Substrate specificity of the bovine and feline neutral α -mannosidases

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Neutral α -mannosidases were prepared from bovine and cat liver. The activities were distinguished from lysosomal and Golgi α -mannosidases by their neutral pH optima, relatively low K_m for their synthetic substrate *p*-nitrophenyl α -D-mannoside, inhibition by Zn^{2+} and absence of inhibition by Co^{2+} , EDTA, low concentrations of swainsonine, or deoxymannojirimycin. The cytosolic α -mannosidases were not retained by concanavalin A-Sepharose. They were able to degrade efficiently a variety of oligosaccharides with structures corresponding to certain high-mannose glycans or the oligomannosyl parts of hybrid and complex glycans. However, unlike lysosomal α -mannosidases from the same species these enzymes were not able to degrade $Man_6GlcNAc_2$ efficiently, and the bovine neutral α -mannosidase was not able to degrade a hexasaccharide with a structure analogous to $Man_5GlcNAc_2$ -PP-dolichol. Sharp differences were noted for the bovine and cat enzymes with regard to the specificity of degradation. The bovine neutral α -mannosidase degraded the substrates by defined pathways, but the cat neutral α -mannosidase often produced complex mixtures of products, especially from the larger oligosaccharides. Therefore the bovine enzyme resembled the rat and human cytosolic α -mannosidases, but the cat enzyme did not. The bovine and cat neutral α -mannosidases, unlike the corresponding lysosomal activities, did not show specificity for the hydrolysis of the (1 $\rightarrow$ 3)- and (1 $\rightarrow$ 6)-linked mannose residues in the N-linked glycan pentasaccharide core.

INTRODUCTION

The function of cytosolic α -mannosidase, with a neutral pH optimum, originally described by Marsh & Gourlay (1971) and subsequently purified from rat liver by Shoup & Touster (1976), is uncertain. Bischoff & Kornfeld (1986) reported that the enzyme is very closely related to an endoplasmic reticulum α -mannosidase involved in the early stages of N-linked glycan processing (Bischoff & Kornfeld, 1983), and suggested that the cytosolic α -mannosidase is derived from endoplasmic reticulum mannosidase by proteolysis. They therefore called it 'soluble α -mannosidase'.

Cytosolic (neutral) α -mannosidase is not deficient in the lysosomal storage disease α -mannosidosis in humans (Carroll *et al.*, 1972), cattle (Phillips *et al.*, 1974) or cats (Burditt *et al.*, 1980). The structures of the major oligosaccharides that accumulate in the tissues of cattle and cats (Abraham *et al.*, 1983, 1986; Daniel *et al.*, 1985; Warren *et al.*, 1986, 1988a,b) with α -mannosidosis are consistent with the incomplete catabolism of N-linked glycans, resulting from an inherited deficiency of lysosomal α -mannosidase activity (De Gasperi *et al.*, 1991). However, some of the stored compounds have structures resembling those of biosynthetic intermediates (Kornfeld & Kornfeld, 1985), suggesting that they might be formed by a different mechanism.

Tulsiani & Touster (1987) have proposed a role for the cytosolic α -mannosidase in glycan degradation based on a study of the substrate specificities of rat kidney lysosomal and cytosolic α -mannosidases, and on the ability of high concentrations of the indolizidine alkaloid swainsonine to inhibit the cytosolic enzyme. In the present study we have prepared neutral α -mannosidases from bovine and cat liver and determined their specificities toward a large variety of oligosaccharides, with structures corresponding to either high-mannose glycans or the oligo-

mannosyl parts of hybrid or complex glycans. We have also compared the structures of the digestion intermediates with those of the compounds stored and excreted in bovine and cat α -mannosidosis. For a preliminary account of some of the experiments, see De Gasperi *et al.* (1989a).

MATERIALS AND METHODS

Oligosaccharide preparation

The mannosyl oligosaccharides used in this study were isolated as previously described (De Gasperi *et al.*, 1991). The oligosaccharides were chosen so that their structures would correspond to those of the compounds generated by the bidirectional degradation of complex, hybrid, or high-mannose glycans (Brasart *et al.*, 1987; Aronson & Kuranda, 1989), or to the glycan moiety or biosynthetic intermediates (Kornfeld & Kornfeld, 1985). Because bovine and cat tissues do not contain lysosomal endo-N-acetyl- β -glucosaminidase, most of the substrates contained a di-N-acetylchitobiose residue at the reducing terminus. For structures and numbering, see Tables 1 and 2. In this paper a prime denotes substrates containing one GlcNAc at the reducing end.

$Man_6GlcNAc_2$, $Man_5GlcNAc_2$ and $Man_4GlcNAc_2$ were purchased from Oxford Glycosystems, Rosedale, NY, U.S.A., and labelled by reduction with NaB^3H_4 as described (Mellis & Baenziger, 1981). The purity of all the substrates was checked by h.p.l.c. before the enzymic digestions. Bovine liver was obtained from a local slaughterhouse and frozen immediately. Frozen cat liver was purchased from Pel-Freez, Rogers, AR, U.S.A.

Isolation of α -mannosidase

(a) Bovine or cat liver was thawed and homogenized in 5 vol. of cold distilled water and the homogenate centrifuged at

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Table 1. Structures and sources of oligosaccharides containing terminal di-*N*-acetylchitobiose, employed as substrates for preparations of neutral α -mannosidase, or as reference compounds for h.p.l.c. analysis of digestion products

Numbering of the oligosaccharides is based on the number of mannose residues. To distinguish the isomeric structures, a, b, c, etc. were arbitrarily assigned. In this paper, only a small number of substrates contained a single GlcNAc residue at the reducing terminus, and these are distinguished by a prime. Explanation of schematic structures: , Man α (1→2)Man; , Man α (1→6)Man; , Man α (1→3)Man; , Man β (1→4)GlcNAc; , GlcNAc β (1→4)GlcNAc.

Structure	Abbreviation	Number	Reference
Man ₂ GlcNAc ₂ *	2a	Warren <i>et al.</i> (1981)
Man ₂ GlcNAc ₂ †	2b	Warren <i>et al.</i> (1988b)
Man ₃ GlcNAc ₂ ‡	3a	Warren <i>et al.</i> (1988a)
Man ₃ GlcNAc ₂ †	3b	Warren <i>et al.</i> (1988b)
Man ₃ GlcNAc ₂ †	3d	Warren <i>et al.</i> (1988b)
Man ₄ GlcNAc ₂ †	4a	Warren <i>et al.</i> (1988b)
Man ₄ GlcNAc ₂ ‡	4e	Warren <i>et al.</i> (1988a)
Man ₅ GlcNAc ₂ †	5a	Warren <i>et al.</i> (1988b)
Man ₆ GlcNAc ₂ §	6a	Commercial
Man ₈ GlcNAc ₂ §	8a	Commercial
Man ₉ GlcNAc ₂ §	9	Commercial

* Obtained by chemical synthesis from (2a') (see Table 2).

† Isolated from urine of swainsonine-poisoned sheep.

‡ Isolated from feline α -mannosidosis liver.

§ Obtained from Oxford Glycosystems Inc., and radiolabelled by reduction with NaB³H₄.

15000 rev./min for 20 min. The supernatant was saved and the residue re-extracted as above. The supernatants from the two extractions were pooled, and (NH₄)₂SO₄ was added to 30% saturation. The precipitate was collected by centrifugation (27000 g, 20 min). The α -mannosidase with neutral pH optimum was quantitatively precipitated by adding (NH₄)₂SO₄ to 30% saturation, whereas the lysosomal activity remained in the supernatant (Phillips *et al.*, 1976).

(b) Since fresh bovine liver could be obtained at a local slaughterhouse, a cytosolic fraction was prepared by subcellular fractionation. The fresh liver tissue was homogenized in 10 mM-sodium phosphate buffer, pH 6.8, containing 0.25 M-sucrose, 1 mM-mercaptoethanol, 1 μ g of leupeptin/ml, 1 mM-*o*-phenanthroline, 1 mM- ϵ -aminohexanoic acid and 1 mM-phenylmethanesulphonyl fluoride, and the homogenate centrifuged at 15000 g for 30 min. The supernatant was then centrifuged at 100000 g for 1 h and the new supernatant, representing the cytosolic fraction, was saved. The 100000 g supernatant was found to contain neutral α -mannosidase activity, which was concentrated by addition of solid (NH₄)₂SO₄ to 30% saturation.

The neutral α -mannosidase prepared in (a) or (b) was further purified essentially as described for the soluble α -mannosidase from rat liver (Bischoff & Kornfeld, 1986). The enzyme fraction was dissolved in 20 mM-Tris/HCl buffer (pH 7.4)/20% (v/v) glycerol/2 mM-dithiothreitol/1 mM-MnCl₂ and *p*-nitrophenyl α -mannoside was added to a final concentration of 2 mM. The fraction was then heat-treated as described (Bischoff & Kornfeld,

1986). Further purification was achieved by DEAE-Sephacel chromatography (Bischoff & Kornfeld, 1986). Most of the studies with the bovine enzyme were performed with the enzyme prepared as in (a). The specific activity was 0.05 unit/mg of protein for the cat enzyme and 0.12 unit/mg of protein for the bovine activity.

Assay of α -mannosidase activity

The activity was routinely assayed with *p*-nitrophenyl α -mannoside (2 mM) in 50 mM-sodium phosphate, pH 6.8 (released *p*-nitrophenol was measured by its absorbance at 400 nm). One unit was defined as the amount of enzyme that released 1 μ mol of *p*-nitrophenol/min under standard assay conditions.

Incubation of neutral α -mannosidase with oligomannosyl glycans

The incubations with oligomannosyl glycans were carried out as follows. The oligosaccharide (final concentration 0.3 mM) was incubated at 37 °C with 0.06–0.12 unit of α -mannosidase in 150 μ l of 50 mM-sodium citrate/sodium phosphate, pH 6.4, containing 1 mM-CoCl₂. The incubations were stopped by the addition of 1 vol. of 95% ethanol and boiling for 3 min. After centrifugation at 14000 rev./min for 15 min, the supernatant was dried, redissolved in 0.5 ml of water and applied to a column of coupled (0.5 cm each in a Pasteur pipette) AG 1 $\times$ 8 (formate) and AG 50 $\times$ 8 (H⁺) (200–400 mesh) ion-exchange resins (Bio-Rad, Richmond, CA, U.S.A.). The eluate was then freeze-dried, reduced with NaBH₄ and analysed by h.p.l.c. Before analysis, the

Table 2. Structures and sources of oligosaccharides containing a single terminal GlcNAc residue, employed as substrates for preparations of neutral α -mannosidase, or as reference compounds for h.p.l.c. analysis of digestion products

See Table 1 for explanation of numbering and key to schematic structures.

Structure	Abbreviation	Number	Reference
	Man ₂ GlcNAc*	2a'	Warren <i>et al.</i> (1983)
	Man ₂ GlcNAc†	2b'	Warren <i>et al.</i> (1988b)
	Man ₃ GlcNAc‡	3a'	De Gasperi <i>et al.</i> (1989b) Warren <i>et al.</i> (1988b)
	Man ₃ GlcNAc§	3b'	Warren <i>et al.</i> (1988b)
	Man ₃ GlcNAc*	3c'	Warren <i>et al.</i> (1983)
	Man ₄ GlcNAc	4b'	Warren <i>et al.</i> (1989)
	Man ₄ GlcNAc*	4c'	Warren <i>et al.</i> (1983)
	Man ₅ GlcNAc¶	5a'	Warren <i>et al.</i> (1988b)
	Man ₅ GlcNAc	5b'	Warren <i>et al.</i> (1989)
	Man ₆ GlcNAc*	6a'	Warren <i>et al.</i> (1983)
	Man ₆ GlcNAc*	9'	Warren <i>et al.</i> (1983)

* Isolated from human α -mannosidosis urine.† Obtained by treatment of (2b) with endo-*N*-acetyl- β -D-glucosaminidase from human spleen.‡ Obtained by endo-*N*-acetyl- β -glucosaminidase D treatment of (3a).§ Obtained by endo-*N*-acetyl- β -glucosaminidase H treatment of (3b).

|| Isolated from pancreas of swainsonine-poisoned sheep.

¶ Obtained by endo-*N*-acetyl- β -glucosaminidase H treatment of (5a).

samples were filtered through an Amicon Centrifree Micro-partition System (Amicon, Danvers, MA, U.S.A.).

H.p.l.c. analysis was performed with a Varian 5020 instrument equipped with a Varian 4290 computing integrator. The separation was performed on a reversible Hi-Chrom 5 μ m Amino-Spherisorb column (Regis Chemical Co., Morton Grove, IL, U.S.A.) eluted with acetonitrile/15 mM-KH₂PO₄, at a flow rate of 2 ml/min, with detection of absorbance at 195 nm. The proportions of acetonitrile/KH₂PO₄ were adjusted to optimize the separation according to the molecular size, and ranged from 7:3 to 3:1. Peaks in the chromatographic profile were identified by comparison of retention times and/or co-injection with reference oligosaccharides from the same sources as the substrates (see Tables 1 and 2). Compounds corresponding to the peaks were quantified by comparison of integrated areas on the basis of the approximation that absorption at 195 nm is solely due to GlcNAc residues. For radiolabelled substrates, fractions were collected every 30 s and analysed by liquid-scintillation counting.

Acetolysis

Fractions eluted from the h.p.l.c. column were desalted through coupled AG 1 $\times$ 8 and AG 50 $\times$ 8 ion-exchange resins and subjected to acetolysis as described (De Gasperi *et al.*, 1991). The acetolysis products were analysed by h.p.l.c. as described above. The structural information gained from the acetolysis was more limited for digestion intermediates of minor pathways, because it was sometimes difficult to distinguish low-radioactive peaks arising from genuine acetolysis cleavage products from minor

peaks arising from side reactions that occurred during the acetolysis treatment. For each substrate the extent of side reactions was evaluated by a control acetolysis of the starting compound.

RESULTS AND DISCUSSION

Characterization of the neutral α -mannosidase

To make sure that the enzyme preparation contained exclusively neutral α -mannosidase, a thorough characterization of the activity was performed before any study of substrate specificity. The pH optimum was found to be at pH 6.4 for both bovine and cat activities, similar to those reported previously for the rat cytosolic enzyme (5.9) (Shoup & Touster, 1976) and for the rat soluble α -mannosidase (6.3–6.8) (Bischoff & Kornfeld, 1986). The K_m for the synthetic substrate *p*-nitrophenyl α -mannoside was found to be around 0.4 mM for both cat and bovine α -mannosidase, much lower than the K_m of the corresponding lysosomal enzymes, which was determined to be about 2 mM. Rat liver cytosolic and soluble α -mannosidases were also reported to have a K_m much lower than the lysosomal activity (Opheim & Touster, 1978; Bischoff & Kornfeld, 1986). The K_m for human neutral α -mannosidase was also much lower than that for lysosomal α -mannosidase (Hirani & Winchester, 1979). The neutral α -mannosidase was completely inhibited by 2 mM-Zn²⁺, but slightly stimulated or unaffected by Co²⁺, which, along with Mn²⁺, seemed to act as stabilizers of the activity.

Scheme 1. Pathway for hydrolysis of mannose residues from pentasaccharide (3a) by bovine or cat neutral α -mannosidase

Fig. 1. Time course for the hydrolysis by bovine neutral α -mannosidase of mannose residues from pentasaccharide (3a) (a) and from tetrasaccharides (2a) and (2b) (b)

Hydrolysis of pentasaccharide (3a) (○) by bovine neutral α -mannosidase yielded tetrasaccharides (2a) (●) and (2b) (▽) and finally trisaccharide ManGlcNAc₂ (▼) (a). The incubation products were analysed as described in the Materials and methods section. The percentage of total oligosaccharides for each compound was calculated from the integrated areas of the h.p.l.c. peaks. (b) Comparison of the rates of hydrolysis of $\alpha(1 \rightarrow 3)$ -linked mannose residue from (2a) (○) and $\alpha(1 \rightarrow 6)$ -linked mannose residue from (2b) (●) in the presence of bovine neutral α -mannosidase. The percentage hydrolysis was calculated from the integrated areas of the h.p.l.c. peaks for compound (2a) or (2b) and ManGlcNAc₂.

Scheme 2. Pathway for hydrolysis of mannose residues from heptasaccharide (5a) by bovine or cat neutral α -mannosidase

Under our conditions EDTA up to 4 mM did not affect the neutral α -mannosidase activity, in contrast with the result for the rat enzyme (Opheim & Touster, 1978). The neutral α -mannosidase was also quite resistant to known inhibitors of other α -mannosidase activities. Unlike lysosomal and Golgi II α -mannosidases, no significant inhibition of the neutral α -mannosidase was observed with 10–20 μ M-swainsonine, and inhibition could be observed only at higher concentrations (IC_{50} approx. 70 μ M). This result is comparable with that of Tulsiani & Touster (1987) for the rat cytosolic α -mannosidase, who observed a 50% inhibition at 100 μ M-swainsonine. Both cat and bovine neutral α -mannosidases were totally unaffected by deoxymannojirimycin up to 40 μ M. Deoxymannojirimycin is a potent inhibitor of Golgi I α -mannosidase (Bischoff *et al.*, 1986). Swainsonine and deoxymannojirimycin, at 10 μ M and 20 μ M respectively, did not affect the result of incubations with specific oligomannosyl glycans (9, 5a, 3a), which confirmed that our preparation was free of lysosomal and Golgi I α -mannosidases. Unlike lysosomal (Phillips *et al.*, 1976; De Gasperi *et al.*, 1991), Golgi I and Golgi II α -mannosidases, and similar to the rat soluble α -mannosidase (Bischoff & Kornfeld, 1986), the neutral activity was not retained by concanavalin A-Sepharose.

All the above results indicated that our preparation contained exclusively neutral α -mannosidase activity, probably of cytosolic origin, since the bovine enzyme prepared by subcellular fractionation of calf liver had the same properties, i.e. pH optimum, sensitivity to cations and inhibitors, as the enzyme prepared from frozen tissue. Moreover, these properties for both the bovine and cat neutral α -mannosidases were very similar to those reported for the well-characterized cytosolic (Opheim & Touster, 1978) and soluble α -mannosidase from rat liver (Bischoff & Kornfeld, 1986), and the human liver neutral α -mannosidase (Hirani & Winchester, 1979). This indicates that the basic properties of the neutral α -mannosidase vary very little among species.

Digestions of substrates from complex glycans

The branched oligosaccharide (3a) would be derived from complex glycans by the action of lysosomal α -fucosidase, neuraminidase, β -galactosidase, and β -hexosaminidase. Both bovine

Fig. 2. H.p.l.c. profile of oligosaccharides obtained by incubating heptasaccharide (5a) with bovine neutral α -mannosidase (a) or bovine lysosomal α -mannosidase (b)

Heptasaccharide (5a) was incubated for 30 min with bovine neutral α -mannosidase (a) or for 7 h with bovine lysosomal α -mannosidase (b). After the times indicated, the incubation mixture was reduced and analysed as described in the Materials and methods section. Oligosaccharide products were identified by co-injection with reduced reference compounds obtained from the same sources as the substrates (see Table 1). Peak identification (see Table 1): M₂, (2b); M₃, (3b); M₄, (4a) and (4e); M₅, (5a).

Fig. 3. Time course for the hydrolysis by bovine neutral α -mannosidase of mannose residues from heptasaccharide (5a) (a) and pentasaccharide (3b) (b)

The hydrolysis of (5a) (○) yielded hexasaccharides (4a) (●) and (4e) (▽), pentasaccharide (3b) (▼), tetrasaccharide (2b) (□) and finally trisaccharide ManGlcNAc₂ (■) (a). The incubation products were analysed as described in the Materials and methods section. The percentage of each oligosaccharide was calculated from the integrated areas of the h.p.l.c. peaks. Pentasaccharide (3b) (○) was hydrolysed by bovine neutral α -mannosidase to yield tetrasaccharide (2b) (●) and finally trisaccharide ManGlcNAc₂ (▽) (b). Analysis and calculations were as for (a).

and cat neutral α -mannosidases degraded oligosaccharide (3a) quite rapidly down to ManGlcNAc₂. In a 30 min incubation, it was hydrolysed 50 % by the bovine enzyme and 76 % by the cat enzyme. Both $\alpha(1 \rightarrow 6)$ - and $\alpha(1 \rightarrow 3)$ -linked mannose residues were hydrolysed indiscriminately, so that the products (2a) and (2b) were observed in a ratio close to 1:1 (see Scheme 1 and Fig. 1a). The lack of specificity for compounds (2a) and (2b), which correspond respectively to the $\alpha(1 \rightarrow 3)$ and $\alpha(1 \rightarrow 6)$ branches of the invariant N-glycan core, was confirmed when these substrates were digested separately. As indicated in Fig. 1(b), they were hydrolysed at similar rates, with compound (2a) being degraded slightly faster than (2b). These results were in sharp contrast with the results obtained with the lysosomal activities, which showed a remarkable specificity towards the hydrolysis of the $\alpha(1 \rightarrow 3)$ -linked mannose residue (De Gasperi *et al.*, 1991).

Digestion of substrates from hybrid glycans

The heptasaccharide (5a), which contains the oligomannosyl structure found in hybrid glycans, was readily hydrolysed by both enzymes. In a 30 min incubation, it was hydrolysed 50 % by the bovine neutral α -mannosidase and 73 % by the cat neutral α -mannosidase. As shown in Scheme 2, the hydrolysis of this heptasaccharide proceeded down a well-defined pathway. The h.p.l.c. analysis at various time points showed that two Man₄GlcNAc₂ products were generated from compound (5a), whereas further cleavage down to ManGlcNAc₂ yielded only one isomer of Man₃GlcNAc₂ and Man₂GlcNAc₂. This result is in contrast with the hydrolysis of this compound by lysosomal α -mannosidase, which yielded one Man₄GlcNAc₂ isomer and two

Table 3. Percentage of individual oligosaccharides produced from undecasaccharide (9) by the action of bovine and cat neutral α -mannosidases

Incubation products were analysed by h.p.l.c. as described in the Materials and methods section. Percentages were calculated from the counts in the peaks corresponding to each oligosaccharide; ND, not detected. The number in parentheses indicates the number of isomeric products that were identified.

	Bovine α -mannosidase	Feline α -mannosidase
Man ₉ GlcNAc ₂	60	41.3
Man ₈ GlcNAc ₂	33 (2)	30.8 (3)
Man ₇ GlcNAc ₂	7 (1)	15.1 (1)
Man ₆ GlcNAc ₂	ND	8.9 (2)
Man ₅ GlcNAc ₂	ND	3.9*

* Not enough material could be obtained to identify the product(s).

Scheme 3. Major pathway for hydrolysis of mannose residues from undecasaccharide (9) by bovine neutral α -mannosidase

For further degradation of compound (8a), see Scheme 4.

Scheme 4. Major pathway for hydrolysis of mannose residues from undecasaccharide (9) by cat neutral α -mannosidase

Compounds arising from further breakdown of compounds (8a, 6a and 6b) were not identified owing to the small amounts of material available.

Scheme 5. Major pathways for hydrolysis of mannose residues from decasaccharide (8a) and octasaccharide (6a) by bovine neutral α -mannosidase

Fig. 4. Acetolysis of oligosaccharides resulting from the incubation of ^3H -labelled decasaccharide (8a) with bovine neutral α -mannosidase

Fractions corresponding to the peaks in the h.p.l.c. profile of the incubation products were subjected to peracetylation, acetolysis for 4 h at 33 °C and *O*-deacetylation, and the acetolysis products identified by comparison of the elution times with those of reduced reference compounds. (a) Man₉GlcNAc₂; (b) Man₈GlcNAc₂; (c) Man₇GlcNAc₂; (d) Man₆GlcNAc₂. The elution times vary between panels because the proportions of acetonitrile/ KH_2PO_4 were varied to optimize the resolution; the proportions were 73:17 (a and b) and 5:1 (c and d). In each panel the late peak corresponds to the undegraded starting material. The occurrence of at least two acetolysis products from each fraction results from the differential rates of cleavage for the core and external $\alpha(1 \rightarrow 6)$ -mannosyl linkages, so that each profile is diagnostic for the structures shown in Scheme 5.

Scheme 6. Major pathway for hydrolysis of mannose residues from deca-saccharide (8a) by feline neutral α -mannosidase

Composite derived from the results of separate digestions of compounds (8a) and (6a).

Man₃GlcNAc₂ isomers [see Fig. 2 and De Gasperi *et al.* (1991)]. The structures of the two Man₄GlcNAc₂ isomers were found to correspond to compounds (4a) and (4e). The structural assignment was based on co-chromatography with authentic standards and by the fact that compound (4a) was sensitive to hydrolysis by endo-*N*-acetyl- β -glucosaminidase H, whereas compound (4e) was resistant to this enzyme (Warren *et al.*, 1988b). Both compounds (4a) and (4e) were further hydrolysed by the neutral α -mannosidases to yield only a single Man₃GlcNAc₂ isomer. This intermediate was found to correspond to pentasaccharide (3b), on the basis of co-chromatography with an authentic (3b) standard and by its sensitivity to endo-*N*-acetyl- β -glucosaminidase H and resistance to endo-*N*-acetyl- β -glucosaminidase D (Warren *et al.*, 1988b). As expected, compound (3b) was then degraded to yield (2b) and finally ManGlcNAc₂. Interestingly, we found that the hydrolysis of compound (3b) was a rate-limiting step in the degradation of compound (5a) (see Fig. 3a), since this oligosaccharide resisted rapid hydrolysis. This was confirmed by a separate time-course experiment with the bovine enzyme (see Fig. 3b). In a 30 min incubation, pentasaccharide (3b) was only 8 % hydrolysed, much less than that observed for (3a).

Digestion of substrates from high-mannose glycans

Although the bovine and cat neutral α -mannosidases had very similar specificities toward substrates derived from complex and hybrid glycans, major differences in their specificities were observed when substrates containing high-mannose glycan structures were tested. Since the incubations were carried out in the presence of swainsonine and deoxymannojirimycin, it is unlikely that the observed differences are due to the presence of other contaminating α -mannosidases, the presence of which could theoretically have gone undetected in our initial characterization of the enzyme preparations. These differences are more

likely therefore to represent genuine differences in the substrate specificities for the neutral α -mannosidases, which would have gone undetected by the traditional characterization performed with only the synthetic substrates.

Man₆GlcNAc₂ (9), which corresponds to the largest high-mannose glycan normally found in mammalian glycoproteins, was found to be quite resistant to the action of both bovine and cat neutral α -mannosidases, even with multiple enzyme addition and prolonged incubations. After 24 h, this oligosaccharide was degraded 60 % by the cat α -mannosidase and only 40 % by the bovine enzyme (Table 3). In both cases the major product was Man₆GlcNAc₂. With the cat enzyme, further cleavage down to Man₅GlcNAc₂ was observed (see below), whereas with the bovine enzyme only a small amount of Man₇GlcNAc₂ could be detected. As shown in Scheme 3, two Man₆GlcNAc₂ isomers were formed by the bovine enzyme, a minor product, (8a), and a major product, (8b). Interestingly, (8b) was also the product of digestion of compound (9) with the bovine lysosomal α -mannosidase and was also inefficiently degraded by this enzyme (De Gasperi *et al.*, 1991). Further hydrolysis by bovine neutral α -mannosidase yielded a single Man₇GlcNAc₂ isomer (7a), since the enzyme did not seem to be able to remove any of the (1 $\rightarrow$ 2)-linked mannose residues on the branch linked (1 $\rightarrow$ 3) to the core mannose residue (see below). Furthermore, because the major Man₆GlcNAc₂ isomer (8b) was resistant to further attack, the overall breakdown of compound (9) was very inefficient. In contrast with the bovine enzyme, the cat neutral α -mannosidase cleaved mannose residues from compound (9) in a randomized fashion, so that all the three possible Man₆GlcNAc₂ isomers were formed (see Scheme 4). However, further hydrolysis yielded only one Man₇GlcNAc₂ species (7c) from which two Man₆GlcNAc₂ isomers were formed (6a and 6b). Cleavage of further mannose residues from Man₆GlcNAc₂ was inefficient, so that the material obtained was not enough for characterization.

In contrast with compound (9), deca-saccharide (8a), with a structure commonly found in glycoproteins (Kobata, 1984), was more readily degraded by the neutral α -mannosidases. Again the bovine enzyme hydrolysed compound (8a) by a definite route, whereas the cat enzyme hydrolysed it in a more random way. The pathway for degradation of compound (8a) by the bovine α -mannosidase is shown in Scheme 5, and Fig. 4 illustrates the determination of the structures of the digestion intermediates by acetolysis. The final product was heptasaccharide (5b).

In contrast, the cat neutral α -mannosidase cleaved deca-saccharide (8a) to yield both $\text{Man}_7\text{GlcNAc}_2$ isomers (7a) and (7b), from which only one octasaccharide (6a) was formed (Scheme 6). This oligosaccharide, although structurally related to (5a) by the presence of an α -(1 $\rightarrow$ 2)-linked mannose residue on the (1 $\rightarrow$ 3) branch, was hydrolysed by what appeared to be a non-specific pathway into complex mixtures of $\text{Man}_5\text{GlcNAc}_2$, $\text{Man}_4\text{GlcNAc}_2$, and $\text{Man}_3\text{GlcNAc}_2$ isomers. It is noteworthy that among the Man_3 isomers formed, (3a), the major oligosaccharide stored in cat α -mannosidosis (Abraham *et al.*, 1986; Warren *et al.*, 1988a), was not formed in any appreciable amount. In regard to its activity towards high-mannose glycans, the cat neutral α -mannosidase appears to behave similarly to the soluble endoplasmic-reticulum α -mannosidase from rat liver (Bischoff & Kornfeld, 1986), which degraded compound (9) to a random mixture of Man_6 , Man_7 , and Man_8 isomers.

At first sight, the formation of the heptasaccharide (5b) from high-mannose glycans by the bovine neutral α -mannosidase might seem to offer an explanation for the storage of the similar compound (5b') in bovine α -mannosidosis (Abraham *et al.*, 1983; Daniel *et al.*, 1985), because compound (5b') could theoretically be derived from compound (5b) by the action of an endo-*N*-acetylglucosaminidase. Our previous study of the substrate specificity of the bovine lysosomal α -mannosidase did not provide an explanation for its accumulation (De Gasperi *et al.*, 1991), because compound (5b) was not a digestion product from any class of glycan, and being readily degraded by the lysosomal activity, would be easily hydrolysed by any residual α -mannosidase. Compound (5b') is also stored in the tissue of cats with α -mannosidosis (Abraham *et al.*, 1986; Warren *et al.*, 1988a), yet compound (5b) is not a major product of the action of cat neutral α -mannosidase on any high-mannose glycan and, furthermore, it can be degraded by the cat enzyme (see below). Therefore the properties of the cat neutral α -mannosidase are not consistent with its playing a significant role in the accumulation of compound (5b') in α -mannosidosis, and this casts a doubt on the likelihood that the bovine neutral enzyme is functioning in this way either. Hexasaccharide (5b') was also found to be the end product of the degradation of compound (9') by human (Al Daher *et al.*, 1992) and rat (Haeuw *et al.*, 1991) neutral α -mannosidases along a defined pathway. Therefore the bovine, human and rat enzymes all degraded high-mannose glycans to yield oligosaccharides with structures similar to the biosynthetic intermediate $\text{Man}_5\text{GlcNAc}_2$ -PP-dolichol (Kornfeld & Kornfeld, 1985).

Digestion of substrates resembling biosynthetic intermediates

Hexasaccharide (5b'), as discussed above, was resistant to the action of bovine neutral α -mannosidase. In contrast, it was degraded by the cat enzyme to yield mainly $\text{Man}_4\text{GlcNAc}$, (4b'). When the incubation was performed in the presence of swainsonine, further hydrolysis yielded compound (3a'), but surprisingly, in the absence of swainsonine, the major tetrasaccharide product was compound (3c'). This was the only case in the study where the presence of swainsonine altered the course of events, but importantly, the result cannot be explained by contamination of the enzyme preparation with lysosomal α -mannosidase, be-

cause, if this was the case, the contamination would have been observed also with other substrates. Compounds (3c') and (4c'), which are both stored in human α -mannosidosis (Yamashita *et al.*, 1980; Matsuura *et al.*, 1981), were quite resistant to the action of bovine neutral α -mannosidase, a result that agrees with earlier findings for the rat enzyme (Tulsiani & Touster, 1987), but were also slowly hydrolysed by the cat enzyme.

From the results discussed above, it can be concluded that the neutral α -mannosidases isolated from bovine and cat liver are able to degrade a wide variety of oligomannosyl glycans efficiently. As was determined for the lysosomal α -mannosidases from the same sources (De Gasperi *et al.*, 1991), the enzymes displayed no apparent specificity for (1 $\rightarrow$ 6), (1 $\rightarrow$ 3) or (1 $\rightarrow$ 2) linkages, or for 'branched' versus 'linear' structures. For some substrates the reactions were faster than for the lysosomal activities, and usually proceeded more readily to completion, in the sense that the ultimate product was the trisaccharide $\text{Man}\beta(1 \rightarrow 4)\text{GlcNAc}\beta(1 \rightarrow 4)\text{GlcNAc}$ rather than the tetrasaccharide (2b), containing a residual α -(1 $\rightarrow$ 6)-linked mannose, obtained with the lysosomal enzyme. The behaviour of the cat neutral enzyme towards smaller substrates, i.e. those with less than six mannose residues, derived from hybrid and complex glycans, was very similar to that of the bovine neutral α -mannosidase, and both were very different from the lysosomal α -mannosidases. However, significant differences between the bovine and cat neutral α -mannosidases were noted for some classes of oligosaccharide substrates. Thus substrates having α -(1 $\rightarrow$ 2)-linked mannose residues on the (1 $\rightarrow$ 3) branch, like compounds (5b) or (5b'), were resistant to the action of the bovine enzyme, but were degraded by the cat enzyme, and larger substrates, i.e. those with more than six mannose residues, were degraded at different rates and along different pathways.

The marked differences in specificity observed between the cytosolic and lysosomal α -mannosidases in both cattle and cats illustrate the value of using natural substrates to differentiate between mannosidase activities. Furthermore, in this study we have shown that the specificities of the neutral α -mannosidases, prepared from bovine and cat liver, toward oligomannosyl glycans containing different patterns of linkages and branching, are quite different, suggesting that the analogous isoenzymes may have different functions in different species. Differences in specificity between the human neutral α -mannosidase and both the corresponding bovine and cat enzymes support this conclusion (Al Daher *et al.*, 1992). This distinction would have been difficult to achieve with artificial substrates.

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