Properties and prenatal ontogeny of β -D-mannosidase in selected goat tissues

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β -D-Mannosidase activity in selected normal adult, neonatal and foetal goat tissues and in tissues from animals affected with caprine β -mannosidosis was examined with the use of 4-methylumbelliferyl β -D-mannopyranoside as substrate. The enzyme in normal adult thyroid, kidney and brain exhibited a sharp unimodal pH optimum at pH 5.0, whereas the enzyme in both normal adult and mutant liver exhibited broad pH ranges of activity (pH 4.5–8.0). No residual enzyme was detectable in mutant kidney or brain; in contrast, residual activity in mutant liver was 52% of that in a neonatal control. Concanavalin A–Sephacrose 4B (Con A–Sephacrose) fractionation of normal adult liver β -D-mannosidase resolved the enzyme into an unbound (non-lysosomal) form (52%) with a broad pH range of activity (pH 4.5–8.0) and a bound (lysosomal) form (48%) with a sharp pH optimum of 5.5. The enzyme in mutant liver consisted entirely of the unbound (non-lysosomal) form. β -D-Mannosidase activity in normal adult thyroid, kidney and brain was resolved by chromatofocusing into two major isoenzymes, with pI 5.5 and 5.9, and traces of a minor isoenzyme, with pI 5.0. In normal adult liver the enzyme was also resolved into three isoenzymes with similar pI values; however, that with pI 5.0 predominated. The predominant form of the enzyme in 60-day-foetal liver was bound by Con A, exhibited a unimodal pH optimum (5.0) and was resolved into two isoenzymes, with pI 5.4 and 5.8; only traces of an isoenzyme with pI 5.0 were detectable. Total hepatic β -D-mannosidase activity increased progressively towards adult values during the last 90 days of gestation as a result of increasing non-lysosomal isoenzyme activity (pI 5.0). Lysosomal β -D-mannosidase was shown to occur in all normal goat tissues studied as multiple isoenzymes, which are genetically and developmentally distinct from the non-lysosomal isoenzyme occurring predominantly, if not exclusively, in liver.

INTRODUCTION

β -Mannosidosis is an autosomal recessive disease of oligosaccharide catabolism observed in Anglo-Nubian goats (Hartley & Blakemore, 1973; Healy *et al.*, 1981; Jones & Dawson, 1981; Jones & Laine, 1981). The disease is caused by deficiency of lysosomal β -D-mannosidase (EC 3.2.1.25), which results in the accumulation of *O*- β -D-mannopyranosyl-(1 $\rightarrow$ 4)-2-acetamido-2-deoxy-D-glucopyranose and *O*- β -D-mannopyranosyl-(1 $\rightarrow$ 4)-*O*-(2-acetamido-2-deoxy- β -D-glucopyranosyl)-(1 $\rightarrow$ 4)-2-acetamido-2-deoxy-D-glucopyranose in various tissues and urine (Jones & Laine, 1981; Matsuura *et al.*, 1981; Jones *et al.*, 1984; Matsuura & Jones, 1985). Storage of the oligosaccharides is associated with extensive neurovisceral pathology and death in the first few weeks of life (Hartley & Blakemore, 1973; Healy *et al.*, 1981; Jones *et al.*, 1983; Lovell & Jones, 1983, 1985).

Dawson (1982) has shown that normal adult goat liver contains two forms of β -D-mannosidase which can be distinguished by virtue of their pH optima, affinity for concanavalin A (Con A), and ability to hydrolyse storage trisaccharide. Only the lysosomal form of the enzyme was deficient in liver from goats affected with β -mannosidosis. More recently, partial purification and

some of the properties of the two forms of β -D-mannosidase from normal adult goat liver have been reported (Cavanagh *et al.*, 1985).

The present paper reports the presence of different forms of β -D-mannosidase in selected tissues of normal goats, of goats affected with β -mannosidosis, and also in foetal-goat liver at various stages of gestation.

MATERIALS AND METHODS

Materials

Normal adult, neonatal and mutant goat (*Capra hircus*) tissues were obtained *post mortem* from animals in the research herd. In order to obtain foetal tissues, donor female goats were superovulated, bred by artificial insemination, and the embryos were surgically transferred to surrogate recipient goats as previously described (Armstrong *et al.*, 1983). At defined intervals of gestation the foetuses were delivered by hysterotomy and killed. Animals were assessed as being normal or heterozygotes for β -D-mannosidosis by measurement of plasma β -D-mannosidase activity (Healy & McCleary, 1982). All tissues were stored at -20°C until use.

4-Methylumbelliferyl β -D-mannopyranoside (4 MU- β -D-Man) and concanavalin A–Sephacrose 4 B (Con

Abbreviations used: ConA, concanavalin A; 4 MU- β -D-Man, 4-methylumbelliferyl β -D-mannopyranoside.

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A-Sepharose) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Polybuffer 94 Exchanger gel and Polybuffer 74 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Buffer A consisted of 10 mM-sodium phosphate, pH 6.8, containing 0.5 M-NaCl, 0.1 mM-MnCl₂ and 0.1 mM-CaCl₂. All other chemicals and reagents were of reagent grade or better.

Preparation of tissue extracts

Tissues were homogenized (Brinkmann homogenizer, model PT 10 35) in distilled water at 4 °C and sonicated (model W140, Heat Systems Ultrasonics) for 60 s. High-speed supernatants were obtained by centrifugation at 100000 *g* for 60 min. In the preparation of supernatants for affinity chromatography, the tissues were homogenized in Buffer A instead of distilled water.

Enzyme assays

β -D-Mannosidase activity was measured with the use of the fluorogenic substrate 4MU- β -D-Man. Incubations were carried out at 37 °C for 30–60 min in the presence of 4 mM-4MU- β -D-Man, 0.05 M-sodium acetate buffer, pH 5.0, or citrate/phosphate buffer (McIlvaine, 1921) at various pH values, and enzyme in a final volume of 0.2 ml. The reaction was terminated by addition of 1.8 ml of 0.1 M-2-amino-2-methylpropane-1,3-diol containing 1.0 mM-EDTA, pH 10.4, and free 4-methylumbelliferone was determined spectrofluorimetrically (excitation, 365 nm; emission, 450 nm). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Concanavalin A-Sepharose 4B chromatography

All procedures were carried out at 4 °C. Each tissue supernatant was chromatographed essentially as described by Dawson (1982) on columns (0.7 cm $\times$ 4.0 cm) of Con A-Sepharose which had been equilibrated with Buffer A. The columns were eluted with Buffer A until the A_{280} became zero. Elution was then continued with Buffer A containing 0.5 M-1-*O*-methyl α -D-glucopyranoside. The eluate was collected in 1.0 ml fractions, and samples were assayed for β -D-mannosidase activity.

Chromatofocusing

All procedures were carried out at 4 °C. Each tissue supernatant was chromatofocused on columns (0.7 cm $\times$ 15.0 cm) of Polybuffer Exchanger 94 gel

equilibrated with 0.025 M-histidine/HCl buffer, pH 6.2. The column was eluted with Polybuffer 74/HCl, pH 4.0, diluted 1:8 with distilled water according to the manufacturer's directions; 1 ml fractions were collected, and the β -D-mannosidase activity, pH and A_{280} of each were measured.

Cellulose acetate electrophoresis

Cellulose acetate strip electrophoresis was performed in the presence of 0.01 M-citric acid/0.02 M-sodium phosphate buffer, pH 7.0 (McIlvaine, 1921). Separations were typically carried out for 20 min at room temperature and a constant current of 7.5 mA (model 51156; Gelman Instrument Co., Ann Arbor, MI, U.S.A.). Bands of enzyme activity were located by incubation with 8.0 mM-4MU- β -D-Man in 0.2 M-sodium acetate buffer, pH 5.0, for 60 min at 37 °C, exposure of the strips to ammonia vapour, and observation with the aid of long-wave u.v. light (365 nm).

RESULTS

Total β -D-mannosidase activities (nmol/h per mg of protein) in various tissues are shown in Table 1. Enzyme activity in normal adult and neonatal tissue was highest in thyroid; activities were lower in liver, kidney and brain, in descending order of specific activity. The specific activity in neonatal thyroid was approx. 3 times that in adult thyroid tissue. Residual β -D-mannosidase activity in mutant liver was 52% of control. In contrast, no residual activity was detectable in supernatants prepared from mutant kidney or brain tissue. No mutant thyroid tissue was available for analysis. Specific activity in foetal brain was comparable with that in normal neonatal and adult tissues at all intervals of gestation studied. On the other hand, β -D-mannosidase activity in 60-day-foetal kidney was higher than that in neonatal and adult tissues. Specific activity in 60-day-foetal liver was 31% of that in normal adult tissue and increased during the last 90 days of gestation to reach 93% of the adult value at 3 days *post partum*. This progressive increase in activity throughout gestation was not observed in kidney or brain tissues, although specific activity in 117-day-foetal thyroid was only 30% of that in normal neonatal tissue.

Fig. 1 shows the pH/activity profiles of β -D-mannosidase in various normal and mutant goat tissues.

Table 1. β -D-Mannosidase activities in various goat tissues

Tissue extracts were prepared and enzyme assays performed with acetate buffer, pH 5.0, as described in the text. Except for studies done on one or two animals, all values represent means $\pm$ S.D., with the numbers of animals studied shown in parentheses; ND, not determined.

Age	Enzyme activity (nmol of 4-MU- β -D-Man hydrolysed/h per mg of protein)			
	Thyroid	Liver	Kidney	Brain
60-day foetus	ND	12.0 $\pm$ 1.2 (4)	23.7 $\pm$ 4.9 (4)	5.5 $\pm$ 1.0 (4)
89-day foetus	ND	14.3	18.3	4.5
117-day foetus	59.5 $\pm$ 6.2 (3)	22.1 $\pm$ 5.7 (3)	20.7 $\pm$ 1.8 (3)	4.2 $\pm$ 0.5 (3)
Neonatal normal kid	195.4 (1)	36.5	14.6	4.0
Adult	56.7, 71.6 (2)	39.3	7.8	3.0
Neonatal mutant kid	ND	19.0	< 0.1	< 0.1

Fig. 1. pH-dependence of β -D-mannosidase in normal and mutant goat tissues

(a) ■, Thyroid; ◆, kidney; ●, brain; (b) ■, adult liver; ◆, mutant liver; (c) ■, 60-day, ◆, 89-day, ●, 117-day normal foetal liver; ▲, 3-day neonatal liver; (d) ■, Con A-bound β -D-mannosidase; ◆, Con A-unbound β -D-mannosidase. Enzyme assays were done with the use of citrate/phosphate buffers (McIlvaine, 1921) at various pH values.

The enzyme activity in normal adult thyroid, kidney and brain supernatants exhibited a sharp unimodal pH optimum (pH 5.0) (Fig. 1a). The enzyme activity in both normal adult and mutant liver supernatants exhibited a broad pH range of activity (pH 4.5–8.0) (Fig. 1b). However, the relative specific activity at pH 7.0–8.0 was significantly higher than that at pH 5.0 in mutant liver compared with the activity in normal tissue. Fig. 1(c) shows the pH-activity profiles of β -D-mannosidase activity in foetal liver supernatants at various stages of gestation. Enzyme activity at 60 days of gestation exhibited a unimodal pH optimum at 5.0, but the relative activity at pH 7.0–8.0 increased progressively throughout gestation towards adult values.

Fractionation of normal adult liver supernatant on Con A-Sepharose resolved β -D-mannosidase activity into unbound and bound forms with different pH-activity profiles (Fig. 1d). The bound enzyme exhibited a sharp unimodal pH optimum at pH 5.5; the unbound enzyme showed two distinct pH optima, at pH 5.0 and 7.0–8.0. Fractionation of mutant liver supernatant showed that all the residual β -D-mannosidase was unbound, confirming that bound β -D-mannosidase is the form deficient in β -mannosidosis (Dawson, 1982). Fractionation of normal 60-day-foetal liver and normal adult thyroid, kidney and brain supernatants showed that > 80% of the activity was the bound form.

Fig. 2 shows the electrophoretic mobilities of β -D-mannosidase in neonatal mutant, normal 60-day-foetal and normal adult liver. Normal adult liver contained approximately equal amounts of two electrophoretically separable isoenzymes. The more anodic isoenzyme was present in mutant liver, but not in 60-day-foetal liver. On

Fig. 2. Cellulose acetate electrophoresis of goat liver 100000 g supernatants

Electrophoresis was performed in 0.01 M-citric acid/0.02 M-sodium phosphate buffer, pH 7.0, for 20 min at constant current of 7.5 mA. Lane 1, neonatal mutant; lane 2, normal 60-day foetus; lane 3, normal adult.

Fig. 3. Chromatofocusing of 100000 g supernatant from goat liver

Supernatant (100000 g) was chromatofocused and assayed for β -D-mannosidase activity as described in the text. (a) Normal adult; (b) neonatal mutant; (c) Con A-unbound fraction from normal adult; ●, activity; ▲, pH; —, A_{280} .

the other hand, the only isoenzyme detectable in 60-day-foetal liver co-migrated with the less anodic isoenzyme in adult tissue. The results indicate that the non-lysosomal form of β -D-mannosidase is more negatively charged at neutral pH than the lysosomal form of the enzyme.

Chromatofocusing of normal adult liver supernatant revealed a major peak with pI 5.0, and two minor peaks, with pI 5.4 and 5.8 (Fig. 3a). The pI of enzyme from mutant liver (pI 5.0) was indistinguishable (Fig. 3b) from that of normal adult liver β -D-mannosidase that did not bind to Con A (Fig. 3c). Owing to instability of the isolated Con A-bound form of the enzyme, reliable estimates of its pI in mature goat liver were not possible.

In 60-day-foetal liver, isoenzymes with pI values of 5.4 and 5.8 predominated, although a shoulder on the side of the pI 5.4 peak suggested the presence of trace amounts of an isoenzyme with a lower pI (Fig. 4a). At all stages of gestation examined, the isoenzymes with pI 5.4 and 5.8 were present in approximately the same relative proportions as at 60 days. In contrast, the pI 5.0 isoenzyme, which was barely detectable at 60 days gestation, became increasingly prominent at 89 and 117 days gestation and was the dominant isoenzyme in neonatal liver (Fig. 4).

In normal adult thyroid, kidney, and brain, at least three isoenzymes of β -D-mannosidase with different pI values were found (Fig. 5). In kidney and brain, the

Fig. 4. Chromatofocusing of 100000 g supernatants from normal foetal and neonatal goat liver

Supernatants (100000 g) were chromatofocused and assayed for β -D-mannosidase as described in the text. (a) 60-day, (b) 89-day, (c) 117-day foetal; (d) 3-day newborn. ●, Activity; ▲, pH; —, A_{280} .

Fig. 5. Chromatofocusing of 100000 *g* supernatants from normal adult tissues

Supernatants (100000*g*) were chromatofocused and assayed for β -D-mannosidase activity as described in the text. (a) Thyroid; (b) kidney; (c) brain. ●, Activity, ▲, pH; —, A_{280} .

predominant isoenzyme had a pI of 5.8–5.9, with much smaller amounts of pI 5.5 isoenzyme and only trace amounts of an isoenzyme with pI 5.0 (Figs. 5*b* and 5*c*). In thyroid, the distribution of isoenzymes was more uniform, with the pI 5.5 form predominating (Fig. 5*a*). In contrast with normal adult or mutant liver, the thyroid was the only non-hepatic tissue that contained significant amounts of an isoenzyme with pI 5.0.

DISCUSSION

Mature goat liver contains at least two major,

genetically distinct, forms of β -D-mannosidase (Dawson, 1982). One of the isoenzymes exhibits a sharp unimodal acidic pH optimum (5.0–5.5), is bound by Con A-Sepharose, and is almost totally deficient in animals affected with β -mannosidosis. Dawson (1982) and Cavanagh *et al.* (1985) have also shown that it catalyses the hydrolysis of the natural oligosaccharide substrates, *O*- β -D-mannopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose and *O*- β -D-mannopyranosyl-(1→4)-*O*-D-(2-acetamido-2-deoxy- β -D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-D-glucopyranose. The results of the studies reported here show that this form of the enzyme

is widely distributed in goat tissues and is present at high specific activity in foetuses at all stages of gestation studied.

Cavanagh *et al.* (1985) reported partial purification of both the lysosomal and non-lysosomal forms of the enzyme from adult goat liver. The lysosomal form was reported to have an apparent M_r of 127000 and pI 6–7. The non-lysosomal form was reported to have an apparent M_r of 43000 and pI 5.5. The results of isoelectric focusing reported by these workers suggested the existence of more than one isoenzymic form of lysosomal β -D-mannosidase, but the resolution of the method used was not sufficient to establish accurately the number or their pI values. We have shown by chromatofocusing that the lysosomal enzyme occurs in at least three forms, differing in pI. The isoenzymic heterogeneity of lysosomal β -D-mannosidase may be due to differences in the extent of sialylation (Needleman & Koenig, 1974); this possibility requires investigation.

Two of the major lysosomal β -D-mannosidase isoenzymes clearly have pI values different from that of the non-lysosomal isoenzyme occurring in liver. The third has a pI (5.0) similar to that of non-lysosomal β -D-mannosidase. Although the presence of this isoenzyme in non-hepatic tissues might indicate the presence of non-lysosomal enzyme, the observation that mutant kidney and brain contained no demonstrable β -D-mannosidase activity argues against this. A more likely possibility is the existence in some non-hepatic tissues, notably thyroid, of a lysosomal isoenzyme with pI 5.0. However, the presence of trace amounts of non-lysosomal isoenzyme in non-hepatic tissues cannot be eliminated.

The results reported here also show that total β -D-mannosidase activities in foetal liver are high at 60 days of gestation and increase progressively during the second half of gestation. Observations on the changing pH profile of enzyme activity and the distribution of isoenzymes as determined by chromatofocusing indicate that this increase in enzyme activity during gestation is attributable largely to increasing relative and absolute amounts of non-lysosomal β -D-mannosidase.

The properties and biological function of the non-lysosomal form of β -D-mannosidase are still unknown, but it has been shown by others (Dawson, 1982) as well as ourselves to account for about half of the total enzyme activity in adult goat liver. The results of cellulose acetate electrophoresis and chromatofocusing of the adult liver enzyme confirmed that the non-lysosomal enzyme is more acidic than the lysosomal form. It has a broad or bimodal pH profile, with optima at pH 5.0 and pH 7.0–8.0. It is not deficient in animals with β -mannosidosis, nor does it catalyse the hydrolysis of the storage trisaccharide accumulating in the tissues of affected animals (Dawson, 1982; Cavanagh *et al.*, 1985), indicating that it is genetically distinct from the lysosomal isoenzyme. It is not bound by Con A–Sepharose, indicating that it is not a high-mannose-containing glycoprotein. Our studies showed that the tissue distribution and ontogeny of the enzyme are also different from those of the lysosomal enzyme. On the

basis of the results of Con A–Sepharose binding studies and the results of studies on tissues from goats affected with β -mannosidosis, the non-lysosomal form of the enzyme appears to be localized almost exclusively to liver. In this and other respects it resembles other glycosidases, which have been shown to exist in lysosomal and non-lysosomal forms (Norden & O'Brien, 1974; Peters *et al.*, 1976; Phillips *et al.*, 1976).

In summary, we have shown that goat liver, kidney, thyroid and brain contain multiple forms of lysosomal β -D-mannosidase, which are all deficient in caprine β -mannosidosis. Normal and mutant neonatal liver, as well as adult liver, also contain a genetically distinct non-lysosomal isoenzyme which is absent from, or present in only trace amounts in, non-hepatic tissues and 60-day foetal liver. Our studies also showed that the two major forms of β -D-mannosidase have different prenatal developmental profiles, which is consistent with other evidence that they are under independent genetic control.

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