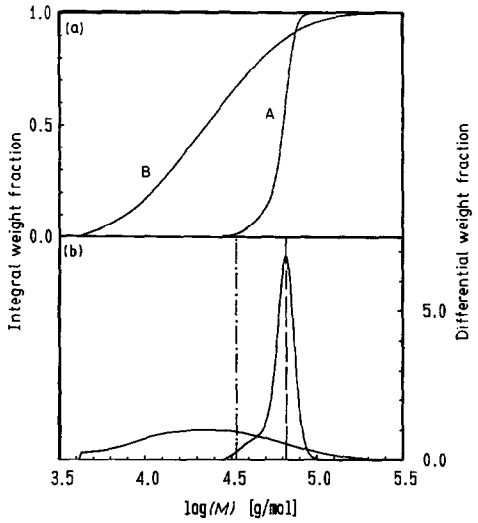


Fig. 6. Molecular weight distributions (a) integral and (b) differential weight fractions: A, synthetic amylose; B, product due to the action of Q-enzyme on A; ----, $M_w$ 67 600 ± 3% (A); -·-·-, $M_n$ 33500 ± 3% (B).

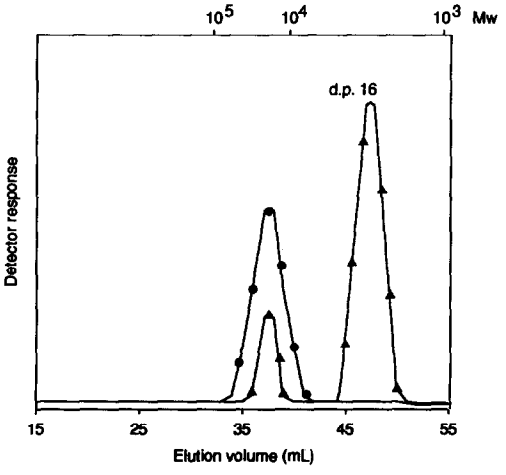


Fig. 7. Elution profiles on Superose 12 and Fractogel HW-40 of synthetic amylose B-360 (●), and the products (▲) obtained on treatment with Q-enzyme and then hydrolysis with isoamylase.

malised l.a.l.l.s.-detector and d.r.i.-detector for amylose (**A**) and the product (**B**), and the molecular weight distributions in terms of integral and differential are shown in Fig. 6. The amylose (**A**) is higher in molecular weight averages ($M_w$ 67 600 ± 3%, $M_n$ 60 500 ± 5%) and less polydisperse ($M_w/M_n$ 1.04 ± 5%) than the reaction product (**B**) ($M_w$ 33 500 ± 3%, $M_n$ 17 100 ± 5%, $M_w/M_n$ 1.96 ± 5%). The product (**B**) was debranched with isoamylase, and the fragments were analysed by s.e.c. and h.p.l.c. The results in Fig. 7 show a distribution pattern with a preferred chain length of d.p. 16. Incubation of phosphorylase and branching enzyme with malto-oligosaccharides as primer and D-

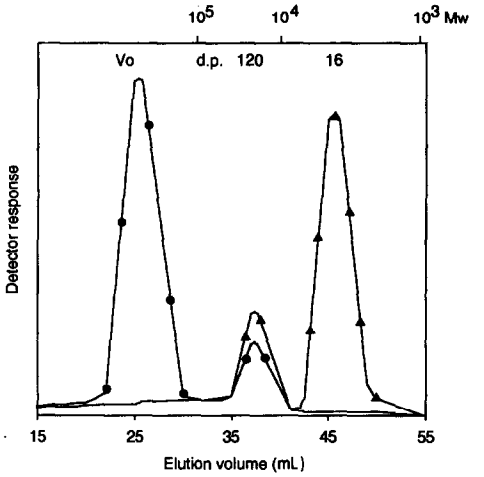


Fig. 8. Elution profiles on Superose 12 and Fractogel HW-40 of the branched product synthesised by simultaneous action of potato phosphorylase and Q-enzyme (see Experimental), before (●) and after (▲) treatment with isoamylase.

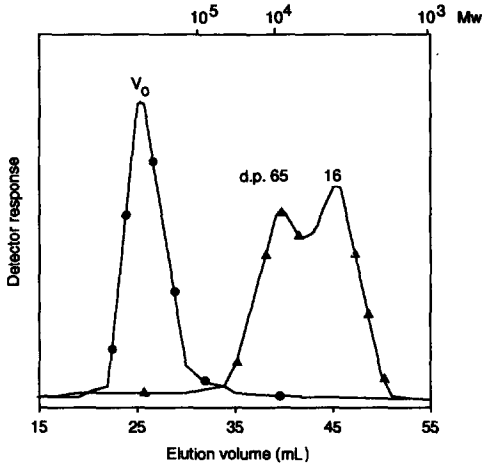


Fig. 9. Elution profiles on Superose 12 and Fractogel HW-40 of potato amylopectin before (●) and after (▲) debranching with isoamylase (see Experimental).

glucose 1-phosphate yielded an amylopectin-type glucan of high molecular weight which was eluted in the void volume (Fig. 8). The low molecular weight material with d.p. ~ 120 reflects the formation of linear less- or non-branched chains due to the side action of Q-enzyme at higher concentrations of phosphorylase.

After debranching with isoamylase, the chain-length distribution of the branched chains was determined by s.e.c. and h.p.l.c. (Fig. 9). The maximum occurred at d.p. 16 and there was an increase of the material with d.p. 120. This observation indicates the parallel formation of branched products of high molecular weight, due to the transfer of short chains to the high molecular weight glucan, and of linear products of low molecular weight by transfer of short chains to short chains.

Native amylopectin (Fig. 10) from potato starch has a bimodal chain-length distribution with maxima at d.p. 16 and d.p. 65 after repetitive debranching with isoamylase. H.p.l.c. of the low molecular weight fraction (d.p. 16) confirmed the chain-length distribution of native amylopectin and the *in vitro* glucans (Fig. 9).

The activities of Q-enzyme against several substrates are shown in Table II and depended on the degree of branching. Only a few additional branches were introduced into amylopectin. Under the conditions of beta-amylolysis used (37°, 24 h, 12 U of beta-amylase), the branched products were found between 50–60% beta-amylolysis, and the $\lambda_{max}$ of the iodine stain was shifted to shorter wavelengths. Amylopectin is a substrate for Q-enzyme and the number of (1→6) linkages is increased, as shown by the increased resistance towards beta-amylolysis.

TABLE II

The action of Q-enzyme on linear and branched α-D-glucans

| *Substrate* | *Beta-amylolysis limit (%)* | | *$\lambda_{max}$ of iodine stain (nm)* | |
|---|---|---|---|---|
| | *Before branching* | *After branching* | *Before branching* | *After branching* |
| *De novo* product (Q + phosphorylase)[a] | – | 51 | – | 575 |
| Amylose (potato, "Sieglinde") | 68 | 50 | 630 | 575 |
| Pea amylose (high amylose starch) | 73 | 55 | 615 | 548 |
| Amylose, d.p. 613 (TA-7, synthetic) | 98 | 56 | 630 | 560 |
| Amylose, d.p. 360 (B-360, synthetic) | 96 | 62 | 612 | 550 |
| Amylopectin (potato, "Sieglinde") | 60 | 55 | 577 | 540 |
| Amylopectin (potato, "Conny") | 57 | 50 | 572 | 525 |
| Waxy maize starch | n.d.[b] | n.d.[b] | 580 | 538 |

[a] Maltoheptaose + α-D-glucose 1-phosphate was the substrate (see Experimental). [b] Not determined.

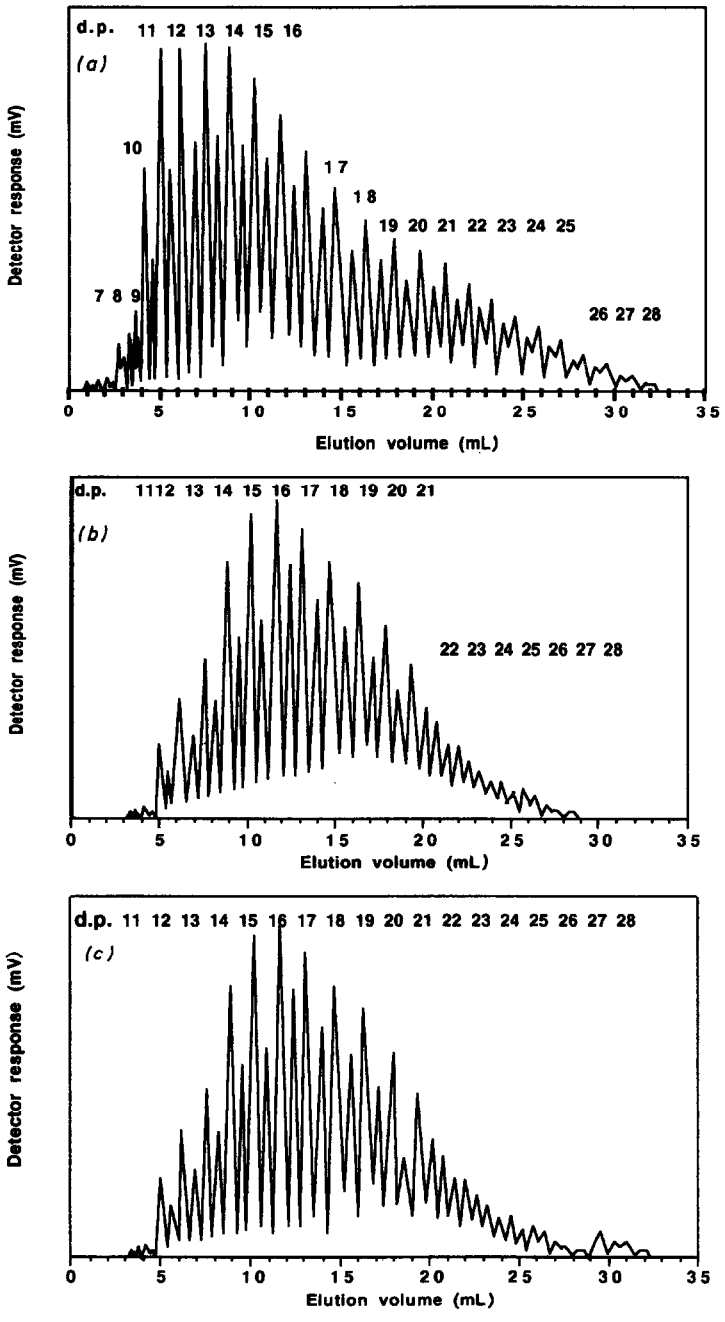


Fig. 10. Reverse-phase h.p.l.c. of *a* (Hayashibara amylose), *b* the debranched product formed by *de novo* synthesis with phosphorylase and branching enzyme, and *c* debranched potato amylopectin (see Experimental).

Thus, Q-enzyme preferentially transfers chains of d.p. 13–20 with a maximum for d.p. 16. Further studies are required to elucidate the relationship between Q-enzymes of different plant sources and their effects on native and synthetic substrates.

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