Partial purification of goat kidney β -mannosidase

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1. Goat kidney β -mannosidase was purified 8500-fold to a specific activity of 65000 nmol/h per mg of protein with a 6% yield by using multiple steps including cation-exchange and anion-exchange fast protein liquid chromatography. This is the first description of a highly purified preparation from goat tissue; however, it was not homogeneous, as judged by silver-stained SDS/polyacrylamide-gel electrophoresis.
2. The enzyme exhibited microheterogeneity when analysed by isoelectric focusing (pI 5.5–6.5).
3. Purified β -mannosidase hydrolysed the terminal β -(1→4)-linkage of oligosaccharides that accumulate in β -mannosidosis.

INTRODUCTION

Lysosomal β -D-mannosidase (EC 3.2.1.25) hydrolyses β -mannopyranoside-*N*-acetylglucosamine linkages in the core structure of the *N*-linked oligosaccharides of glycopeptides and glycoproteins. Genetic disorders associated with deficient activity of lysosomal β -mannosidase have been described in goats [1–4] and recently in humans [5,6]. Caprine β -mannosidosis is a well-documented, lethal, lysosomal storage disorder [1,3,7–12]. Affected goats are characterized by severe neurological deficits associated with myelin abnormalities and accumulation of the disaccharide Man β 1-4GlcNAc and the trisaccharide Man β 1-4GlcNAc β 1-4GlcNAc in tissues and urine [3,7,9,10]. A mixture of two tetrasaccharides and the pentasaccharide Man β 1-4GlcNAc β 1-4Man β 1-4GlcNAc β 1-4GlcNAc accumulate to a lesser extent [12].

β -Mannosidase has been partially purified and incompletely characterized from several mammalian sources [13–18], and the reported yield, stability and activity are low in sources other than guinea-pig liver, from which the enzyme was purified to electrophoretic homogeneity [19].

We have undertaken the purification and characterization of lysosomal β -mannosidase from normal goat kidney to make possible further biochemical and immunological studies of the defective enzyme in the disease state. Kidney tissue was chosen for study because it is free of a separate, non-lysosomal, β -mannosidase activity [4,14]. A purification scheme for lysosomal β -mannosidase, incorporating f.p.l.c., is described. Selected physical and kinetic properties are also presented. This is the first report of a procedure yielding highly purified lysosomal β -mannosidase from goat tissue. Preliminary results were presented previously [18].

EXPERIMENTAL

Materials

Goat kidney tissue was stored at -20°C . ConA–Sephadex, Sephadex G-150, methyl α -D-mannopyranoside

(grade III), 4-methylumbelliferyl β -D-mannopyranoside, 4-nitrophenyl β -D-mannopyranoside, ammonium persulphate, BSA (fraction V, 96–99% albumin, and fraction V, 98–99% albumin) and M_r standards for SDS/PAGE (MW-SDS-200) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Acrylamide, *NN*'-methylenebisacrylamide and SDS were from Bio-Rad Laboratories, Richmond, CA, U.S.A. Leupeptin and pepstatin were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A. Ultrafiltration membranes were purchased from Amicon, Danvers, MA, U.S.A. Ampholines were obtained from LKB Instruments, Gaithersburg, MD, U.S.A. Whatman Linear K High Performance TLC plates (10 cm $\times$ 10 cm) for t.l.c. were obtained from the Anspec Co. Ann Arbor, MI, U.S.A. The Gelcode Silver Staining System was purchased from Pierce Chemical Co., Rockford, IL, U.S.A. Other reagents and chemicals were analytical grade. In substrate specificity studies, Man α 1-4Man β 1-4GlcNAc, Man β 1-3GlcNAc and Man β 1-6GlcNAc were generously given by Chris Warren, Massachusetts General Hospital, Boston, MA, U.S.A. Other β -linked oligosaccharides were isolated from β -mannosidosis tissues as previously described [9,10,12].

Enzyme assay

β -Mannosidase activity was determined at pH 5.0 with 4-methylumbelliferyl β -D-mannopyranoside as previously described [3], except that the substrate solution contained 0.2 mg of BSA (fraction V, 96–99%)/ml. One unit of enzyme activity was defined as that amount of enzyme which hydrolysed 1 nmol of substrate/h at 37°C . Activity with *p*-nitrophenyl β -D-mannopyranoside was determined as described previously [1].

Protein determinations

Protein concentration was determined according to the method of Bradford [20], with BSA (fraction V, 96–99%) as the standard. Protein eluted from columns was monitored by absorbance at 280 nm.

Abbreviations used: ConA, concanavalin A; BSA, bovine serum albumin; f.p.l.c., fast protein liquid chromatography; PAGE, polyacrylamide-gel electrophoresis.

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Electrophoresis

The method of O'Farrell [21] was used for SDS/PAGE analysis. The stacking and separating gels (1.5 mm) had polyacrylamide concentrations of 4% and 10% respectively. The gels were run at 40 V for 14–16 h and stained by using the Gelcode Silver Staining System. Flat-bed isoelectric focusing was performed for 4800 V·h at 4 °C over pH ranges of either 4–7 or 5–7 in 5% polyacrylamide gels. Immediately after focusing, the pH across the gel was determined with a surface electrode. Bands of enzyme activity were located after incubation at 42 °C for 30–60 min, as described previously [16].

Substrate specificity

Substrate-specificity studies were performed by incubating the various substrates with highly purified β -mannosidase in 0.1 M-sodium acetate buffer, pH 5, containing 1 mg of BSA (fraction V, 98–99%)/ml for 36 h. BSA (fraction V, 98–99%) was required because lower grades contained endo-*N*-acetyl- β -glucoaminidase as a contaminant. Final evaluation of reaction products was performed by t.l.c. with butanol/acetic acid/butyl acetate/water (27:27:17:28, by vol.) as developing solvent with orcinol/H₂SO₄ reagent used for detection of hexose-containing compounds [9,10].

Determination of kinetic parameters

Kinetic parameters with 4-methylumbelliferyl β -D-mannopyranoside (0.1–3.9 mM) and *p*-nitrophenyl β -D-mannopyranoside in the range 0.7–21.0 mM were determined by the Lineweaver–Burk method by using a weighted non-linear-regression method [22].

F.p.l.c.

A system including Pharmacia's gradient programmer GP-250, two P-500 pumps, a UV-1 single-path monitor and Mono S HR 10/10 (cation-exchange) and Mono Q HR 5/5 (anion-exchange) columns was used for f.p.l.c. The solutions were prepared with triple-deionized water, filtered with a 0.22 μ m-pore-size filter and degassed under vacuum before use.

High-speed supernatant preparation

Thawed goat kidney tissue (80–100 g) was minced and homogenized with a Waring blender (3 × 30 s) in 1 vol. of buffer A [50 mM-NaCl/1 mM-MnCl₂/1 mM-MgCl₂/10% (v/v) glycerol/10 mM-sodium citrate buffer, pH 5.5] containing 0.2 mg of leupeptin/l and 0.7 mg of pepstatin/l. The crude homogenate was centrifuged (38000 *g* for 30 min) and the supernatant was kept. The pellet was then resuspended twice in buffer A containing proteinase inhibitors and centrifuged as described above, and the three supernatant pools were combined. All procedures were carried out at 4 °C except for the ultrafiltration, ConA–Sephacrose chromatography and f.p.l.c., which were conducted at room temperature. All buffers contained 0.02% NaN₃ except those used for f.p.l.c.

Concentration

Selected column fractions were concentrated by ultrafiltration in an Amicon stirred cell with either a PM-10 or a PM-30 membrane.

RESULTS

Good recovery and storage stability for goat kidney β -mannosidase was obtained at pH 5.5 with 10% glycerol. For example, 90% recovery was obtained after 2 months storage at 4 °C in 50 mM-NaCl/10% (v/v) glycerol/10 mM-sodium citrate buffer, pH 5.5. Thus the reported β -mannosidase instability [15,16] that has hampered enzyme purification was significantly diminished.

Lysosomal β -mannosidase purification

The high-speed supernatant was heated at 50 °C for 10 min and occasionally stirred. The suspension was centrifuged (38000 *g* for 30 min), and (NH₄)₂SO₄ was slowly added to the supernatant to a concentration of 50% saturation. The suspension was centrifuged (38000 *g* for 20 min) after stirring on ice for 1 h. The pellets were resuspended in buffer A containing proteinase inhibitors, pooled and stored at –20 °C. Several tissue preparations, after (NH₄)₂SO₄ fractionation, were pooled to ensure sufficient enzyme activity at later stages. The dissolved (NH₄)₂SO₄-precipitated pellets were centrifuged (38000 *g* for 20 min) and the supernatant was mixed with ConA–Sephacrose (10–15 mg of protein/ml of gel) previously equilibrated in buffer A. After 2 h, the suspension was poured into a column and washed with buffer A until the absorbance of the eluate at 280 nm was less than 0.05. The bound glycoproteins, including lysosomal β -mannosidase, were then eluted with 1.0 M-methyl α -D-mannopyranoside in buffer A containing 0.55 M-NaCl. Fractions (5.8 ml) containing most of the enzyme activity were pooled, concentrated to about

Fig. 1. Gel filtration of β -mannosidase

Fractions from ConA–Sephacrose chromatography, after concentration to 10 ml, were applied to a Sephadex G-150 column (120 cm × 4.5 cm) equilibrated with buffer B. Fractions (5.6 ml) were monitored for absorbance at 280 nm (●), and samples were assayed for β -mannosidase activity (▲). Pooled fractions are indicated by a bar.

Fig. 2. F.p.l.c. cation-exchange chromatography of β -mannosidase

Concentrated fractions from the Sephadex G-150 gel filtration were chromatographed on a Mono S HR 10/10 column equilibrated in buffer B. Enzyme ($\blacktriangle$) was eluted with a multi-step NaCl gradient (-----). The flow rate was 4 ml/min. Fractions (3 ml) were monitored for absorbance at 280 nm (-----). Pooled fractions are indicated by a bar.

10 ml and applied to a Sephadex G-150 column (120 cm $\times$ 4.5 cm) equilibrated with buffer B [50 mM-NaCl/10% (v/v) glycerol/10 mM-sodium citrate buffer, pH 5.5]. Enzyme was eluted (Fig. 1) at a flow rate of 20 ml/h. Peak fractions (5.6 ml) were pooled, concentrated to about 8 ml and then chromatographed on a Mono S HR 10/10 column equilibrated with buffer B. With a multi-step NaCl gradient at a flow rate of 4.0 ml/min, β -mannosidase activity was eluted at 0.25 M-NaCl, as a broad non-symmetrical peak (Fig. 2). Peak fractions (3.0 ml) were pooled, dialysed for 12–14 h against buffer C [20% (v/v) glycerol/20 mM-Tris/HCl buffer, pH 7.5] and then chromatographed on a Mono Q HR 5/5 column equilibrated with buffer C. With a linear NaCl gradient at a flow rate of 0.5 ml/min, β -mannosidase activity was eluted at 80 mM-NaCl as a single peak (Fig. 3). The peak fractions (0.5 ml) from this final chromatography step were pooled before further analyses. Multiple bands were detected in samples of the pooled fractions after SDS/PAGE (results not shown).

Fig. 3. F.p.l.c. anion-exchange chromatography of β -mannosidase

Peak fractions from the f.p.l.c. cation-exchange chromatography were dialysed against buffer C and then applied to a Mono Q HR 5/5 column equilibrated with the same buffer. β -Mannosidase activity ($\blacktriangle$) was eluted with a linear NaCl gradient (-----). The flow rate was 0.5 ml/min. Fractions (0.5 ml) were monitored for absorbance at 280 nm (-----). Pooled fractions are indicated by a bar.

The highly purified lysosomal β -mannosidase was purified 8500-fold, to a specific activity of 65000 units/mg of protein, with a 6% yield (Table 1).

Isoelectric point

A broad band of β -mannosidase activity over the range pH 5.5–6.5 was observed after isoelectric focusing, with the most prominent activity at pH 5.5 and 6.1 (results not shown).

Substrate specificity

Lysosomal β -mannosidase was able to hydrolyse the pentasaccharide Man β 1-4GlcNAc β 1-4Man β 1-4GlcNAc β 1-4GlcNAc to mannose and the tetra-saccharide GlcNAc β 1-4Man β 1-4GlcNAc β 1-4GlcNAc,

Table 1. Purification of goat kidney β -mannosidase

For full experimental details see the text. Activity was monitored as described with 4-methylumbelliferyl β -D-mannopyranoside (1.8 mM) as substrate.

	Activity (units)	Protein (mg)	Sp. activity (units/mg of protein)	Yield (%)	Purification (fold)
High-speed supernatant (38000 g, 30 min)	211000	27600	7.6	100	1.0
Heat fractionation (50 °C, 10 min)	172000	16900	10.2	82	1.3
0–50%-satn. (NH ₄) ₂ SO ₄ fractionation	126400	7200	17.5	60	2.3
ConA–Sephadex chromatography	101000	216	470	48	62
Sephadex G-150 chromatography	63400	32.9	1900	30	290
F.p.l.c.: Mono S chromatography	36400	2.17	17000	17	2200
F.p.l.c.: Mono Q chromatography	13000	0.20	65000	6	8500

Fig. 4. T.L.C. of β -mannosidase-hydrolysis reaction products

All incubations were in 0.1 M-sodium acetate buffer, pH 5.0, containing 0.1% BSA, and containing 100 units of purified goat kidney β -mannosidase and 20 μ g of natural substrates isolated from β -mannosidosis goat kidney tissues. Plates (Whatman LHP-K) were developed in butanol/acetic acid/butyl acetate/water (27:27:17:28, by vol.) and developed with orcinol/ H_2SO_4 reagent. Lane 1, oligomer; lane 2, standards (mannose, disaccharide, trisaccharide, pentasaccharide); lane 3, disaccharide incubated with enzyme; lane 4, disaccharide incubated without enzyme; lane 5, trisaccharide incubated with enzyme; lane 6, trisaccharide incubated without enzyme; lane 7, pentasaccharide incubated with enzyme; lane 8, pentasaccharide incubated without enzyme.

on the basis of the results in Fig. 4. The enzyme was reactive towards the β -(1 $\rightarrow$ 4)-linked trisaccharide but was weakly reactive towards the β -(1 $\rightarrow$ 4)-linked disaccharide (Fig. 4). Goat kidney β -mannosidase failed to hydrolyse the internal β -(1 $\rightarrow$ 4)-linkage of Man α 1-4Man β 1-4GlcNAc or Man β 1-3GlcNAc but was weakly reactive towards Man β 1-6GlcNAc (results not shown).

Kinetic parameters

The apparent K_m for 4-methylumbelliferyl β -D-mannopyranoside was 0.8 mM and that for *p*-nitrophenyl β -D-mannopyranoside was 9.0 mM. The ratio of V_{max} values for the two substrates was 2.25, with the *p*-nitrophenyl substrate providing greater activity.

DISCUSSION

A protocol including sequential ConA-Sephadex, Sephadex G-150, and f.p.l.c. cation-exchange and anion-exchange chromatographies was used to prepare a highly purified goat kidney lysosomal β -mannosidase. This protocol produced a specific activity of 65000 units/mg of protein with a 6% yield and an 8500-fold purification. Despite this high degree of purity, the enzyme was not homogeneous, as assessed by silver-stained SDS/PAGE (results not shown). Some of the observed bands may arise from multiple forms of the enzyme that are associated with different stages of enzyme processing or degrees of proteolysis, since isoelectric focusing showed a broad band of β -mannosidase activity in the pH range 5.5–6.5. Pearce *et al.* [4] also found multiple isoelectric points for goat β -mannosidase. Likewise, the broad skewed peak of β -mannosidase activity from f.p.l.c. cation-exchange suggests that multiple enzyme forms are

present in the enzyme preparations. Other bands in the SDS/PAGE gel may be caused by a variation in charge, because of an uneven uptake of SDS by the enzyme as is often seen with glycoproteins [23]. Nevertheless, other bands were derived from contaminants that did not possess β -mannosidase activity.

Substrate-specificity studies suggest that goat kidney β -mannosidase was more reactive towards the larger oligosaccharides (i.e. penta- and tri-saccharides) than to the disaccharide (Fig. 4). Rat, human and goat liver lysosomal β -mannosidases give similar results [13,16,24]. β -Mannosidase was unreactive towards the β -(1 $\rightarrow$ 3)-linked disaccharide as well as an internal β -(1 $\rightarrow$ 4)-linkage, but the enzyme did hydrolyse the β -(1 $\rightarrow$ 6)-linked disaccharide. These substrate-specificity characteristics are consistent with the criteria for an 'inverting' glycosidase [16,25].

The apparent K_m was 0.8 mM with the synthetic substrate 4-methylumbelliferyl β -D-mannopyranoside and 9.0 mM for *p*-nitrophenyl β -D-mannopyranoside. In comparison, the K_m of the human serum enzyme with the *p*-nitrophenyl substrate was 2.2 mM [17], and the guinea-pig liver enzyme demonstrated non-Michaelis-Menten kinetics [19].

Recently, β -mannosidase was purified from guinea-pig liver by using ConA-Sephadex, Sephadex G-200, GlcNAc-agarose and conventional anion-exchange and cation-exchange chromatographies [19]. An 8000-fold purification with a 28% yield was obtained, and it was judged to be homogeneous on the basis of SDS/PAGE results. When compared, the guinea-pig liver and goat kidney enzymes appear to differ in their properties. For example, rabbit antibody to guinea-pig liver β -mannosidase is unreactive towards human and goat β -mannosidases in immunoinhibition studies, indicating immunological non-identity between guinea-pig liver and the goat kidney enzymes [26].

Therefore the present protocol, with the use of f.p.l.c. cation-exchange and anion-exchange chromatographies, to prepare β -mannosidase is unique, and it is the first report of a highly purified lysosomal β -mannosidase from goat tissue.

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