Bovine kidney β -mannosidase: purification and characterization

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Lysosomal β -mannosidase was purified 160000-fold in 24% yield from bovine kidney by a four-step purification procedure, which included concanavalin A-Sepharose, immunoaffinity, TSK-butyl and h.p.l.c. cation-exchange chromatography. When analysed by SDS/PAGE and detected by Coomassie Blue or silver staining, the purified enzyme preparation consists of two prominent peptides (100 and 110 kDa) and a third minor peptide (84 kDa). These three peptides are immunologically related

and are consistently associated with β -mannosidase activity in all chromatographic steps. Removal of N-linked carbohydrate from the 84, 100 and 110 kDa peptides decreases their molecular sizes to 75, 86 and 91 kDa respectively. Bovine kidneys lacking β -mannosidase activity, acquired from calves affected with β -mannosidosis, do not contain detectable quantities of the three β -mannosidase peptides, as judged by monoclonal- and polyclonal-antibody reactivity.

INTRODUCTION

At least five different lysosomal glycosidases act in a progressive manner to degrade asparagine-linked oligosaccharides from their non-reducing end. The absence of any one of these glycosidases results in the storage and excretion of the undegraded substrate(s). Genetic disorders associated with β -mannosidase deficiency (β -mannosidosis) have been described in Nubian goats [1–4], Salers cattle [5–7] and humans [8–12]. The disaccharide Man β 1-4GlcNAc, the trisaccharide Man β 1-4GlcNAc β 1-4GlcNAc and other more complex oligosaccharides accumulate in the tissues and urine of affected goats and calves [13–18]. In humans, the disaccharide Man β 1-4GlcNAc is the major storage and excretion product [9,19–21].

The clinical and pathological abnormalities of calves and goats with β -mannosidosis are extremely similar [1,5–7,13,17,22–25], with one noted exception. Calves, unlike goats, respond to loud noises and have intact auditory brainstem-evoked potentials [26]. Goats and calves with β -mannosidase deficiency are most notably characterized by severe neurological deficits associated with myelin abnormalities [27–30]. Without intensive care after birth, affected calves and goats die within the first 1–2 days of the neonatal period. In contrast, clinical manifestations of β -mannosidosis in humans can be mild, and in the cases reported thus far there is considerable clinical heterogeneity [12]. In general, affected individuals are characterized by mental retardation, deafness and in older patients angiokeratoma.

β -Mannosidase has been partially purified and incompletely characterized from several mammalian sources [31–37]. The reported molecular mass of the enzyme, as estimated by a variety of techniques, ranges from 80 to 120 kDa.

β -Mannosidase has been purified from guinea-pig liver [38] and goat kidney [39]. The enzyme from guinea-pig liver is a monomer of 110 kDa. In goat kidney, β -mannosidase activity is associated with two related peptides of 100 and 90 kDa. These two peptides are absent in kidneys from β -mannosidosis affected goats.

To overcome difficulties associated with the structural characterization of this low-abundance enzyme from a limited tissue source (goat kidney), we have undertaken the purification

and characterization of bovine β -mannosidase from normal kidney. To characterize bovine β -mannosidosis further, monoclonal and polyclonal antibodies were used to analyse β -mannosidase-deficient kidneys.

MATERIALS AND METHODS

Materials

Kidneys from mature cattle were obtained from Ada Beef Co., Ada, MI, U.S.A. Kidneys from calves with β -mannosidosis were acquired from Bruce Abbott at the Texas Veterinary Medical Diagnostic Laboratory, College Station, TX, U.S.A. All tissues were stored at -20°C or -80°C until needed. Concanavalin A (Con A)-Sepharose 4B (10–15 mg of ligand/ml), 4-methylumbelliferyl β -D-mannopyranoside, and methyl- α -glucoside were from Sigma (St. Louis, MO, U.S.A.). TSK-butyl resin was from Pharmacia (Uppsala, Sweden), and Endoglycosidase H and recombinant N-Glycosidase F were from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). All electrophoresis reagents were from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Enzyme assay

β -Mannosidase activity was assayed at pH 5.0 with 4-methylumbelliferyl β -D-mannopyranoside as previously described [3]. One unit of enzyme activity was defined as that amount of enzyme which hydrolysed 1 μmol of substrate/h at 37°C .

Protein determinations

Protein concentrations were measured by using bicinchoninic acid (Pierce, Rockford, IL, U.S.A.), with BSA as a standard [40].

Preparative-scale purification of β -mannosidase

β -Mannosidase was purified from bovine kidney as described for the goat enzyme [39] by immunoaffinity chromatography using the anti-(goat β -mannosidase) monoclonal antibody 43F10S. In brief, a crude homogenate was prepared, bound to Con A-Sepharose, and eluted with methyl α -glucoside. The pooled

Table 1 Summary of the purification of β -mannosidase from normal bovine kidney

β -Mannosidase was isolated from 15 kg of bovine kidney. Similar results were obtained with two independent experiments.

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	Yield (%)	Purification (fold)
High-speed supernatant	18 700	1 460 000	0.0128	100	1
Con A-Sepharose chromatography	10 000	6050	1.65	53	129
Immunoaffinity chromatography	6000	7.0	860	32	67 000
TSK-butyl Chromatography	4700	3.3	1400	25	110 000
H.p.l.c.: Mono S chromatography	4500	2.3	2000	24	160 000

enzyme was precipitated with saturated $(\text{NH}_4)_2\text{SO}_4$, extracted with CCl_4 and applied to the immunoaffinity column. The immunoaffinity-purified protein was chromatographed on the TSK-butyl resin with a $(\text{NH}_4)_2\text{SO}_4$ gradient (1.25–0 M). The fractions with activity were pooled and then subjected to h.p.l.c. cation-exchange chromatography (Mono S) as described [35,39].

Analytical-scale purification of β -mannosidase

Kidneys from normal and affected calves (60 g each) were subjected to a two-step purification procedure (Con A-Sepharose followed by immunoaffinity purification) as described for kidneys from normal and β -mannosidase-deficient goats [39].

SDS/PAGE analysis

Gel electrophoresis, gel staining, transfers and Western analysis were performed by established protocols [41]. The SDS/PAGE sample buffer contained 2.5% (v/v) 2-mercaptoethanol. The second antibodies used in the Western analysis were alkaline phosphatase-conjugated goat anti-rabbit IgG.

Production of polyclonal antiserum

Anti-(bovine β -mannosidase) antiserum was produced as described for the goat enzyme [39], with one modification. Before preparative SDS/PAGE, the pooled enzyme fractions from Mono S chromatography (1 mg) were digested with recombinant N-Glycosidase F as described previously [39]. Removal of the carbohydrate enhanced the resolution of the peptide of interest. One rabbit received three 50 μg injections of the SDS/PAGE-purified and deglycosylated 100 kDa peptide (86 kDa after deglycosylation). This antiserum is referred to throughout the text as anti- β -mannosidase antiserum.

Anti-80 rabbit serum, generated against the 80 kDa goat peptide [39], was used in these studies to characterize the corresponding bovine peptide.

RESULTS

Purification of bovine β -mannosidase

An immunoaffinity purification procedure developed to purify goat kidney β -mannosidase [39] was used to prepare mg quantities of purified bovine β -mannosidase. The procedure, summarized in Table 1, has produced an enzyme preparation with a specific activity of 2000 units/mg of protein with a 160 000-fold purification in 24% yield.

The results obtained in the final Mono S purification step are shown in Fig. 1. Most of the protein and enzyme activity were

eluted as coincident peaks from the Mono S column (Figure 1a). Fractions from the activity peak were assessed by Coomassie Blue-stained SDS/PAGE (Figure 1b). Two peptides of 100 and 110 kDa predominate, and a smaller amount of an 84 kDa peptide is also present.

The relationship of the 84, 100 and 110 kDa peptides was determined by Western analysis. Rabbit antiserum raised against deglycosylated gel-purified 100 kDa peptide was used to probe an immunoblot of the Mono S pool (Figure 2a). Before deglycosylation (Figure 2a, lane 2) and after deglycosylation (Figure 2a, lane 3) all three of the peptides in the Mono S pool reacted with the anti- β -mannosidase antiserum. All of the anti- β -mannosidase immunoreactive peptides are consistently associated with β -mannosidase activity during chromatography (size exclusion, hydrophobic interaction) as well as cation-exchange.

An approx. 80 kDa peptide co-eluted with β -mannosidase in the immunoaffinity step during enzyme purification from both bovine and goat kidneys. This 80 kDa peptide was relatively abundant in the Con A-Sepharose pool, but only a small percentage (< 5%) was retained in the immunoaffinity column (results not shown). Little or no 80 kDa peptide was detected in the final Mono S purification step for the bovine enzyme, as demonstrated by probing an immunoblot of the Mono S pool with anti-80 serum (Figure 2b, lanes 2 and 3). To assess the specificity of these antisera, 5 μg of Con A-Sepharose pool was subjected to Western-blot analysis and probed with the anti-80 serum (Figure 2b, lane 1) and the anti- β -mannosidase serum (Figure 2a, lane 1). The anti- β -mannosidase serum shows no reactivity with the abundant 80 kDa peptide (Figure 2a, lane 1).

Carbohydrate composition of bovine β -mannosidase

To assess the amount and type of N-linked carbohydrate attached to the purified bovine peptides, the TSK pool was digested with Endoglycosidase H and N-Glycosidase F (Figure 3). Endoglycosidase H, which removes the high-mannose N-linked oligosaccharides from glycoproteins, lowered the molecular mass of the 84 and 100 kDa peptides to 82 and 96 kDa (Figure 3, lane 3). The 110 kDa peptide was decreased to a slightly smaller heterogeneous peptide. N-Glycosidase F, which removes all N-linked oligosaccharides from glycoproteins, decreased the molecular size of these peptides to 75, 86 and 91 kDa respectively (Figure 3, lane 1). Additional deglycosylation experiments using protein eluted early in Mono S chromatography (primarily 100 and 110 kDa) and late (primarily 100 kDa) resulted in similar molecular-mass assignments (results not shown). The oligosaccharide composition of bovine β -mannosidase appears to be

Figure 1 H.p.l.c. cation-exchange chromatography of bovine β -mannosidase

β -Mannosidase activity (6.7×10^3 units) from the TSK-butyl procedure (see Table 1) was pooled and dialysed into 10 mM citrate/50 mM NaCl/10% glycerol buffer, pH 5.5. The dialysed sample was subjected to Mono S chromatography (a). The enzyme was eluted with a multi-step NaCl gradient (0.05–1 M NaCl). Protein was monitored at 280 nm in a 10 mm flow cell. Activity is expressed in total units/1 ml fraction. Samples (25 μ l) from selected fractions were subjected to SDS/PAGE analysis (b). Protein bands were made visible by Coomassie Blue staining. The positions of molecular-mass standards (in kDa) are indicated on the right of panel (b).

primarily of the complex type, although 1–2 high-mannose residues are apparently present.

Analytical-scale purification of β -mannosidase from normal and affected tissues

To characterize the genetic defect in calves affected with β -mannosidosis, kidney from affected and control animals was subjected to Con A–Sepharose and immunoaffinity chromatography. Equivalent amounts of kidney (60 g each) from age-matched normal and affected calves were homogenized, bound and eluted from Con A–Sepharose. The recovery of glycoproteins at this step was similar in normal and affected tissue, as indicated

Figure 2 Immunological characterization of purified bovine β -mannosidase by Western-blot analysis

Purified β -mannosidase (1 μ g of the Mono S pool, lane 2) and the same amount of protein incubated with N-Glycosidase F (lane 3) were subjected to SDS/PAGE (7.5% acrylamide), transferred to Immobilon P and probed with anti- β -mannosidase rabbit serum (a) and anti-80 rabbit serum (b). To evaluate the specificity of the rabbit sera, lane 1 in each panel contains 5 μ g of the Con A–Sepharose pool. The positions of molecular-mass standards (in kDa) are indicated on the right of panel (b).

Figure 3 Glycosylation of affinity-purified β -mannosidase

Purified β -mannosidase (2 μ g of the TSK-butyl pool) was deglycosylated with N-Glycosidase F (lane 1), no glycosidase (lane 2) or Endoglycosidase H (lane 3). The digestion mixtures were resolved on SDS/PAGE (7.5% acrylamide), and protein bands were made visible by silver staining. The low-molecular-mass peptide in lane 1 is N-Glycosidase F. The positions of molecular-mass standards (in kDa) are indicated on the right of the Figure.

Figure 4 Comparative analysis of β -mannosidase purified from normal and affected goat tissues

Kidney from normal and affected calves were subjected to Con A-Sepharose and immunoaffinity chromatography. In panels (a) and (b), equal volumes of the Con A-Sepharose pools from normal (N) and β -mannosidase-deficient (D) tissue were subjected to Western-blot analysis (91 and 75 μ g of protein respectively). Panel (a) was probed with anti-80 rabbit serum, and panel (b) was probed with anti- β -mannosidase rabbit serum. In panels (c) and (d) equal volumes of the immunoaffinity pools from normal (N) and β -mannosidase-deficient (D) tissue were subjected to SDS/PAGE and Western-blot analysis. Panel (c) is a Western blot probed with anti- β -mannosidase rabbit serum, and panel (d) is a polyacrylamide gel stained with silver. The positions of molecular-mass standards (in kDa) are indicated on the right of the panels. The above results are representative of two separate experiments.

by comparable levels of protein and α -mannosidase activity. The glycoprotein pools from the Con A-Sepharose step (normal and affected) were subjected to immunoaffinity purification using two analytical-sized columns (0.5 ml of resin). After elution, equal volumes of eluate from normal and affected tissues were analysed by SDS/PAGE. Western analysis (using anti-80 rabbit serum as a probe) of the Con A-Sepharose pool indicated that the anti-80 immunoreactive peptide was absent from affected tissue (Figure 4a, lane D). Using anti- β -mannosidase antiserum, Western-blot analysis of the Con A-Sepharose (Figure 4b) and immunoaffinity-purified (Figure 4c) pools indicates that the 84, 100 and 110 kDa peptides are present in normal calf tissue and absent from tissue from affected animals. Silver staining of the immunoaffinity-purified pools (Figure 4d) also shows that the 80, 84, 100 and 110 kDa peptides are absent from affected tissue.

DISCUSSION

In this paper we describe the purification and partial characterization of β -mannosidase in normal and β -mannosidase-deficient bovine kidney. Bovine β -mannosidase was purified 160 000-fold to a specific activity of 2000 units/mg of protein, with a 24% yield. In comparison, the goat enzyme was purified 120 000-fold to a specific activity of 1190 units/mg with a similar yield [39]. When assessed by Coomassie Blue or silver staining for SDS/PAGE, the bovine enzyme consists of three peptides (84, 100 and 110 kDa), whereas the corresponding goat peptides were 90 and 100 kDa. All of these peptides are consistently associated with β -mannosidase activity during all chromatographic steps.

An 80 kDa peptide that is not associated with β -mannosidase activity is present in immunoaffinity chromatographic fractions for both bovine and goat enzymes. From Western-blot analysis (using anti-80 rabbit serum as a probe), most of the 80 kDa peptide in the Con A-Sepharose pool (> 95%) does not bind to

the immunoaffinity column. The anti-80 immunoreactive peptide that does bind to the immunoaffinity column is not associated with activity in subsequent chromatography.

Deglycosylation of the bovine peptides with Endoglycosidase H decreased the size of the bovine peptides (84, 100 and 110 kDa) to 82, 96 and approx. 106 kDa. Deglycosylation with N-Glycosidase F decreased the size of the bovine peptides to 75, 86 and 91 kDa. Previously we reported that deglycosylation of the analogous goat peptides (90/100 kDa) with Endoglycosidase H and N-Glycosidase F decreased the size of these peptides to 86/96 kDa and 86/91 kDa respectively [39]. This suggests that the bovine and goat peptides contain similar amounts of high-mannose carbohydrate (approx. 2–4 kDa, as estimated by SDS/PAGE) and indicates that the 10 kDa increase in size between the bovine and analogous goat peptides is largely due to the presence of additional complex carbohydrate on the bovine peptides.

The 84, 100 and 110 kDa peptides are most likely related via protease processing. The size of the native bovine enzyme, as estimated by Superose h.p.l.c., is approx. 120 kDa (results not shown). This suggests that the enzyme probably does not exist as a multimer.

When extracts of kidney tissue from calves with β -mannosidase deficiency are subjected to Con A-Sepharose and immunoaffinity chromatography, the 84, 100 and 110 kDa peptides are not detected in either the Con A-Sepharose or the immunoaffinity pools, as assessed by silver staining and Western-blot analysis. This implies that either these peptides are absent from affected kidney tissue or they are structurally altered in such a way that Con A-Sepharose or the polyclonal and monoclonal antibodies do not bind to them. These findings are similar to those reported for goats with β -mannosidase deficiency [39].

We previously reported that the anti-80 immunoreactive peptide was present in kidney from affected goats [39]. The anti-80 immunoreactive peptide was not detected in β -mannosidase-deficient bovine kidney. The reason for this variance between affected goat and bovine tissues is unclear. Characterization of the bovine and goat mutations may lead to insights regarding the absence of the 80 kDa peptide in β -mannosidase-deficient bovine kidney.

We now have available mg quantities of β -mannosidase, which will allow a partial amino acid sequence to be obtained and should advance our efforts to clone the β -mannosidase gene. Cloning of this gene will facilitate the characterization of the β -mannosidase enzyme and the β -mannosidosis disease mutation.

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