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β-D-MANNOSIDASE FROM *Helix pomatia*

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ABSTRACT

β-D-Mannosidase (β-D-mannoside mannohydrolase EC 3.2.1.25) was purified 160-fold from crude gut-solution of *Helix pomatia* by three chromatographic steps and then gave a single protein band (mol. wt. 94,000) on SDS-gel electrophoresis, and three protein bands (of almost identical isoelectric points) on thin-layer isoelectric focusing. Each of these protein bands had enzyme activity. The specific activity of the purified enzyme on *p*-nitrophenyl β-D-mannopyranoside was 1694 nkat/mg at 40° and it was devoid of α-D-mannosidase, β-D-galactosidase, 2-acetamido-2-deoxy-D-glucosidase, (1→4)-β-D-mannanase, and (1→4)-β-D-glucanase activities, almost devoid of α-D-galactosidase activity, and contaminated with $<0.02\%$ of β-D-glucosidase activity. The purified enzyme had the same K_m for borohydride-reduced β-D-manno-oligosaccharides of d.p. 3–5 (12.5mM). The initial rate of hydrolysis of (1→4)-linked β-D-manno-oligosaccharides of d.p. 2–5 and of reduced β-D-manno-oligosaccharides of d.p. 3–5 was the same, and *o*-nitrophenyl, methylumbelliferyl, and naphthyl β-D-mannopyranosides were readily hydrolysed. β-D-Mannobiose was hydrolysed at a rate ~25 times that of 6^1-α-D-galactosyl-β-D-mannobiose and 6^3-α-D-galactosyl-β-D-mannotetraose, and at ~90 times the rate for β-D-mannobi-itol.

INTRODUCTION

Only limited studies have been performed on β-D-mannosidase[1], despite the occurrence of (1→4)-linked β-D-mannosyl residues in several reserve and structural plant polysaccharides, including galactomannans, glucomannans, and galactoglucomannans[2], and in the oligosaccharide moieties of glycoproteins[3]. β-D-Mannosidase has been reported to occur in a wide range of plant[4–12] and animal[13–17] tissues, and in culture filtrates or mycelia of several micro-organisms[13,18–22]. However, due to the low levels of activity in the source materials, and, in some cases, the instability of the enzyme, only a few highly purified preparations of β-D-mannosidase have been reported. β-D-Mannosidase has been partially purified from hen oviduct[16], pineapple bromelain[6], the snail *Achatina fulica*[15], the marine gastropod *Turbo cornutus*[14], barley[12], the fungus *Rhizopus niveus*[18], and lucerne seed[8]. *Polyporus sulfureus*[19] was reported as a good source of this enzyme for use in structural studies of core glyco-

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peptides and was obtained in an essentially homogeneous form with a specific activity of 4.2 U/mg, and essentially devoid of other glycosidase activities, except that of α-D-galactosidase. The purified β-D-mannosidase from *Tremella fuciformis*[20] appeared as a single protein band on gel electrophoresis and had a final specific activity of 20.0 U/mg, but the overall yield of purified enzyme was very low. β-D-Mannosidase purified (to 14.3 U/mg) from culture filtrates of *Aspergillus niger*[21] was homogeneous on polyacrylamide gel electrophoresis and had a mol. wt. of 120,000. The enzyme hydrolysed (1→4)-β-D-mannobiose at approximately two-thirds of the rate for (1→4)-β-D-mannotriose, and the K_m for each substrate was ~mM. Large-scale purification of β-D-mannosidase from *A. niger*[22] gave a homogeneous enzyme having a mol. wt. of 130,000 and a specific activity of 188 U/mg, and which was free from other glycosidase activities; α-D-galactosidase was removed by using a column of *N*-ε-aminocaproyl-α-D-galactopyranosylamine–Sepharose 4B. The β-D-mannosidase was relatively specific for the (1→4)-linked β-D-mannosyl residues; (1→3)- and (1→6)-linked β-D-mannosyl residues were hydrolysed at rates 0.04 and 0.1% of that for (1→4)-linked β-D-mannosyl residues.

The purification and some of the properties of a β-D-mannosidase from the gut solution of *Helix pomatia* are now described.

EXPERIMENTAL

Substrates. — *p*-Nitrophenyl, *o*-nitrophenyl, naphthyl, and methylumbelliferyl β-D-mannopyranosides, *N*-ε-aminocaproyl-β-D-mannopyranosylamine, β-D-manno-oligosaccharides, and reduced β-D-manno-oligosaccharides were prepared as previously described[23]. 6^1-α-D-Galactosyl-β-D-mannobiose and 6^1-α-D-galactosyl-β-D-mannotriose were purified from the β-D-mannanase hydrolysate of carob D-galacto-D-mannan and were characterised enzymically, by n.m.r. spectroscopy, and by methylation analysis[24]. 6^3-α-D-Galactosyl-β-D-mannotetraose was purified from the β-D-mannanase (lucerne seed) hydrolysate of carob galactomannan. Details of the purification and characterisation of this and a range of other galactose-containing β-D-manno-oligosaccharides will be described elsewhere.

Chromatography and electrophoresis. — Isoelectric focusing, SDS-gel electrophoresis, and t.l.c. were performed as previously described[23].

Enzyme assays. — α-D-Galactosidase and β-D-mannanase were routinely assayed, as previously described[23], using *p*-nitrophenyl α-D-galactopyranoside or carob galactomannan dyed with Remazo Brilliant Blue. β-D-Mannosidase was assayed by incubating enzyme preparation (0.1 mL) with *p*-nitrophenyl β-D-manno-pyranoside (0.1 mL, 10mM) in 0.1M sodium acetate buffer (pH 4.5) at 40°. The reaction was terminated and the colour developed by the addition of aqueous 2% sodium carbonate (3 mL). A similar procedure was employed for the assay of other glycosidases. Endo-(1→4)-β-D-glucanase was assayed[25] by using CM-cellulose 4M6F dyed with Remazol Black as substrate.

Properties of H. pomatia *β-D-mannosidase.* — The mol. wt. of the β-D-mannosi-

dase was determined[23] by gel-permeation chromatography and SDS-gel slab electrophoresis. The pH optimum was determined by incubating enzyme preparation (0.05 mL, 16 nkat/mL) with 10mM *p*-nitrophenyl β-D-mannopyranoside (0.2 mL) in 0.1M acetate–phosphate buffer (pH 3.5–8.0). The pH stability was determined by incubating enzyme (0.1 mL, 68 nkat/mL) with acetate–phosphate buffer (0.1 mL; 0.2M, pH 3.5–8.0) for 90 min at 40°. Acetate buffer (0.4 mL; 0.2M, pH 4) was added and aliquots (0.05 mL) were removed for assay of β-D-mannosidase by incubation with 10mM *p*-nitrophenyl β-D-mannopyranoside (0.2 mL) in 0.2M acetate buffer (pH 4.5). The temperature–activity curve was obtained by incubating β-D-mannosidase (0.05 mL, 17 nkat/mL) with 10mM *p*-nitrophenyl β-D-mannopyranoside (0.2 mL) in sodium acetate buffer (0.2M, pH 4.5) for 2 min. Temperature stability was measured by incubating aliquots (0.1 mL) of enzyme in acetate buffer (0.1M, pH 4.5) at 25–79° for 15 min. Residual activity on *p*-nitrophenyl β-D-mannopyranoside was then assayed at 40°. Protein levels were determined by the Folin–Lowry method[26].

Helix pomatia β-D-*mannosidase*. — (*a*) *Purification*. Crude, snail-gut solution (10 mL, Sigma G0876) was diluted to 20 mL with ice-cold phosphate buffer (200mM, pH 6.5), dialysed against ice-cold phosphate buffer (10mM, pH 6.5) for 16 h, and applied to a column (3 × 22 cm) of DEAE-cellulose equilibrated with the same buffer. Protein was eluted with a linear NaCl gradient (0→0.2M) in phosphate buffer (10mM, pH 6.5). The fractions containing β-D-mannosidase activity were combined, concentrated by dialysis against polyethylene glycol 4000, and applied to a column (2.5 × 88 cm) of Ultrogel AcA 44 pre-equilibrated with 20mM sodium acetate buffer (pH 5.0). Active fractions were combined, the pH was adjusted to 4.0, and the solution was applied to a column (1.2 × 12 cm) of CM-cellulose equilibrated with 20mM acetate buffer (pH 4.0). Protein was eluted with a linear gradient of NaCl (0→0.2M) in acetate buffer (20mM, pH 4). The active fractions were combined, concentrated in a Diaflo ultrafiltration cell with a UM 10 membrane, washed with 5mM acetate buffer (pH 5), and stored in the frozen state.

(*b*) *Hydrolysis of* β-D-*manno-oligosaccharides and reduced* β-D-*manno-oligosaccharides*. — Aqueous oligosaccharide (0.2 mL, 15 mg/mL) and sodium acetate buffer (0.1 mL; 10mM, pH 4.5) were incubated with β-D-mannosidase (0.10 mL, 10.8 nkat on *p*-nitrophenyl β-D-mannopyranoside at 40°). Samples (10 μL) were subjected to t.l.c. during 0–4 h. Other samples (20 μL) were heated to denature the β-D-mannosidase and diluted with water (0.5 mL), and aliquots (0.1–0.2 mL) were used for measurement of reducing activity[27] and total carbohydrate[28]. An allowance was made for mannitol which was not detected by the phenol–sulphuric acid method[28].

For the determination of K_m and V_{max} values, reduced manno-oligosaccharide (0.2 mL, 1–30mM) in acetate buffer (pH 4.5, 0.1M) was incubated at 40° with β-D-mannosidase (0.05 mL). The reaction was terminated by the addition of *p*-hydroxybenzohydrazide solution[27] (5 mL) and the colour was developed during 6 min at 100°.

RESULTS

Helix pomatia *β*-D-*mannosidase*. — (*a*) *Purification*. A typical scheme used for the purification is shown in Table I. Chromatography of the crude solution, after dialysis, on DEAE-cellulose (Fig. 1) removed most of the contaminating α-D-galactosidase and (1→4)-*β*-D-mannanase. The remaining *β*-D-mannanase was removed by chromatography of the active fraction on Ultrogel AcA 44 (Fig. 2). A further two-fold increase in the specific activity of the *β*-D-mannosidase was obtained by chromato-

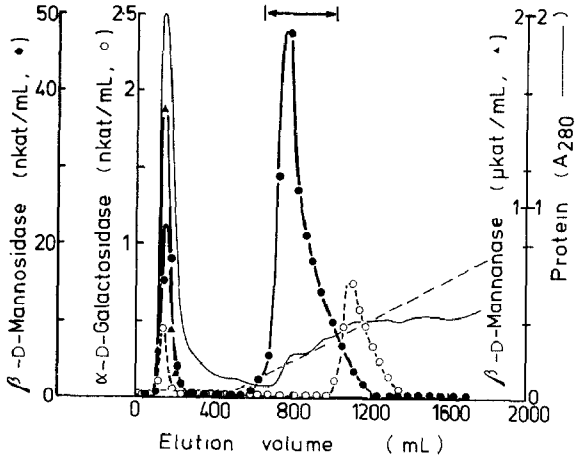

Fig. 1. Chromatography of crude gut-solution (10 mL) of *H. pomatia* diluted 2-fold with 0.2M phosphate buffer (pH 6.5) and dialysed against 10mM phosphate buffer (pH 6.5) on a column (3 × 22 cm) of DEAE-cellulose with 10mM phosphate buffer (pH 6.5) at 4° and a 0→200mM NaCl gradient.

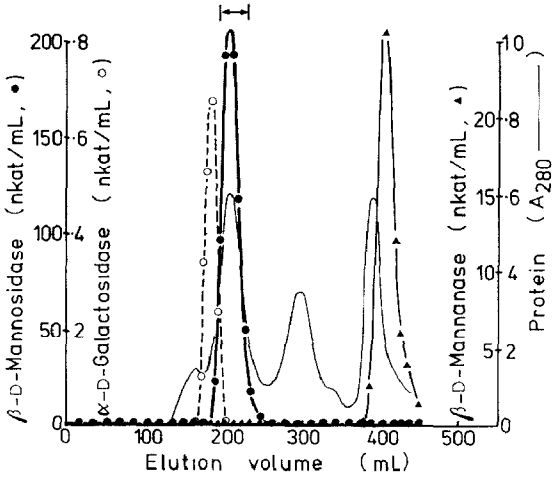

Fig. 2. Chromatography of *H. pomatia* *β*-D-mannosidase (see Fig. 1) on a column (2.5 × 88 cm) of Ultrogel AcA 44 with 20mM acetate buffer (pH 5.0) at 4°.

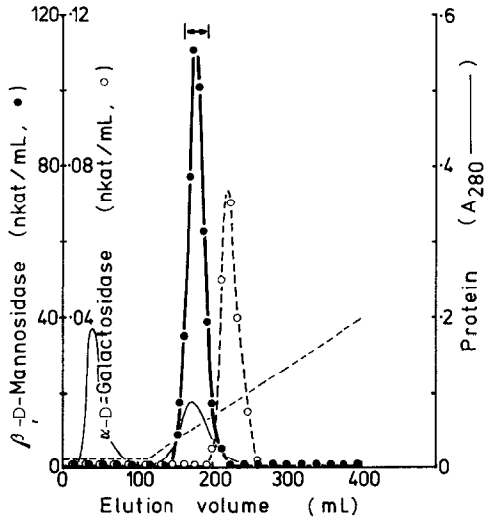

Fig. 3. Chromatography of *H. pomatia* β-D-mannosidase (see Fig. 2) on a column (1.2 × 12 cm) of CM-cellulose with 20mM acetate buffer (pH 4.0) at 4° and a 0→200mM NaCl gradient.

graphy of the active fraction on CM-cellulose (Fig. 3). The isoelectric-focusing patterns of the β-D-mannosidase fraction at each stage of purification are shown in Fig. 4, and the levels of contaminating α-D-galactosidase, β-D-mannanase, and α-D-mannosidase in these fractions are shown in Table II.

The degree of contamination with α-D-galactosidase of β-D-mannosidase recovered from CM-cellulose chromatography was $<0.01\%$ (assayed by using *p*-nitrophenyl α-D-galactopyranoside). Contamination with β-D-glucosidase was $\sim 0.02\%$, and β-D-galactosidase, β-D-xylosidase, and 2-acetamido-2-deoxy-D-glucosidase activities (assayed with the appropriate *p*-nitrophenyl glycosides) were $<0.001\%$. The enzyme appeared as a single protein band on SDS gel electrophoresis, but three protein bands with very similar isoelectric points were detected on isoelectric focusing. Each of the protein bands had enzyme activity.

(*b*) *Properties.* Some of the properties are shown in Table III. The enzyme was stable on extended incubation to 45°, but there was a rapid loss of activity above 50°. With very short incubation times, the temperature optimum was 55° (Fig. 5). The optimum pH was 4.0, but, on extended incubation, the enzyme slowly lost activity at this pH (Fig. 6). Consequently, the enzyme was routinely assayed and used at pH 4.5. Snail β-D-mannosidase, unlike the exo-β-D-mannanase from guar seeds[23], was unstable at pH >7. Chromatography of the enzyme on DEAE-cellulose at pH 8 resulted in considerable loss of activity, and consequently poor recoveries. Of numerous metal ions tested, only Hg^{2+}, Ag^{+}, and Cu^{2+} caused appreciable loss of activity; with mM metal ion at 40° for 10 min, the activity losses were 50, 70, and 27%, respectively. mM *N*-ε-Aminocaproyl-β-D-mannopyranosylamine caused no inhibition and thus was of no value for the preparation of affinity-chromatography materials.

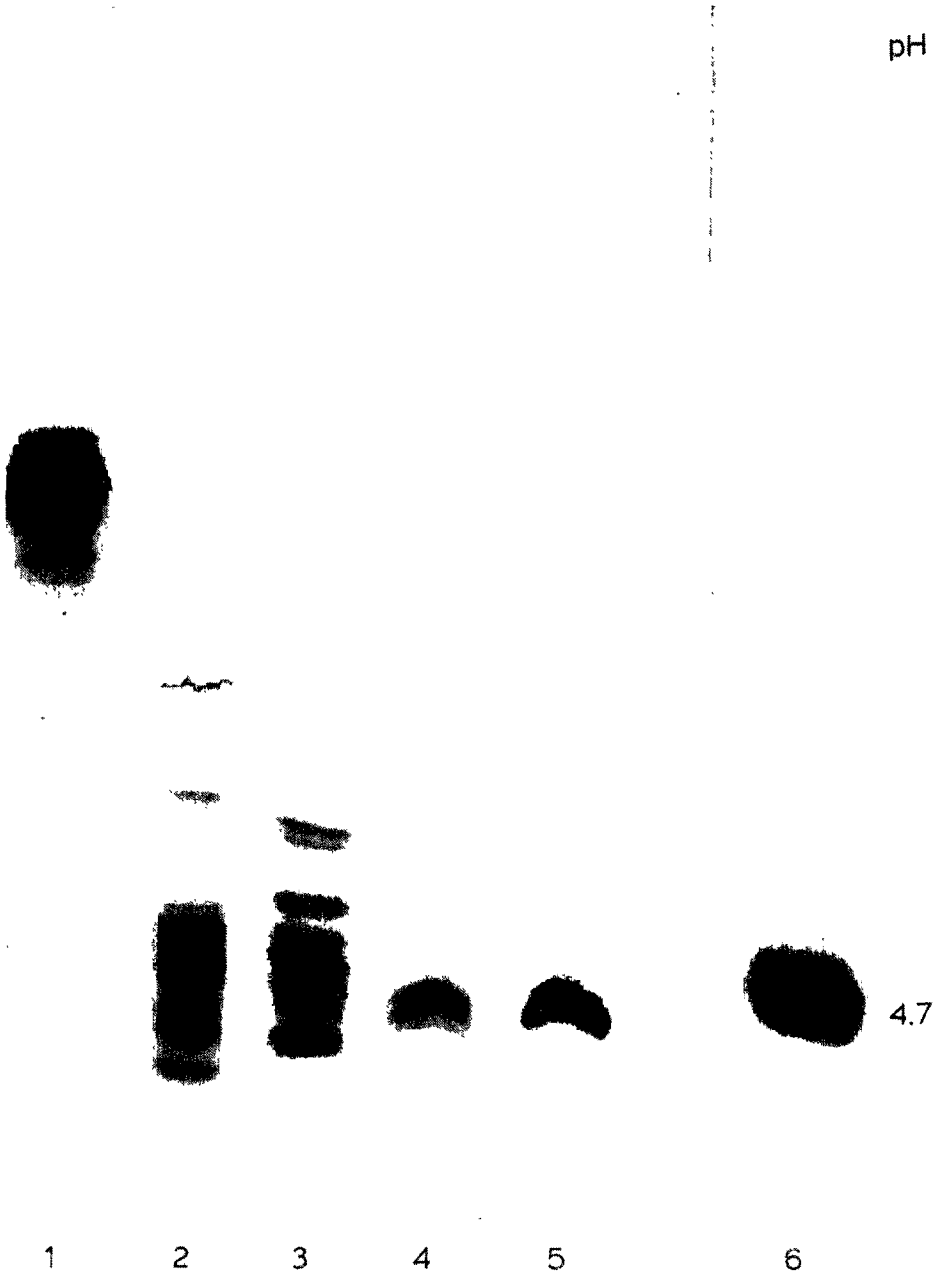

Fig. 4. Thin-layer, isoelectric focusing of *H. pomatia* β-D-mannosidase at various stages of purification: 1, haemoglobin standard; 2, crude solution; 3, enzyme recovered from DEAE-cellulose; 4, enzyme recovered from Ultrogel AcA 44; 5 and 6, β-D-mannosidase recovered from CM-cellulose chromatography. Samples 1–5 were stained for protein with Coomassie Brilliant Blue R 250, and sample 6 was stained for β-D-mannosidase activity with naphthyl β-D-mannopyranoside/Fast Blue BB (salt).

TABLE I

PURIFICATION OF β-D-MANNOSIDASE FROM *Helix pomatia* GUT-SOLUTION

Stage of purification	*Protein (mg)*	*Activity[a] (nkat)*	*Specific activity (nkat/mg)*	*Recovery (%)*	*Purification (-fold)*
Crude extract	800	8320	10.4	—	1
Dialysis (pH 8)	569	8250	14.5	99	1.4
DEAE-Cellulose (pH 6.5)	51.5	6060	117.7	73	11.3
Ultrogel AcA 44 (pH 5)	4.9	4837	987.1	58	94.9
CM-Cellulose (pH 4)	1.8	3049	1693.9	37	162.9

[a]Assayed with *p*-nitrophenyl β-D-mannopyranoside as substrate.

TABLE II

REMOVAL OF CONTAMINATING ACTIVITIES DURING THE PURIFICATION OF β-D-MANNOSIDASE

Stage of purification	*Level of enzyme activity[a]*		
	α-D-Galactosidase	*β-D-Mannanase*	*α-D-Mannosidase*
Crude preparation	5.1	505.4	3.9
DEAE-Cellulose	3.1×10^{-1}	11.5	0
Ultrogel AcA 44	4.5×10^{-2}	0	0
CM-Cellulose	$<1.0 \times 10^{-4}$	0	0

[a]Percentage of β-D-mannosidase activity.

TABLE III

PROPERTIES OF β-D-MANNOSIDASE

Property	*β-D-Mannosidase*
Molecular weight[a]	94,000 ±2,000
pI	4.7
Carbohydrate (%)	3.1
pH optimum	4.0
p-Nitrophenyl β-D-mannopyranoside	
V_{max} (40°, pH 4.5) (nkat/mg)	1694
K_m (mM)	1.43

[a]SDS gel-slab electrophoresis.

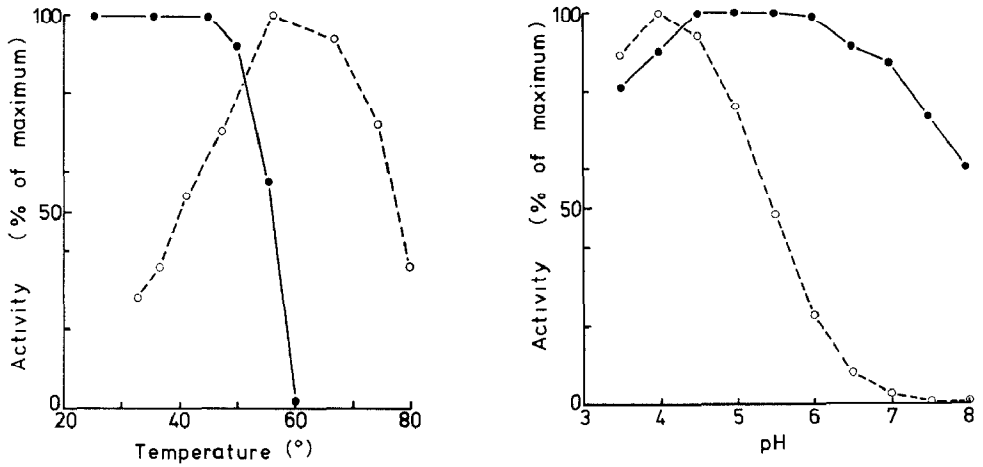

Fig. 5. Temperature stability (—●—) and activity (—○—) of *H. pomatia* β-D-mannosidase.

Fig. 6. pH Stability (—●—) and activity (—○—) of *H. pomatia* β-D-mannosidase.

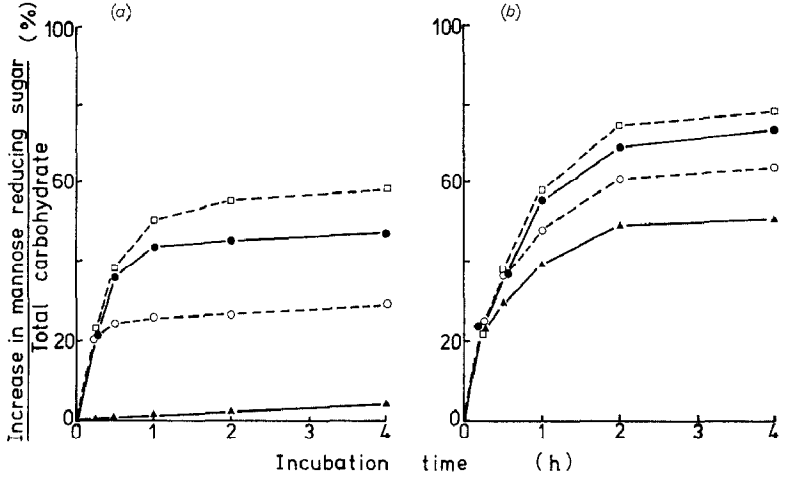

Fig. 7. Hydrolysis of (*a*) reduced β-D-manno-oligosaccharides (3.0 mg, 7.5 mg/mL) and (*b*) β-D-manno-oligosaccharides (3.0 mg, 7.5 mg/mL) by *H. pomatia* β-D-mannosidase (10.8 nkat for *p*-nitrophenyl β-D-mannopyranoside at 40°); d.p. 2 (—▲—), 3 (—○—), 4 (—●—), and 5 (—□—).

Similar compounds have been used in the purification of other glycoside hydrolases[29].

(*c*) *Mode of action.* The hydrolysis of β-D-manno-oligosaccharides and reduced β-D-manno-oligosaccharides is shown in Fig. 7. Values were reported as the increase in mannose as a percentage of total carbohydrate, to allow direct comparison to be made. Thus, the maximum theoretical values for reduced and non-reduced penta-, tetra-, tri-, and di-saccharides are 80, 75, 67, and 50%, respectively. Under the incubation conditions described in Fig. 7, the β-D-manno-oligosaccharides, but not the

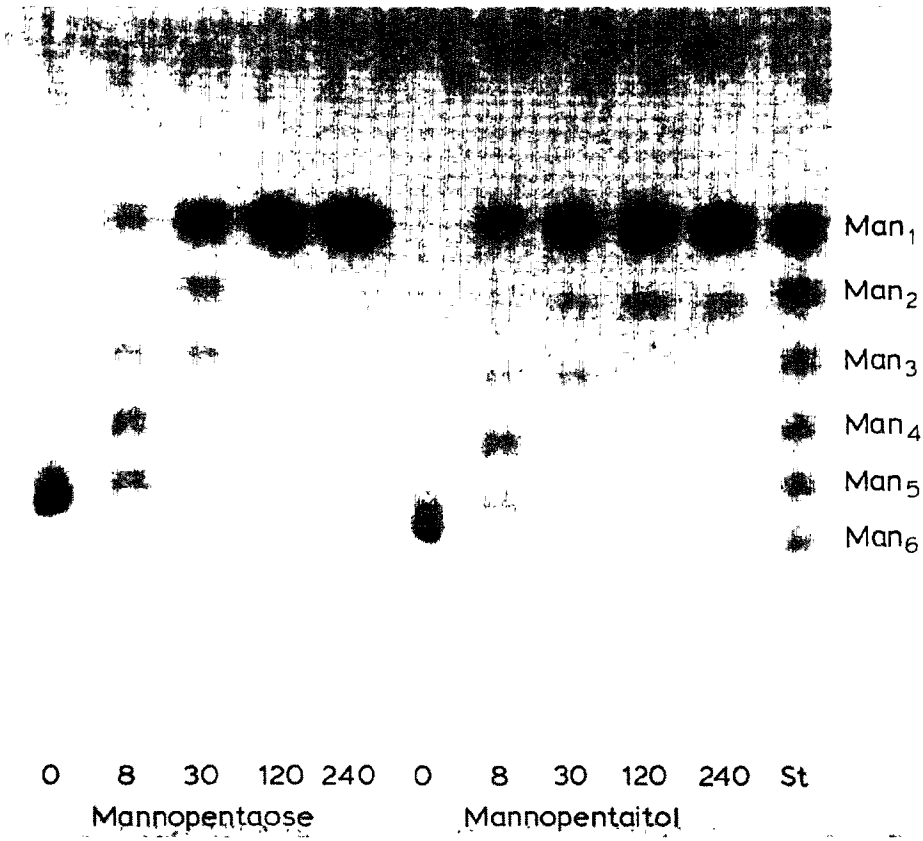

Fig. 8. T.l.c. of the hydrolysis products of mannopentaose and mannopentaitol by *H. pomatia* β-D-mannosidase. Incubation conditions are described in Fig. 7, and aliquots were removed at 0–240 min for chromatography; St, mannose–mannohexaose.

reduced forms, were essentially completely hydrolysed during 2 h (see Fig. 8). The relative initial rates of hydrolysis of β-D-manno-oligosaccharides of d.p. 2–5 were the same. The reduced and non-reduced oligosaccharides of d.p. 3–5 were hydrolysed at the same initial rate, but the hydrolysis rate for β-D-mannobi-itol was one-ninetieth of that for β-D-mannobiose.

The action pattern of *Helix pomatia* β-D-mannosidase was distinctly different from that of the exo-β-D-mannanase from guar seeds[23]. The guar enzyme hydrolysed β-D-mannopentaose at a rate ~8 times that of β-D-mannobiose and 5 times that of β-D-mannotriose. Consequently, hydrolysis of the pentasaccharide was marked by an accumulation of β-D-manno-oligosaccharides of lower d.p., and, on hydrolysis of β-D-mannopentaitol, β-D-mannotri-itol accumulated.

The effect of branch points was studied by comparing the rates of hydrolysis of β-D-mannobiose, β-D-mannotriose, 6^1-α-D-galactosyl-β-D-mannobiose, and 6^1-α-D-galactosyl-β-D-mannotriose[24] (see Figs. 9 and 10). Under the incubation conditions

described, β-D-mannobiose and β-D-mannotriose were rapidly and completely hydrolysed to D-mannose. 6^1-α-D-Galactosyl-β-D-mannotriose was rapidly hydrolysed to 6^1-α-D-galactosyl-β-D-mannobiose, which was cleaved at a rate ~4% of that of β-D-mannobiose. However, with an excess of enzyme, 6^1-α-D-galactosyl-β-D-mannobiose was completely hydrolysed to α-D-galactosyl-D-mannose and D-mannose[24]. In contrast, under similar conditions, the exo-β-D-mannanase from guar seeds had no effect on this oligosaccharide, although it readily hydrolysed 6^1-α-D-galactosyl-β-D-mannotriose to 6^1-α-D-galactosyl-β-D-mannobiose plus D-mannose. 6^3-α-D-Galactosyl-β-D-mannotetraose (1 mg, 5.0 mg/mL) was resistant to guar exo-β-D-mannanase (8.5 nkat on *p*-nitrophenyl β-D-mannopyranoside) during 20 h at 40° and pH 5.5, whereas, under the same incubation conditions, β-D-mannotetraose was hydrolysed

TABLE IV

KINETIC CONTANTS OF *H. pomatia* β-D-MANNOSIDASE

Substrate	K_m *(mM)*	V_{max} *(nkat/mg)*
p-Nitrophenyl β-D-mannopyranoside	1.43	1694
O-Nitrophenyl β-D-mannopyranoside	2.33	2536
Methylumbelliferyl β-D-mannopyranoside	0.91	842
Naphthyl β-D-mannopyranoside	3.22	1283
β-D-Mannobi-itol	—	—
β-D-Mannotri-itol	12.5	672
β-D-Mannotetraitol	12.5	642
β-D-Mannopentaitol	12.5	672
β-D-Mannohexaitol	12.5	662

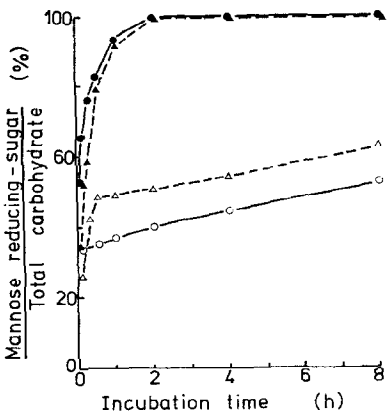

Fig. 9. Hydrolysis of β-D-mannobiose (—●—), β-D-mannotriose (—▲—), 6^1-α-D-galactosyl-β-D-mannobiose (—○—), and 6^1-α-D-galactosyl-β-D-mannotriose (—△—) (3.0 mg, 7.5 mg/mL) by *H. pomatia* β-D-mannosidase (12.1 nkat for *p*-nitrophenyl β-D-mannopyranoside at 40°).

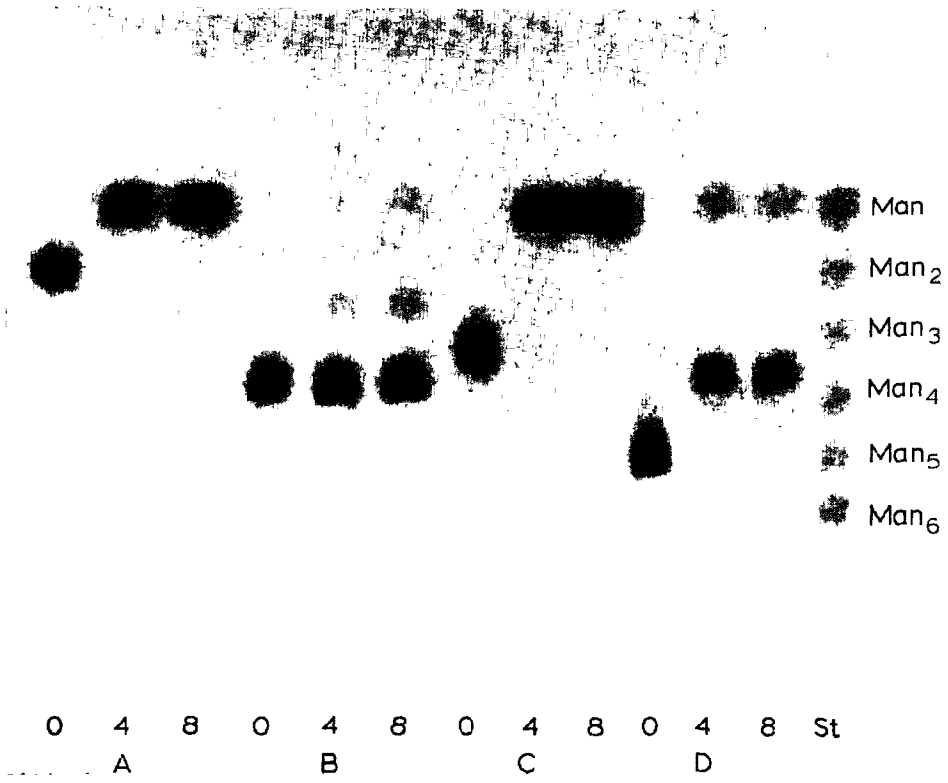

Fig. 10. T.l.c. of the hydrolysis products of A, β-D-mannobiose; B, 6^1-α-D-galactosyl-β-D-mannobiose; C, β-D-mannotriose; and D, 6^1-α-D-galactosyl-β-D-mannotriose by *H. pomatia* β-D-mannosidase. Incubation conditions are described in Fig. 9. Aliquots were removed at 0, 4, and 8 h; St, mannose-mannohexaose.

to a mixture of D-mannose and β-D-mannobiose. 6^3-α-D-Galactosyl-β-D-manno-tetraose was ~60% hydrolysed to 6^3-α-D-galactosyl-β-D-mannotriose and D-mannose by *H. pomatia* β-D-mannosidase (8.5 nkat on *p*-nitrophenyl β-D-mannopyranoside), and 6^1-α-D-galactosyl-β-D-mannobiose was hydrolysed at the same rate.

The kinetic constants for synthetic substrates and reduced β-D-manno-oligosaccharides are given in Table IV. The K_m and V_{max} values for reduced oligosaccharides of d.p. 3–6 were essentially identical. Hydrolysis of β-D-mannobi-itol was so slow that accurate kinetic data could not be obtained. *p*-Nitrophenyl, *o*-nitrophenyl, methylumbelliferyl, and naphthyl β-D-mannopyranosides were readily hydrolysed, and the enzyme had a greater affinity for these substrates than for the β-D-manno-oligosaccharides.

DISCUSSION

β-D-Mannosidase has found considerable use in characterising the structure of the core glycopeptides of glycoproteins and the oligosaccharides accumulated or

excreted in various storage disorders in animals[30]. The enzyme is also useful in the characterisation of the galactose-containing (1→4)-β-D-manno-oligosaccharides produced on enzymic or acid hydrolysis of legume-seed galactomannans[24] and has similar potential for the oligosaccharides produced on partial hydrolysis of glucomannans and galactoglucomannans. However, with few exceptions[19-22], the β-D-mannosidases used to date have been only partially purified and had quite low specific activities[5,6,14-16]. Reports on the substrate specificity and kinetics of β-D-mannosidase are very limited. The β-D-mannosidase partially purified from pineapple bromelain[6] readily hydrolysed the β-D-linked mannosyl residue in Man-$(GlcNAc)_2$-Asn, a core glycopeptide. The relative rates of hydrolysis of *p*-nitrophenyl β-D-mannopyranoside, core glycopeptide, methyl β-D-mannoside, 4-*O*-β-D-mannopyranosyl-D-mannitol, and 4-*O*-β-D-mannopyranosyl-D-glucopyranoside were ~60:20:1:1:1. The β-D-mannosidase from hen oviduct[16] also hydrolysed *p*-nitrophenyl β-D-mannopyranoside at a rate ~3 times that of Man-$(GlcNAc)_2$-Asn and Man-$(GlcNAc)_2$. A β-D-mannosidase, obtained in a highly purified form from *Polyporus sulfureus*[19], readily hydrolysed 2-acetamido-2-deoxy-4-*O*-β-D-mannopyranosyl-D-glucose, 4-*O*-β-D-mannopyranosyl-D-galactopyranose, β-D-mannobiose, and β-D-mannotriose, but the relative rates were not reported. A purified β-D-mannosidase from *Aspergillus niger*[21] hydrolysed β-D-mannotriose, β-D-mannotri-itol, β-D-mannobiose, and β-D-mannobi-itol at the relative initial rates of 100:68:22:1.4. Partially degraded and native guar and carob galactomannans were slowly hydrolysed, but this may have been due to the separate or combined action of a contaminating α-D-galactosidase. In separate studies[22] of *A. niger* β-D-mannosidase, α-D-galactosidase activity could only be completely removed by specific affinity-binding to columns of *N*-ε-aminocaproyl-α-D-galactopyranosylamine–Sepharose 4B.

Results obtained by other workers clearly demonstrate the marked effect of the aglycon group on the susceptibility of the (1→4)-linked β-D-mannosyl residues to hydrolysis by β-D-mannosidases. Similar results were obtained in the studies reported here. Conversion of β-D-mannobiose into the sugar alcohol resulted in a 90-fold decrease in the susceptibility to hydrolysis; similar results have been reported[13,21]. The susceptibility of the (1→4)-β-D-linkage in β-D-mannobiose to hydrolysis is also significantly affected by substitution at either position 6 by α-D-galactopyranose. 6^1-α-D-Galactosyl-β-D-mannobiose was hydrolysed at a rate only 4% of that of β-D-mannobiose (Fig. 9). However, in the presence of sufficient enzyme, quantitative hydrolysis to 6-*O*-α-D-galactosyl-D-mannopyranose and D-mannose occurred[24]. 6^2-α-D-Galactosyl-β-D-mannobiose was totally resistant to the β-D-mannosidase. β-D-Mannobiose was hydrolysed at the same initial rate as β-D-mannotetraose, and substitution of either oligosaccharide by an α-linked D-galactose at position 6 on the second mannose from the non-reducing end, resulted in a 25-fold decrease in the initial rate of hydrolysis.

The relative rates of hydrolysis of β-D-manno-oligosaccharides by the β-D-mannosidase differed significantly from those reported for the β-D-mannosidases from *Rhizopus niveus*[18] and *Tremella fuciformis*[20]. With the latter two enzymes,

β-D-mannobiose was hydrolysed more rapidly than β-D-mannotriose and β-D-mannotetraose. However, in each case, the K_m and V_{max} values for these substrates were not determined; for the *Rhizopus niveus* enzyme[18], the level of substrate employed was undoubtedly sub-saturating.

The action pattern of *Helix pomatia* β-D-mannosidase was quite different from that[23] of an exo-β-D-mannanase from guar seeds. The latter enzyme readily hydrolysed a range of natural and synthetic substrates, but the preferred substrates were the β-D-manno-oligosaccharides of d.p. $\geqslant 5$. β-D-Mannotri-itol was resistant to the guar enzyme, but was hydrolysed at the same rate as β-D-mannohexaitol by *Helix pomatia* β-D-mannosidase. Also, the latter enzyme had the same K_m value (12.5mM) for β-D-mannotri-itol and β-D-mannohexaitol; for guar exo-β-D-mannanase, these values were 80.0 and 2.8mM respectively. With the synthetic substrates studied, *Helix pomatia* β-D-mannosidase had the lowest K_m with methylumbelliferyl β-D-mannopyranoside and the highest V_{max} with *o*-nitrophenyl β-D-mannopyranoside. Similar results were obtained with guar exo-β-D-mannanase.

Thus, commercially available, gut solution of *Helix pomatia* is a very useful source of β-D-mannosidase. The levels of activity in the crude solution are very high (832 nkat/mL at 40°) and the enzyme can be obtained readily in an almost homogeneous form (specific activity, 1694 nkat/mg) by three simple chromatographic steps.

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