Carbohydrate Research, 101 (1982) 75–92
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

PURIFICATION AND PROPERTIES OF A β-D-MANNOSIDE MANNOHYDROLASE FROM GUAR

BARRY V. MCCLEARY
Biological and Chemical Research Institute, N.S.W. Department of Agriculture, Rydalmere 2116, N.S.W. (Australia)
(Received May 13th, 1981; accepted for publication, July 14th, 1981)

ABSTRACT

A β-D-mannoside mannohydrolase enzyme has been purified to homogeneity from germinated guar-seeds. Difficulties associated with the extraction and purification appeared to be due to an interaction of the enzyme with other protein material. The purified enzyme hydrolysed various natural and synthetic substrates, including β-D-manno-oligosaccharides and reduced β-D-manno-oligosaccharides of degree of polymerisation 2 to 6, as well as *p*-nitrophenyl, naphthyl, and methylumbelliferyl β-D-mannopyranosides. The preferred, natural substrate was β-D-mannopentaose, which was hydrolysed at twice the rate of β-D-mannotetraose and five times the rate of β-D-mannotriose. This result, together with the observation that α-D-mannose is released on hydrolysis, indicates that the enzyme is an exo-β-D-mannanase.

INTRODUCTION

The biodegradative hydrolysis of galactomannan during legume-seed germination is thought to require the presence of at least three enzymes: α-D-galactosidase (EC 3.2.1.22), β-D-mannanase (EC 3.2.1.78), and β-D-mannoside mannohydrolase (EC 3.2.1.25; *i.e.*, β-D-mannosidase or exo-β-D-mannanase)[1]. The essential role of α-D-galactosidase and β-D-mannanase has been confirmed[2], but the importance of β-D-mannoside mannohydrolase has yet to be established. The combined action of α-D-galactosidase and β-D-mannanase converts most galactomannans into D-galactose, β-D-mannobiose, β-D-mannotriose, and traces of D-mannose[3]. The conversion of the β-D-manno-oligosaccharides into D-mannose requires the presence of a further enzyme.

In the conversion of galactomannans into D-galactose and D-mannose by microbial-enzyme preparations, the third enzyme was shown to be β-D-mannosidase[4]. Initial studies of legume-seed extracts indicated the absence of β-D-mannosidase[5]. However, this enzyme was subsequently shown to be present in both aleurone and cotyledonary tissue of seeds of fenugreek[1], guar[6], carob[6,7], lucerne[6], and honey locust[6].

Attempts to purify legume-seed β-D-mannoside mannohydrolase have had

limited success. The enzymes from lucerne seed were only partially soluble and very unstable, and recoveries from ion-exchange and gel-filtration media were low[6]. An enzyme tentatively termed exo-β-D-mannanase was partially purified[8] from extracts of germinated guar-seed and shown to release D-mannose from the non-reducing end of reduced ivory-nut β-D-mannan, but detailed studies of its properties and substrate requirements were not performed.

Clearly, to determine the importance of β-D-mannoside mannohydrolases in the mobilisation of galactomannan in germinating legume-seeds, it is essential to purify and characterise these enzymes, and we now report on this aspect.

MATERIALS AND METHODS

Plant material. — Seeds of guar (*Cyamopsis tetragonolobus* (L) Taub. c.v. CP 177) were obtained from Mr. I. Parkin, Fielder–Gillespie Ltd., Albion, Queensland. The seeds (1980 harvest) were stored over a desiccant at 4°. Carob seeds were obtained from Mr. R. Thompson, Cowra, N.S.W. Other seeds were obtained from local suppliers. Seeds were germinated in the dark. After soaking in 0.5% aqueous NaOCl for 10 min and washing, the seeds were soaked in distilled water for 8 h (with frequent changes), and then germinated between layers of filter paper, in trays at 25°.

T.l.c. — This was performed on Merck DC-Alufolien Kieselgel 60 (0.2 mm) plates, which were developed with 1-propanol–nitromethane–water (5:2:3). Spots were detected by spraying with 5% H_2SO_4 in ethanol and heating to 110°.

Isoelectric focusing. — This was performed with an LKB Multiphor, and slab gels prepared using ampholines made as previously reported[9]. Gels were prepared by mixing freshly prepared solutions of: acrylamide (3.0 g) plus bis-acrylamide (0.08 g) in ice-cold water (40 mL); ampholine solution (4 mL, 40% w/v); and ammonium persulphate (80 mg) plus *N,N,N′,N′*-tetramethylethylenediamine (0.1 mL) in ice-cold water (16.9 mL). Gels were poured immediately, and they polymerised within 15 min. The pI of β-D-mannoside mannohydrolase was determined by reference to standard proteins (Pharmacia Fine Chemicals, Isoelectric Focusing Calibration Kit, pI 3.5–9.3), and also by slicing the gel into 5-mm sections, which were ground and extracted in water (2 mL) and assayed for pH and enzyme activity.

p-*Nitrophenyl β-D-mannopyranoside.* — The procedure of Garegg *et al.*[10] was employed. After recrystallisation, the preparation was completely devoid of *p*-nitrophenyl α-D-mannopyranoside. Incubation of the substrate (0.1 mL, 5mM) in 0.1M sodium acetate buffer (pH 4.5) with jack-bean α-D-mannosidase (16.7 nkat, Sigma M7257) gave no detectable hydrolysis within 1 h at 40°.

o-*Nitrophenyl, naphthyl, and methylumbelliferyl β-D-mannopyranosides.* — These glycosides were prepared by the procedure employed for *p*-nitrophenyl β-D-mannopyranoside[10], but replacing *p*-nitrophenol with equimolar quantities of *o*-nitrophenol, 1-naphthol, or methylumbelliferone. *o*-Nitrophenyl β-D-mannopyranoside (25%) had m.p. 161–163° (Found: C, 46.44; H, 5.30; N, 4.47. $C_{12}H_{15}NO_8$ calc.: C, 47.84; H, 5.02; N, 4.65%).

Naphthyl β-D-mannopyranoside crystallised in the aqueous phase while this was being extracted with chloroform[10]. The aqueous phase was concentrated to dryness and the residue was twice recrystallised from 50% aqueous acetone at 80° (the acetone concentration was increased to 90% and the solution stored at 4° for 4 days); yield, 30%; m.p. 237–239° (Found: C, 62.61; H, 5.98. $C_{16}H_{19}O_6$ calc.: C, 62.76; H, 5.92%).

Crude methylumbelliferyl β-D-mannopyranoside crystallised on concentration of the aqueous phase (after chloroform extraction)[10]. Recrystallisation from 50% aqueous acetone (0.5 g/30 mL) at 80° (on cooling, the acetone concentration was increased to 80% and the solution stored at −20° for several weeks); yield, 26%; m.p. 240–242° (Found: C, 56.25; H, 5.43. $C_{16}H_{19}O_8$ calc.: C, 56.80; H, 5.67%).

Each of these substrates, after recrystallisation, was devoid of the α anomer as demonstrated by their resistance to hydrolysis by α-D-mannosidase (jack bean, Sigma M7275).

Preparation of N-ε-*aminocaproyl-β-*D-*mannopyranosylamine–Sepharose* 4 B. — β-D-Mannopyranosylamine[11] and *N*-benzyloxycarbonyl-ε-aminocaproic acid[13] were prepared by literature procedures. *N*-(*N*-Benzyloxycarbonyl-ε-aminocaproyl)-β-D-mannopyranosylamine (**1**) was prepared by a procedure similar to that used[12] for the preparation of *N*-(*N*-benzyloxycarbonyl-ε-aminocaproyl)-α-D-galactopyranosylamine. After 20 h, the reaction solution was concentrated below 40°, and the residue was recrystallised twice from ethanol, to give **1** (85%). *N*-ε-Aminocaproyl-β-D-mannopyranosylamine (**2**) was prepared by hydrogenation[13] of **1** over Pd/C in 80% aqueous methanol for 8 h; yield, 85%; m.p. 185–187° (Found: C, 48.62; H, 8.20; N, 9.16. $C_{12}H_{24}N_2O_6$ calc.: C, 49.30; H, 8.28; N, 9.58%). *N*-ε-Aminocaproyl-β-D-mannopyranosylamine–Sepharose 4B was prepared by treating **2** with cyanogen bromide-activated[14] Sepharose 4B.

Assay of α-D-*galactosidase and* β-D-*mannoside mannohydrolase.* — Enzyme preparations (0.1 mL) were incubated, as appropriate, with either *p*-nitrophenyl α-D-galactopyranoside (0.1 mL, 10mM) in 0.1M sodium acetate buffer (pH 5) or with *p*-nitrophenyl β-D-mannopyranoside (0.1 mL, 5mM) in 0.1M sodium acetate buffer (pH 5.5), for 1–5 min. The reaction was terminated and colour developed by the addition of aqueous sodium carbonate (3 mL, 2%). β-D-Mannoside mannohydrolase was also assayed by incubating enzyme preparation (0.05 mL) with mannopentaitol (0.1 mL, 10mM) in 0.2M sodium acetate buffer (pH 5.5) for 1–5 min. The reaction was terminated by the addition of *p*-hydroxybenzohydrazide solution (5 mL)[15], and the colour was developed by incubating the tubes for 6 min at 100°.

Assay of β-D-*mannanase.* — β-D-Mannanase was routinely assayed using carob galactomannan dyed with Remazol Brilliant Blue R (RBB-Carob galactomannan) as substrate[16].

Purification of β-D-*mannoside mannohydrolase.* — Guar seed (500 g) was germinated at 25° for 2.5 days (the bulk of the reserve galactomannan had then been mobilised), homogenised in water at 22° in a Waring Blendor, and incubated at 30° for 1 h. The slurry was rehomogenised and squeezed through fine nylon-mesh. The

filtrate was centrifuged (3,500*g*, 30 min) and the supernatant stored. The pellet was resuspended in a solution of 0.2M NaCl in 50mM Tris buffer (pH 8) and combined with the filter-cake obtained on squeezing the original slurry through the nylon mesh. This material was homogenised and filtered. The filtrate, which contained most of the β-D-mannoside mannohydrolase activity, was centrifuged (3,500*g*, 20 min) and the supernatant (2 L) dialysed against three changes of 20mM Tris buffer (10 L, pH 8) during 48 h. After dialysis, wet-cake DEAE-cellulose (~500 g), pre-equilibrated with the same buffer, was added with gentle stirring. The slurry was poured onto a sintered-glass funnel (in 2 separate lots) and washed with 20mM Tris buffer (4 L, pH 8). β-D-Mannoside mannohydrolase was eluted with 0.2M NaCl plus 20mM Tris buffer (pH 8). The active fraction was dialysed (20 h) against 20mM Tris buffer (10 L, pH 8) and then applied to a column (3.7 × 17 cm) of DEAE-cellulose pre-equilibrated with the same buffer. After washing with 20mM Tris buffer (500 mL, pH 8), protein was eluted with a linear NaCl gradient (0→0.4M) in the same buffer. The active fractions were combined, concentrated by $(NH_4)_2SO_4$ precipitation (80% saturation), dialysed against 0.2M NaCl plus 20mM Tris buffer (pH 8), and applied to a column (2.5 × 88 cm) of Ultrogel AcA 44 equilibrated with the same solution. The eluted enzyme was concentrated by dialysis against poly(ethylene glycol) 4000 and then dialysed against 10mM Tris buffer (pH 8). Immediately before application to a column (1.6 × 12 cm, pre-equilibrated with 25mM diethylamine HCl, pH 11) of Polybuffer Exchanger PBE 118[17], the pH was adjusted to 11 by addition of diethylamine HCl buffer to a final concentration of 25mM. The enzyme recovered from Ultrogel AcA 44 was applied to Polybuffer Exchanger PBE 118 resin in three separate lots. Proteins were eluted with Pharmalyte pH 8–10.5 HCl[8] (diluted 1:45 and adjusted to pH 8). After elution of β-D-mannoside mannohydrolase, the column was washed with M NaCl, to remove remaining proteins, including α-D-galactosidase and β-D-mannanase.

Alternatively, β-D-mannoside mannohydrolase, recovered from Ultrogel AcA 44 and dialysed, was further purified by chromatography on CM-cellulose (pH 6.3). The dialysed enzyme was adjusted to pH 6.3 by addition of acetate buffer to 20mM, and then applied to a column (1.6 × 12 cm) of pre-equilibrated CM-cellulose. The column was washed with 20mM acetate buffer (pH 6.3) to remove α-D-galactosidase and β-D-mannanase, and the β-D-mannoside mannohydrolase was eluted with a linear gradient of sodium chloride (0→0.4M). Recovered enzyme was dialysed against 5mM Tris buffer (pH 8) and stored frozen in polypropylene containers.

Preparation of β-D-manno-oligosaccharides. — *Livistona australis* mannan (15 g), purified as previously described[16], was slowly added with vigorous stirring to conc. HCl (60 mL). The solution was stirred at room temperature (23 ±1°) for 1 min, and then ice-cold, fuming HCl (60 mL) was added[18,19]. The solution was stirred at room temperature for a further 1 h, poured into ice-cold water (1 L), neutralised with $NaHCO_3$, and concentrated to dryness at 40°. The residue was redissolved in the minimum volume of water (200 mL) at 80°, ethanol (200 mL) was added, and the mixture was stored at 4° for 2 days. Insoluble material was removed by

centrifugation, the supernatant solution was concentrated to dryness, and the residue was dissolved in water (100 mL). Aliquots (4.0 mL) of this solution were applied[20] to a column (3.5 × 87 cm) of Bio-Gel P-2 (<400 mesh) equilibrated at 60°, and oligosaccharides were eluted with water. Oligosaccharide concentration was determined by the phenol–sulphuric acid method[21], and the d.p. of the eluted fractions was determined by t.l.c. Reduced manno-oligosaccharides were prepared by treatment[22] of oligomers with $NaBH_4$.

Hydrolysis of β-D-manno-oligosaccharides and reduced β-D-manno-oligosaccharides by β-D-mannoside mannohydrolase. — A solution of each oligosaccharide (0.6 mL, 10 mg/mL) in acetate buffer (5mM, pH 5.5) was incubated with guar β-D-mannoside mannohydrolase (50 μL, 7.3 nkat on *p*-nitrophenyl β-D-mannopyranoside at 40°). Samples (15 μL) were subjected to t.l.c. after 0–120 min. Separate aliquots (50 μL) were heated to denature β-D-mannoside mannohydrolase and diluted by the addition of water (1.5 mL), and aliquots (0.1–0.2 mL) removed for measurement of reducing activity[15] and total carbohydrate[21]. An allowance was made for mannitol, which is not detected by the phenol–sulphuric acid method.

For the determination of K_m and V_{max}, reduced manno-oligosaccharide (0.2 mL, 1–30mM) in acetate buffer (pH 5.5, 0.1M) was incubated at 40° with β-D-mannoside mannohydrolase (0.05 mL). The reaction was terminated by the addition of *p*-hydroxybenzohydrazide[15] (5 mL) and the colour developed by incubating the tubes for 6 min at 100°.

K_i values were determined by using *p*-nitrophenyl β-D-mannopyranoside as substrate in the presence of 10 or 100mM D-mannose, β-D-mannotri-itol, or *N*-ε-aminocaproyl-β-D-mannopyranosylamine.

Properties of guar β-D-mannoside mannohydrolase. — The mol. wt. of β-D-mannoside mannohydrolase was determined by SDS-gel slab electrophoresis[23], using a 10% cross-linked gel and the imidazole–phosphate buffer system. Standard proteins were obtained from Pharmacia Fine Chemical AB (Electrophoresis Calibration Kit; mol. wt. 14,400–94,000). pH Optima were determined for *p*-nitrophenyl β-D-mannopyranoside with 0.1M acetate–phosphate buffer (pH 3.5–8.0). The pH-stability was determined by incubating enzyme (0.1 mL) in acetate–phosphate buffer (0.1M, pH 3.5–8.0) or diethylamine HCl buffer (0.1M, pH 11) for 30 min at 40°. Each sample was adjusted to pH 5.5 by addition of acetate buffer (1.0 mL, 0.2M), and aliquots (0.1 mL) were removed for assay. The effect of time of incubation on stability at pH 4–5 (0.1M acetate buffer) was determined by a similar procedure. The temperature stability was measured by incubating aliquots (0.1 mL) of enzyme in acetate buffer (0.1M, pH 5.5) at 20–55° for 15 min. Residual activity on *p*-nitrophenyl β-D-mannopyranoside was then assayed at 40°. The temperature optimum was determined by incubating enzyme preparation (0.1 mL) with *p*-nitrophenyl β-D-mannopyranoside substrate (0.1 mL), pre-equilibrated to the assay temperature, for 2 min. Protein levels were determined by the Folin–Lowry method[24].

Configuration of the products released during action[25] of β-D-mannoside mannohydrolase. — To a solution of β-D-mannohexaitol (1 mL, 10 mg/mL) was added

50 μL of β-D-mannoside mannohydrolase (6 nkat), and the solution was placed in a polarimeter tube at 27°. Changes in optical rotation (365 nm) were measured over 10 min.

Staining for β-D-mannoside mannohydrolase in polyacrylamide gels. — A technique similar to that used for staining α-D-galactosidase in gels was employed[9] with naphthyl β-D-mannopyranoside as the substrate.

RESULTS AND DISCUSSION

Extraction of β-D-mannoside mannohydrolase. — The effectiveness of three solutions in the extraction of β-D-mannoside mannohydrolase from cotyledons or endospermic tissue of guar seeds, or from whole seeds, is shown in Table I. Extraction of the enzyme from cotyledonary tissue required solutions with salt concentrations in excess of 0.2M. However, extraction from endospermic tissue could be effected with a range of solutions. With acetate buffer, more enzyme was extracted from the isolated endosperms than from the whole seeds (endosperms plus cotyledons). It appeared that solutions from cotyledons contained material that interacted with β-D-mannoside mannohydrolase, rendering it insoluble under these extraction conditions.

Extraction of β-D-mannoside mannohydrolase from seeds of other legume species, including lucerne[6], also required solutions of high pH and salt concentration. A comparison of the effectiveness of two solutions in the extraction of α-D-galactosidase, β-D-mannanase, and β-D-mannoside mannohydrolase from seeds of a range of legumes is shown in Table II. α-D-Galactosidase and β-D-mannanase were readily extracted with either acetate buffer or with Tris buffer plus sodium chloride. However,

TABLE I

EXTRACTION OF β-D-MANNOSIDE MANNOHYDROLASE FROM COTYLEDONS, ENDOSPERM TISSUE, AND WHOLE GUAR-SEEDS[a]

Extraction solution	*β-D-Mannoside mannohydrolase (nkat/30 seeds[b])*		
	Cotyledons	*Endosperms[c]*	*Whole seed[c]*
Water	13	87	60
100mM Acetate buffer (pH 5.0)	7	67	29
200mM Sodium chloride in 50mM Tris buffer (pH 8)	49	121	167

[a]Germinated for 1.5 days at 25°. [b]Original dry-weight of seeds equals 1 g/30 seeds. [c]Endosperms and whole seeds were blended with an Ultraturrax apparatus, and incubated with Driselase β-D-mannanase for 4 h at 30° (to solubilise galactomannan), and then re-blended and adjusted to volume before assaying.

TABLE II

EFFECT OF BUFFER COMPOSITION ON THE EXTRACTION OF α-D-GALACTOSIDASE, β-D-MANNANASE, AND β-D-MANNOSIDE MANNOHYDROLASE FROM GERMINATED LEGUME-SEEDS[a]

Seed material	*Extraction buffer*[b]	*α-D-Galactosidase (nkat/g)*	*β-D-Mannanase (nkat/g)*	*β-D-Mannoside mannohydrolase (nkat/g)*
Trifolium repens	A	80	5	1.8
(New Zealand white clover)	B	60	5	3.3
Trifolium repens	A	77	10	0.9
(Ladino white clover)	B	87	12	2.4
Trifolium pratense	A	220	2	1.5
(New Zealand red clover)	B	229	2	8.0
Medicago sativa	A	217	12	1.2
	B	182	12	15.1
Leucaena leucocephala	A	650	75	26.1
	B	500	80	50.4
Sesbania cannabina	A	247	70	0.1
	B	217	74	0.2

[a]Germinated for 3 days at 25°. [b]A, McIlvane citrate–phosphate buffer (100mM, pH 5.5); B, 200mM sodium chloride in 50mM Tris buffer (pH 8).

TABLE III

PURIFICATION OF β-D-MANNOSIDE MANNOHYDROLASE FROM GERMINATED GUAR-SEEDS

Step	*Protein (mg)*	*Activity (nkat)*	*Specific activity (nkat/mg)*	*Recovery (%)*	*Purification (-fold)*
Crude extract	15,400	38,000	2.5	100	1
Dialysis (pH 8)	12,350	33,100	2.7	87.1	1.1
Centrifugation (3,500*g*)	6,800	20,900	3.1	55	1.2
DEAE-cellulose (bulk)	3,180	13,900	4.4	37	1.8
DEAE-cellulose (column)	327	9,600	29.4	25	11.8
Ultrogel AcA 44	39	4,700	120.5	12	48.2
Polybuffer Exchanger PBE 118	10.8	2,820	261.1	7.4	104.4

without exception, β-D-mannoside mannohydrolase was much more effectively extracted with Tris buffer plus sodium chloride. This limited extractibility of β-D-mannoside mannohydrolase with solutions of low concentration of salt and low pH may explain some of the difficulties experienced by other investigators[5] in detecting this enzyme in legume-seed extracts.

Purification of β-D-mannoside mannohydrolase. — The conditions of extraction of guar β-D-mannoside mannohydrolase had a large effect on the relative ease of purification. Effective extraction was achieved only at salt concentrations in excess

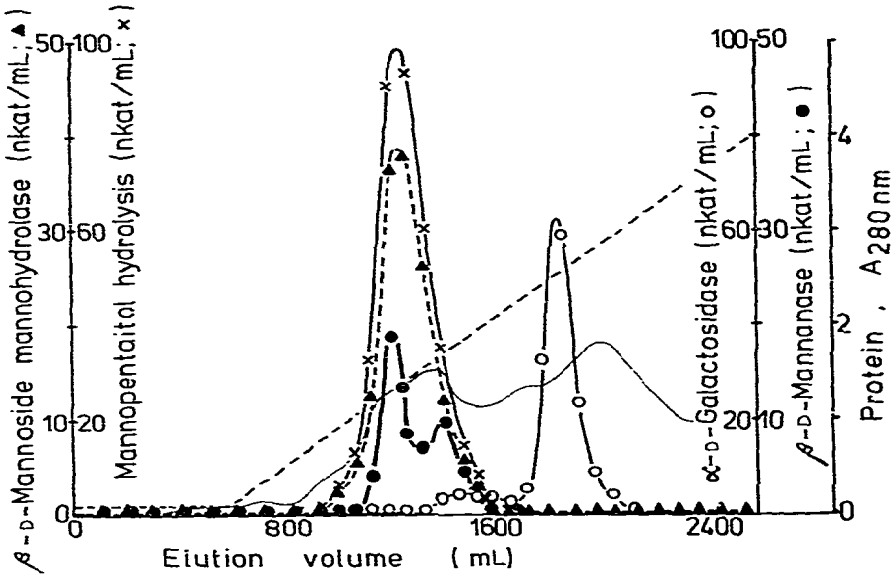

Fig. 1. Chromatography of guar β-D-mannoside mannohydrolase on a column (3.7 × 17 cm) of DEAE-cellulose with 20mM Tris buffer (pH 8) at 4° and a 0 → 400mM KCl gradient. The material chromatographed was obtained by batch DEAE-cellulose purification. β-D-Mannoside mannohydrolase and α-D-galactosidase were assayed with the nitrophenyl glycoside substrates.

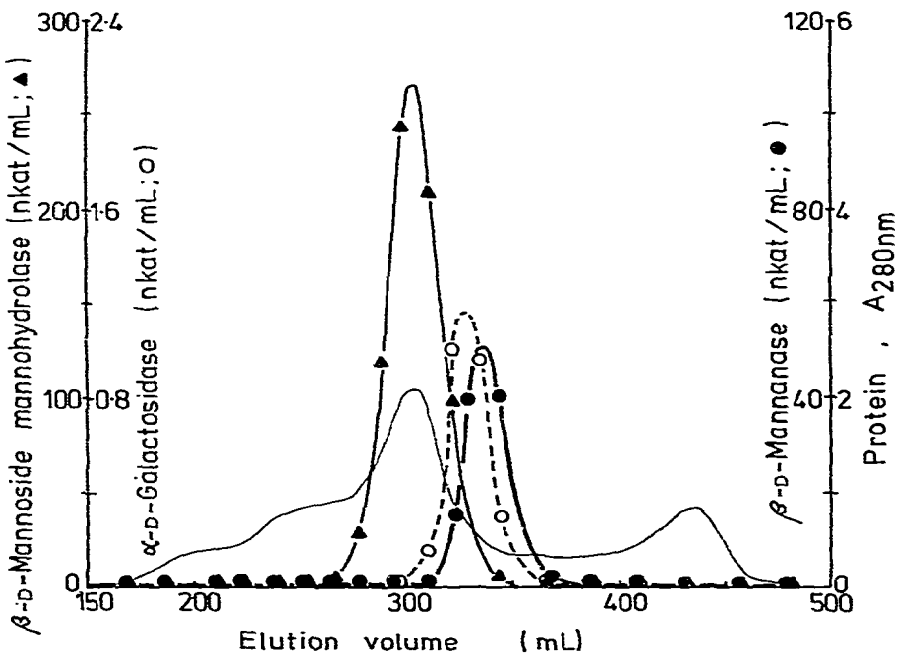

Fig. 2. Chromatography of guar β-D-mannoside mannohydrolase on a column (2.5 × 88 cm) of Ultrogel AcA 44 with 0.2M NaCl plus 20mM Tris buffer (pH 8) at 4°. The sample chromatographed was obtained by chromatography on DEAE-cellulose.

of 0.2M. However, if the seed material was extracted at this salt concentration in a solution buffered at pH 5.0–5.5, essentially all of the enzyme precipitated from solution on dialysis. In contrast, if the enzyme was extracted with 0.2M sodium chloride buffered at pH 8, 60–80% of the β-D-mannoside mannohydrolase remained soluble on dialysis (Table III). The insolubility of the enzyme after extraction and dialysis at pH 5.0–5.5 was considered to be due to interaction with other protein at these pH values. Pure, guar β-D-mannoside mannohydrolase is quite soluble in solutions of low pH and low salt concentration. The extraction procedure finally adopted for the preparation of the enzyme took advantage of its limited solubility (in the crude extract) in water, but solubility in 0.2M sodium chloride solution. Germinated guar-seed was homogenised in water and centrifuged, to give an aqueous extract plus a residue. The aqueous extract contained most of the α-D-galactosidase and β-D-mannanase activity, but only 20–30% of β-D-mannoside mannohydrolase. The rest of this enzyme (70–80%) remained in the insoluble residue and was extracted with a solution of sodium chloride in Tris buffer. This extraction procedure gave β-D-mannoside mannohydrolase having a specific activity of ~2.5 nkat/mg of protein in the crude extract. A much lower specific activity (0.5 nkat/mg) was obtained if the preliminary extraction with water was omitted. The protein which interacted with β-D-mannoside mannohydrolase, rendering it insoluble, was removed by binding the enzyme to DEAE-cellulose which was then washed exhaustively with dilute Tris buffer.

Chromatography of guar β-D-mannoside mannohydrolase on DEAE-cellulose is shown in Fig. 1. The enzyme was eluted as a single, sharp peak. A single peak of enzyme activity was also obtained on gel-permeation chromatography on Ultrogel AcA 44 (Fig. 2), and the elution volume indicated a molecular weight of ~60,000, which is considerably lower than that obtained when the initial extraction was performed at low pH. Under these conditions, the enzyme was eluted as a broad band of activity from both DEAE-cellulose and Ultrogel AcA 44. Also, the peak of activity on Ultrogel was near the column void-volume, indicating a molecular weight in excess of 100,000. Previous studies[8] indicated that guar β-D-mannoside mannohydrolase (termed exo-β-D-mannanase) had a molecular weight in excess of 100,000. However, in these studies, the enzyme was extracted and handled at a pH which has now been shown to be conducive to interaction with other protein material.

β-D-Mannoside mannohydrolase recovered from Ultrogel AcA 44 was still contaminated with α-D-galactosidase and β-D-mannanase. Both of these activities could be removed by chromatography of the enzyme on either CM-cellulose or by chromatofocusing on Pharmacia Polybuffer Exchanger, PBE 118 (Fig. 3). Chromatography on CM-cellulose (Fig. 4) gave complete removal of β-D-mannanase and α-D-galactosidase, but the recovery of activity was quite low (25–40%). A better recovery of activity (~60%) was obtained on chromatofocusing. In both cases, the specific activity of the recovered enzyme was similar.

Guar seeds contain two β-D-mannoside mannohydrolase enzymes, one in the cotyledons and one in the endosperms, which are chromatographically indistinguish-

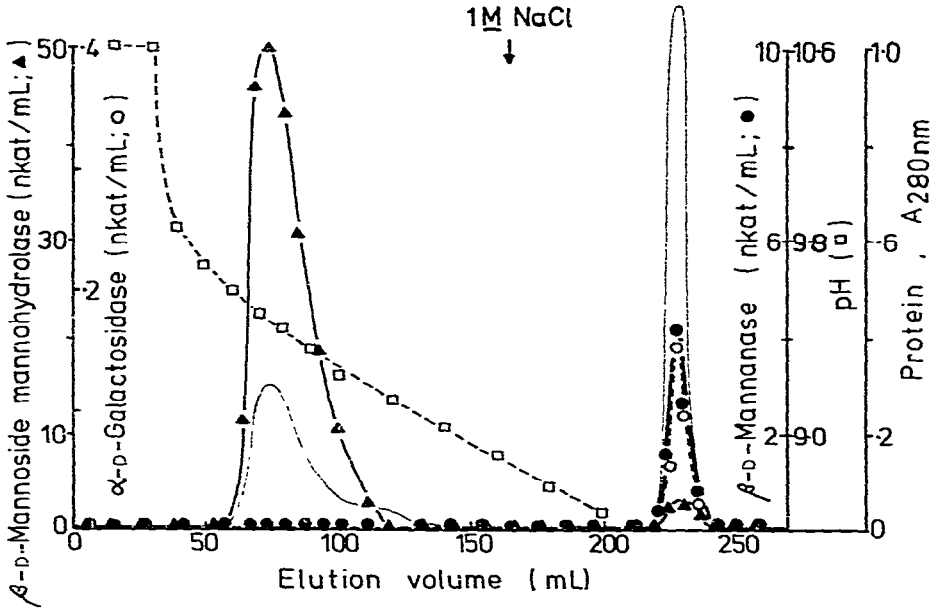

Fig. 3. Chromatofocusing of guar β-D-mannoside mannohydrolase on a column (1.6 × 12 cm) of Polybuffer Exchanger PBE 118. The column was equilibrated with, and the enzyme applied in, 25mM diethylamine HCl buffer and eluted with Pharmalyte pH 8–10.5 HCl (diluted 1:45 and adjusted to pH 8). The sample chromatographed was one third the enzyme obtained by chromatography on Ultrogel AcA 44.

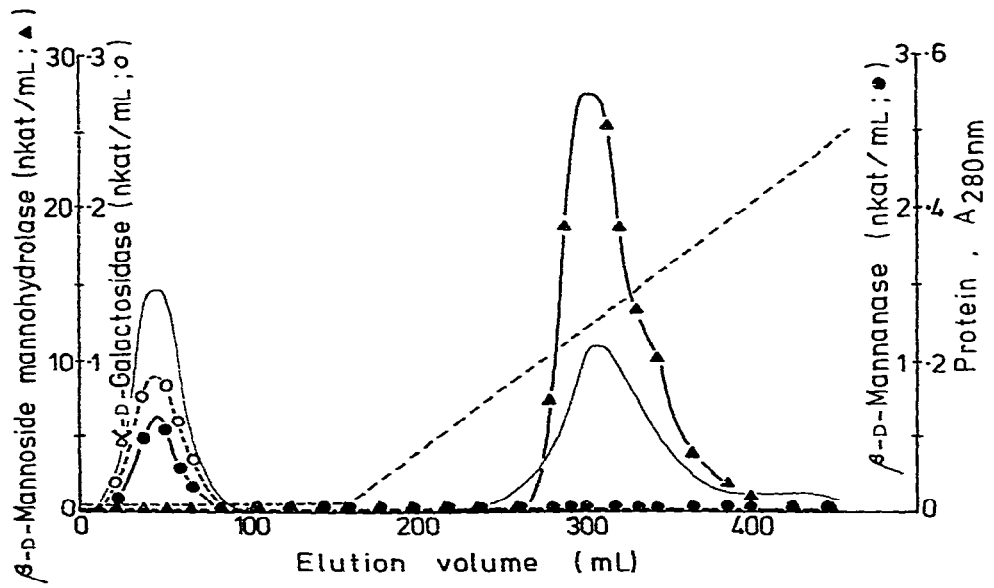

Fig. 4. Chromatography of guar β-D-mannoside mannohydrolase on a column (1.6 × 12 cm) of CM-cellulose with 20mM acetate buffer (pH 6.3) at 4° and a 0 → 400mM NaCl gradient. The material chromatographed was one third the enzyme obtained by chromatography on Ultrogel AcA 44 (but not the preparation described in this paper).

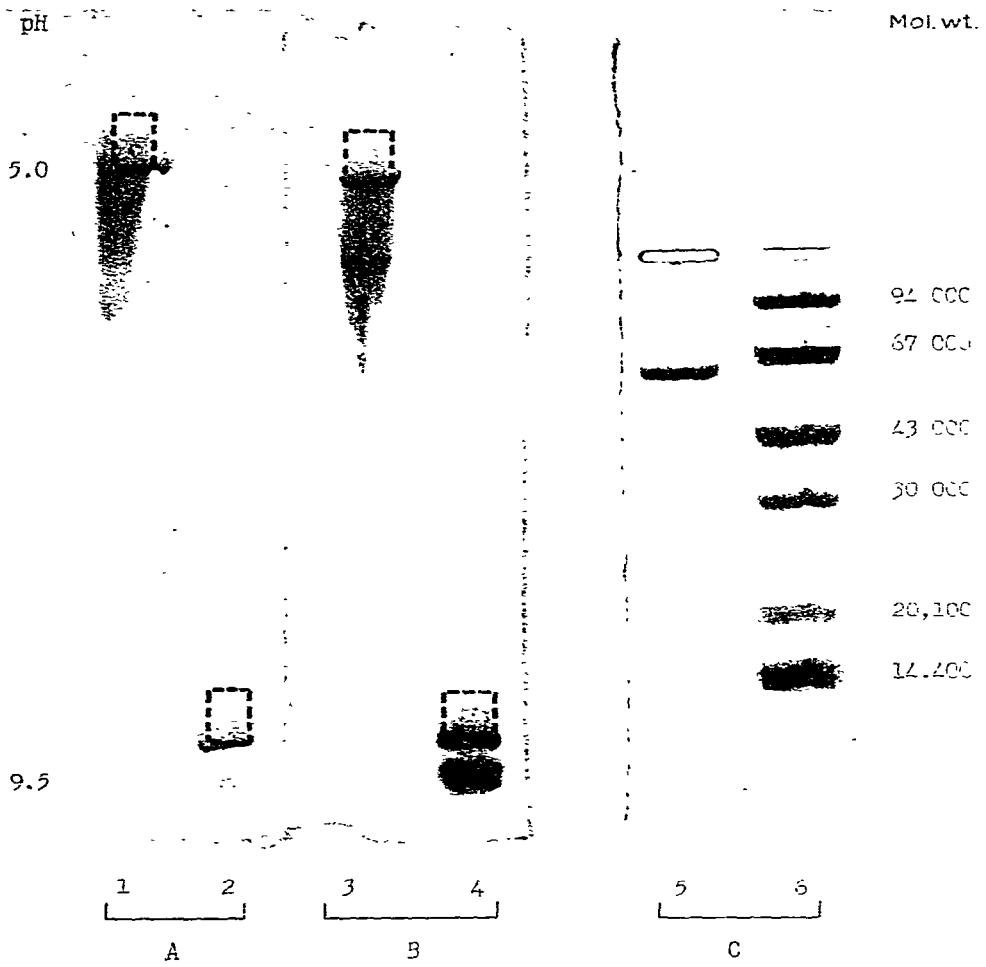

Fig. 5. Isoelectric focusing (A and B) and SDS-electrophoresis (C) of guar β-D-mannoside mannohydrolase. A, stained for activity with naphthyl β-D-mannopyranoside/fast blue BB salt. B, stained for protein with Coomassie Brilliant Blue. Samples 1 and 3 were applied at the anode end and samples 2 and 4 were applied at the cathode end. C, SDS-electrophoresis of guar β-D-mannoside mannohydrolase (5) and a standard protein mixture, mol.wt. 14,400–94,000 (6).

able. In separate experiments, germinated guar-seeds were manually separated into cotyledon and endosperm–seed coat fractions. These were extracted and chromatographed separately, and the β-D-mannoside mannohydrolase enzymes showed identical elution patterns on both DEAE-cellulose and Ultrogel AcA 44. They also had identical properties and action patterns.

Attempts to purify guar β-D-mannoside mannohydrolase by affinity chromatography on *N*-ε-aminocaproyl-β-D-mannopyranosylamine–Sepharose 4B were unsuccessful. The enzyme did not bind to the column material. Subsequent results showed that *N*-ε-aminocaproyl-β-D-mannopyranosylamine is a very poor inhibitor

TABLE IV

PROPERTIES OF β-D-MANNOSIDE MANNOHYDROLASE

Property	*Guar β-D-mannoside mannohydrolase*
Molecular weight	59,000 ±2,000
pI	9.4
Carbohydrate (%)	7.0
pH optimum	5.0–6.0
pH stability (40°, 0.5 h)	5.0–8.0
(4°, 20 h)	5.0–10.0
Temperature optimum	52°
Temperature stability (pH 5.5, 15 min)	up to 45°
Anomeric configuration of the released D-mannose	α
K_i (mannose)	23.8mM
p-Nitrophenyl β-D-mannopyranoside	
V_{max} (40°, pH 5.5; nkat/mg)	261
K_m (mM)	0.5
A_{280} (1 mg/mL)[a]	2.05

[a]Determined by the Folin–Lowry method[24].

of guar β-D-mannoside mannohydrolase; it gave no detectable inhibition at mM concentration. This contrasts with the results obtained with *N*-ε-aminocaproyl-α-D-galactopyranosylamine, which is a potent inhibitor of α-D-galactosidase (K_i 3 × 10^{-4}M)[13].

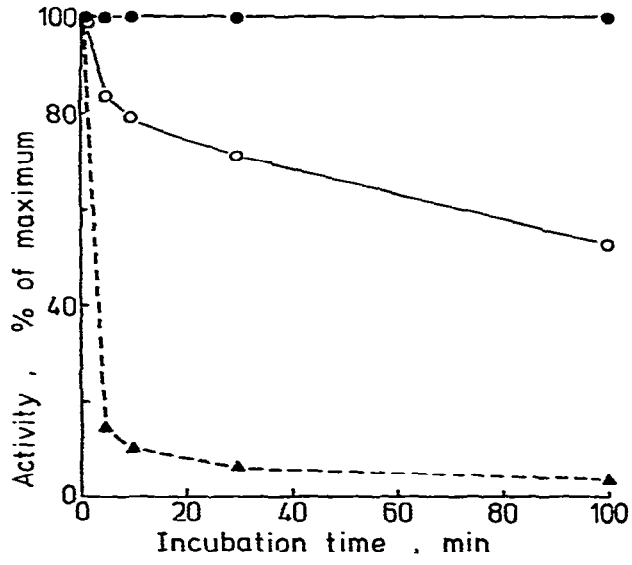

Fig. 6. Stability of guar β-D-mannoside mannohydrolase on incubation for 5–100 min at 40° and at pH 5 (●), 4.5 (○) or 4 (▲).

Properties of β-D-mannoside mannohydrolase. — Some properties of β-D-mannoside mannohydrolase are shown in Table IV. Both SDS–polyacrylamide gel electrophoresis (Fig. 5) and gel filtration on Ultrogel AcA 44 indicated a molecular weight of 59,000 ±2,000. This contrasts with a value of greater than 100,000 previously reported for the same enzyme[8]. The reason for this difference has been discussed. β-D-Mannoside mannohydrolase was unstable to isoelectric focusing (Fig. 5). Sample applied near the cathode side of the gel migrated towards the cathode and partially focused. However, some of the sample protein precipitated from solution at the end of the paper application-tab. Sample applied at the anode side of the gel precipitated from solution as it migrated towards the cathode. The precipitated protein still contained enzyme activity, as shown by its ability to hydrolyse naphthyl β-D-mannopyranoside[9].

Guar β-D-mannoside mannohydrolase released D-mannose from β-D-mannohexaitol in the α-anomeric configuration, indicating that it is an exo-β-D-mannanase, not a β-D-mannosidase. The enzyme was stable at pH 5.5; at pH values below 5.0, there was a rapid loss of enzyme activity (Fig. 6). This contrasts with previous results[8] which indicated that the enzyme was quite stable at pH values as low as 3.5. Of the metal ions tested, Hg^{2+}, Ag^{+}, Cu^{2+}, and Zn^{2+} caused appreciable inactivation at mM concentration. The extent of inhibition was: Hg^{2+}, 100%; Ag^{+}, 100%; Cu^{2+}, 40%; and Zn^{2+}, 18%. β-D-Mannotri-itol at a concentration of 10mM gave no detectable inhibition, whereas D-mannose did (K_i 23.8mM).

Mode of action of guar β-D-mannoside mannohydrolase. — Only limited studies of the mode of action of β-D-mannoside mannohydrolase enzymes have previously been performed, due in part to the lack of availability of suitable substrates. Recently, improved techniques have been developed for the synthesis of *p*-nitrophenyl β-D-mannopyranoside, and, in the current work, one of these[10] was adapted to the preparation of *o*-nitrophenyl, naphthyl, and methylumbelliferyl β-D-mannopyrano-

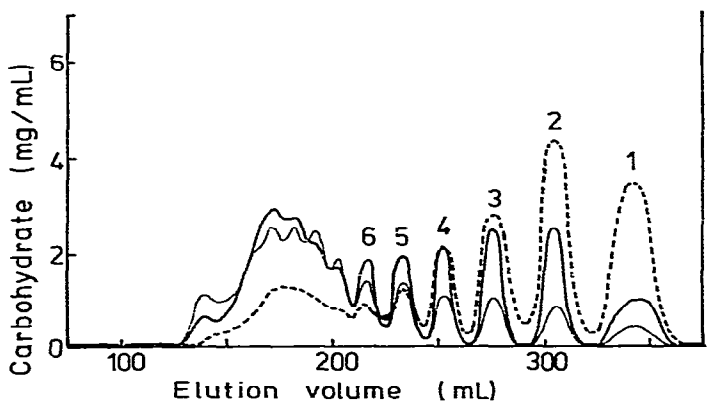

Fig. 7. Chromatography of β-D-manno-oligosaccharides on a column (3.5 × 87 cm) of Bio-Gel P-2 (<400 mesh) with distilled water at 60°. Oligosaccharide preparations were obtained by incubation of β-D-mannan with fuming HCl for 0.5 (———), 1 (———), and 2 h (– – – –).

TABLE V

RELATIVE, INITIAL RATES OF HYDROLYSIS OF β-D-MANNO-OLIGOSACCHARIDES AND REDUCED β-D-MANNO-OLIGOSACCHARIDES BY GUAR β-D-MANNOSIDE MANNOHYDROLASE

Substrate	*Relative rate of hydrolysis*[a] (%)
β-D-Mannobi-itol	0
β-D-Mannobiose	12
β-D-Mannotri-itol	0.8
β-D-Mannotriose	20
β-D-Mannotetraitol	20
β-D-Mannotetraose	52
β-D-Mannopentaitol	100
β-D-Mannopentaose	100
β-D-Mannohexaitol	90
β-D-Mannohexaose	90

[a]Determined by incubating β-D-mannoside mannohydrolase (0.05 mL, 2.0 nkat on mannopentaitol) with β-D-manno-oligosaccharide (0.1 mL, 10 mg/mL) in 0.1M acetate buffer (pH 5.5) at 40° for 2–10 min. The extent of hydrolysis of the oligosaccharides was <5%.

sides. β-D-Manno-oligosaccharides were prepared by hydrolysis of β-D-mannan by fuming hydrochloric acid. The patterns of amounts of β-D-manno-oligosaccharides produced on acid hydrolysis of β-D-mannan are shown in Fig. 7.

The initial rate of hydrolysis of a range of β-D-manno-oligosaccharides and reduced β-D-manno-oligosaccharides by guar β-D-mannoside mannohydrolase is shown in Table V. The greater rate of hydrolysis of β-D-mannopentaose and β-D-mannopentaitol compared to that for β-D-mannohexaose and β-D-mannohexaitol was simply due to the higher molar concentration of the former two substrates in the assay mixtures. The concentration of substrate in these incubations was less than saturating (see Table VI). β-D-Manno-oligosaccharides of d.p. <5 are hydrolysed

TABLE VI

KINETIC CONSTANTS OF GUAR β-D-MANNOSIDE MANNOHYDROLASE

Substrate	K_m *(mM)*	V_{max} *(nkat/mg)*
β-D-Mannobi-itol	—	0
β-D-Mannotri-itol	80.0	35.8
β-D-Mannotetraitol	12.0	532
β-D-Mannopentaitol	2.8	967
β-D-Mannohexaitol	2.8	967
p-Nitrophenyl β-D-mannopyranoside	0.5	261
o-Nitrophenyl β-D-mannopyranoside	3.0	1005
Methylumbeliiferyl β-D-mannopyranoside	0.5	1150

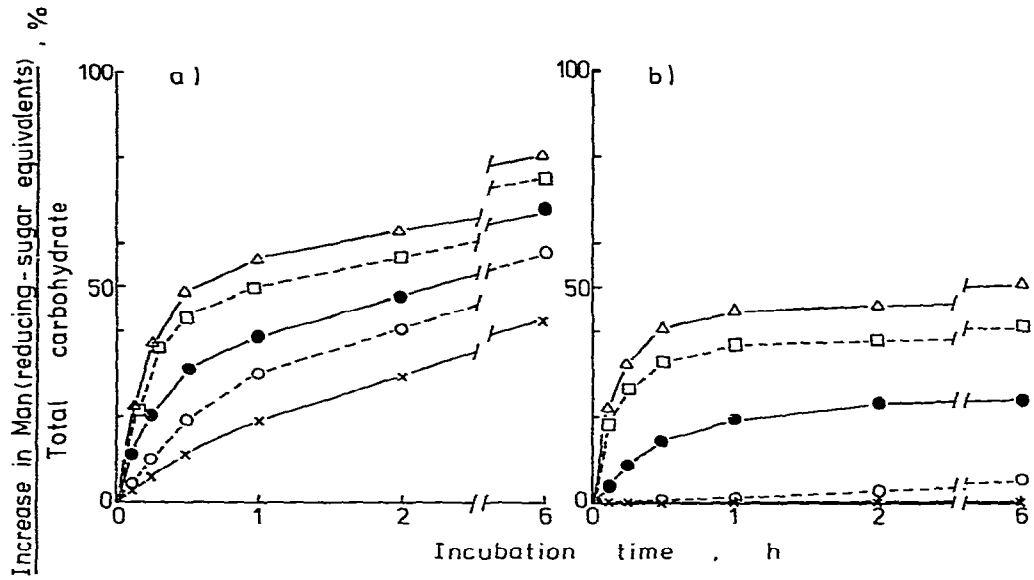

Fig. 8. Hydrolysis of manno-oligosaccharides (9.2 mg/mL) and reduced manno-oligosaccharides (9.2 mg/mL) by guar β-D-mannoside mannohydrolase (11.5 nkat/mL on *p*-nitrophenyl β-D-manno-pyranoside): (a) manno-oligosaccharides; (b) reduced manno-oligosaccharides of d.p. 2 (×), 3 (○), 4 (●), 5 (□), and 6 (△).

at rates considerably less than that for β-D-mannopentaose. Reduction of the terminal β-D-mannosyl residue with borohydride greatly affects the susceptibility of the oligosaccharides to hydrolysis. β-D-Mannobi-itol is totally resistant to hydrolysis, and β-D-mannotri-itol is hydrolysed at only one twenty-fifth the rate for β-D-mannotriose. However, β-D-mannohexaitol is hydrolysed at the same rate as β-D-mannohexaose. The hydrolysis of β-D-manno-oligosaccharides and reduced β-D-manno-oligosaccharides by β-D-mannoside mannohydrolase is shown in Fig. 8. To allow direct comparison of the rates and degrees of hydrolysis of reduced and non-reduced oligosaccharides, values are represented as increase in released mannose (reducing-sugar equivalents) as a percentage of total carbohydrate. Thus, the maximum, theoretical values on complete hydrolysis of β-D-mannohexaose (or β-D-mannohexaitol), β-D-mannopentaose (or β-D-mannopentaitol), β-D-mannotetraose (or β-D-mannotetraitol), β-D-mannotriose (or β-D-mannotri-itol), and β-D-mannobiose (or β-D-mannobi-itol) are 83, 80, 75, 67, and 50%, respectively. It is evident from Fig. 8 that, after incubation for 6 h with β-D-mannoside mannohydrolase, the β-D-manno-oligosaccharides are essentially hydrolysed to the theoretical maxima. However, hydrolysis of the reduced β-D-manno-oligosaccharides is far from complete, reflecting the resistance of β-D-mannotri-itol to hydrolysis. The decrease in rate of hydrolysis of the β-D-manno-oligosaccharides with time is a reflection of the lower d.p. of the available substrate and may also be due, in part, to inhibition by the released D-mannose. There is essentially no loss of enzyme activity over the incubation period employed. The

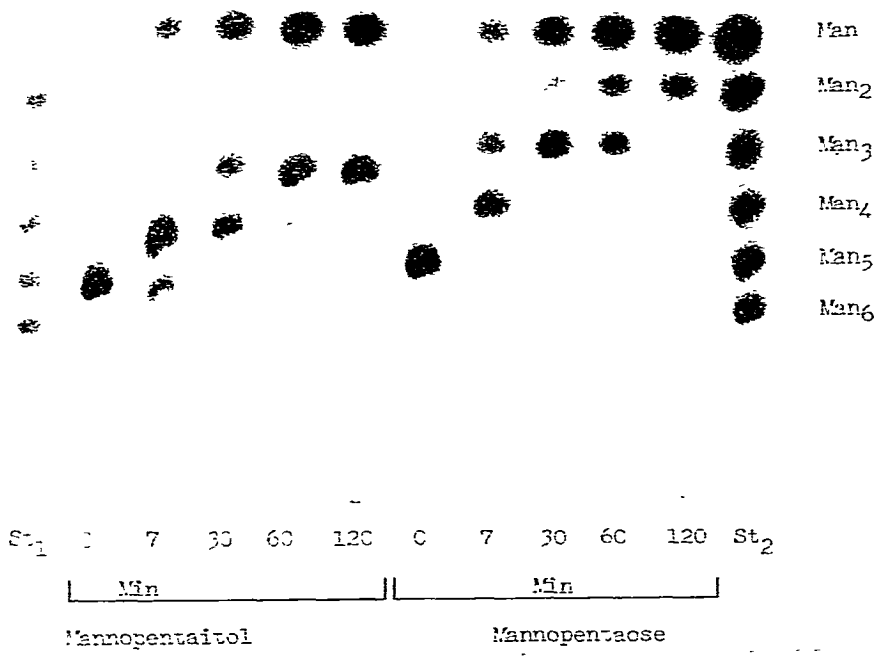

Fig. 9. T.l.c. of the hydrolysis products of mannopentaose and mannopentaitol by guar β-D-mannoside mannohydrolase. Incubation conditions are described in Fig. 8. Aliquots were removed at 0–120 min for chromatography. St_1, mannobiitol–mannohexaitol. St_2, mannose–mannohexaose.

patterns of amounts of oligosaccharides produced on hydrolysis of β-D-mannopentaose and β-D-mannopentaitol by this enzyme are shown in Fig. 9. β-D-Mannopentaose is rapidly hydrolysed to β-D-mannobiose, which is slowly hydrolysed to D-mannose. β-D-Mannopentaitol is hydrolysed to β-D-mannotri-itol, but this is quite resistant to further hydrolysis. Under no circumstances did the enzyme release mannitol from mannobi-itol, as previously reported[8].

The K_m and V_{max} values for guar β-D-mannoside mannohydrolase on a range of reduced β-D-manno-oligosaccharides, and on nitrophenyl and methylumbelliferyl β-D-mannopyranosides, are shown in Table VI. As the d.p. of the reduced β-D-manno-oligosaccharides decreased from 5 to 2, the K_m value increased and V_{max} decreased. The enzyme has a greater affinity for *p*-nitrophenyl and methylumbelliferyl β-D-mannopyranosides than for the reduced β-D-manno-oligosaccharides or *o*-nitrophenyl β-D-mannopyranoside, but the V_{max} for *p*-nitrophenyl β-D-mannopyranoside is only

25% of that for β-D-mannohexaitol and for *o*-nitrophenyl and methylumbelliferyl β-D-mannopyranosides.

CONCLUSIONS

Many of the problems associated with the extraction and purification of β-D-mannoside mannohydrolase from germinated legume-seeds appear to be due to the interaction of these enzymes with other relatively insoluble protein-material present in the cotyledons of these seeds. Furthermore, the interaction appears to be reasonably specific; neither α-D-galactosidase nor β-D-mannanase interact with this protein material.

Studies of β-D-mannoside mannohydrolases are limited[26]. The few enzymes which have been studied in any detail have been shown to be β-D-mannosidases. Reese and Shibata[4] partially purified β-D-mannosidase enzymes from *Penicillium funiculosum*, *P. ochro-chloron*, and *Helix pomatia*, and found that, for each enzyme, the initial rates of hydrolysis of β-D-mannotri-itol and β-D-mannotriose were similar, but that β-D-mannobiose was hydrolysed at approximately ten times the rate for β-D-mannobi-itol. For these enzymes, and for one described by Hashimoto and Fukumoto[27] from *Rhizopus niveus*, β-D-mannobiose was hydrolysed at one-half to two-thirds the rate for β-D-mannotriose. The β-D-mannosidase from *R. niveus* hydrolysed β-D-mannotetraose and β-D-mannotriose at a similar rate, which was approximately twice the initial rate of hydrolysis of β-D-mannobiose, and approximately five and twenty times the initial rates of hydrolysis of β-D-mannopentaose and β-D-mannohexaose, respectively. Clearly, the action patterns of these enzymes are quite different to that of the guar β-D-mannoside mannohydrolase currently described. For this enzyme, the d.p. for maximal rate of hydrolysis is five or greater, and the enzyme has a much lower K_m for β-D-mannopentaitol and β-D-mannohexaitol than for β-D-mannotri-itol. These results indicate that guar β-D-mannoside mannohydrolase is an exo-β-D-mannanase rather than a β-D-mannosidase[28]. This conclusion is supported by the observation that the D-mannose released on hydrolysis of mannohexaitol by guar β-D-mannoside mannohydrolase has the α-anomeric configuration, *i.e.*, the configuration is inverted[28].

The β-D-mannoside mannohydrolase enzyme of guar seeds was first described by Lee[8]. The enzyme was purified nine-fold, had a specific activity of 1.55 U/mg on reduced ivory-nut mannan, and was highly contaminated with α-D-galactosidase (5%) and a range of other glycosidases. Contamination with β-D-mannanase was not accurately determined. The enzyme described here was purified one hundred-fold and had a specific activity of 967 nkat/mg (57.9 U/mg) on mannohexaitol. The enzyme was devoid of α-D-galactosidase and β-D-mannanase (determined viscometrically)[29]. Studies to establish the importance of the enzyme in the mobilisation of reserve galactomannan during seed germination are in progress.

ACKNOWLEDGMENTS

The author thanks Professor H. Neukom (Swiss Federal Institute of Technology) for useful discussions and for arranging micro-analysis of the compounds synthesised, Dr. D. Ridley (University of Sydney) for the use of a Perkin–Elmer 241 Digital Polarimeter, Mr. M. Hill for photography, and Miss C. Lomas for assistance in preparing this manuscript.

REFERENCES

1 J. S. G. Reid and H. Meier, *Planta*, 112 (1973) 301–308.
2 P. M. Dey, *Adv. Carbohydr. Chem. Biochem.*, 35 (1978) 341–376.
3 B. V. McCleary, N. K. Matheson, and D. M. Small, *Phytochemistry*, 15 (1976) 1111–1117.
4 E. T. Reese and Y. Shibata, *Can. J. Microbiol.*, 11 (1965) 167–183.
5 R. Somme, *Acta. Chem. Scand.*, 24 (1970) 72–76.
6 B. V. McCleary and N. K. Matheson, *Phytochemistry*, 14 (1975) 1187–1194.
7 A. Seiler, *Planta*, 134 (1977) 209–221.
8 S. R. Lee, Ph.D. Thesis, University of Minnesota, Minneapolis, 1965; *Chem. Abstr.*, 68 (1968) 111694c.
9 B. V. McCleary, *Carbohydr. Res.*, 71 (1979) 205–230.
10 P. J. Garegg, T. Iversen, and T. Norberg, *Carbohydr. Res.*, 73 (1979) 313–314.
11 H. S. Isbell and H. L. Frush, *J. Org. Chem.*, 23 (1958) 1309–1319.
12 R. Schwyzer, B. M. Iselin, and M. Feurer, Ger. Pat., 1,085,881 (1960); *Chem. Abstr.*, 56 (1962) 4864.
13 N. Harpaz, H. M. Flowers, and N. Sharon, *Biochim. Biophys. Acta*, 341 (1974) 213–221.
14 R. Axen, J. Porath, and S. Ernback, *Nature (London)*, 214 (1967) 1302–1304.
15 M. Lever, *Anal. Biochem.*, 47 (1972) 273–279.
16 B. V. McCleary, *Carbohydr. Res.*, 67 (1978) 213–221.
17 Chromatofocusing with Polybuffer and PBE. Pharmacia Fine Chemicals.
18 Y. S. Wong, M.Sc. Thesis, McGill University, Montreal, 1976.
19 G. L. Miller, J. Dean, and R. Blun, *Arch. Biochem. Biophys.*, 91 (1960) 21–26.
20 K. Kainuma, A. Nogami, and C. Mercier, *J. Chromatogr.*, 121 (1976) 361–369.
21 M Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
22 M. Abdel-Akher, J. K. Hamilton, and F. Smith, *J. Am. Chem. Soc.*, 73 (1951) 4691–4692.
23 H. Fehrnstrom, (1977) LKB Application Note 306, SDS and Conventional Poly-acrylamide Gel Electrophoresis with LKB 2117 Multiplier.
24 O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265–275.
25 M. Streamer, K. E. Eriksson, and B. Pettersson, *Eur. J. Biochem.*, 59 (1975) 607–613.
26 R. F. H. Dekker and G. N. Richards, *Adv. Carbohydr. Chem. Biochem.*, 32 (1976) 277–352.
27 Y. Hashimoto and J. Fukumoto, *Nippon Nogei Kagaku Kaishi*, 43 (1969) 317–322.
28 E. T. Reese, *Recent Adv. Phytochem.*, 11 (1977) 311–367.
29 B. V. McCleary, R. Amado, R. Waibel, and H. Neukom, *Carbohydr. Res.*, 92 (1981) 269–285.