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SPECIFICITY STUDIES ON α-MANNOSIDASES USING OLIGOSACCHARIDES FROM MANNOSIDOSIS URINE AS SUBSTRATES

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Summary

Oligosaccharides containing terminal non-reducing α(1 → 2)-, α(1 → 3)-, and α(1 → 6)-linked mannose residues, isolated from human and bovine mannosidosis urines were used as substrates to test the specificities of acidic α-mannosidases isolated from human and bovine liver. The enzymes released all the α-linked mannose residues from each oligosaccharide and were most effective on the smallest substrate. Enzyme A in each case was less active on the oligosaccharides than α-mannosidase B_2, even though the apparent K_m value for the substrates was the same with each enzyme. The human acidic α-mannosidases were also found to be more active on substrates isolated from human rather than bovine mannosidosis urine. Human α-mannosidase C, which has a neutral pH optimum when assayed with a synthetic substrate, did not hydrolyse any of the oligosaccharides at neutral pH, but was found to be active at an acidic pH.

Introduction

Mannosidosis is a lysosomal storage disorder caused by the simultaneous loss of two types of acidic α-mannosidase (pH optimum between 4.0 and 4.5) designated as A and B. A third type of α-mannosidase, called the C form, with a pH optimum between 6.0 and 6.5 is unaffected in the disease [1]. Mannose-containing oligosaccharides have been shown to accumulate in tissues [2] and are also excreted in urine as a result of these enzyme deficiencies [3,4]. An almost identical situation regarding these enzymes and their deficiencies prevails also in mannosidosis of Angus Cattle [5]. However, the most abundant oligosaccharide isolated from calf mannosidosis urine is different from those obtained from human urine [6]. A study of the distribution of α-mannosidases

Abbreviations: Man*p*, mannopyranoside; GlcNAc, 2-acetamido-2-deoxyglucose.

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in different human tissues has shown that form B can be resolved further into two acidic components B_1 and B_2. The activities of A, B_2 and C were found to be high in liver [7]. Consequently this tissue was chosen as a source of these enzymes for the purpose of studying their specificities using isolated mannose-rich oligosaccharides as substrates. The acidic enzymes from calf liver were also isolated for an identical study.

Materials

A post-mortem sample of human liver was obtained 10 h after death. Calf liver was purchased from a slaughter house. Both samples were stored at −20° C. Sephadex G-200 fine (Pharmacia Fine Chemicals, Uppsala, Sweden), DEAE- and CM-celluloses (Types 52, W. and R. Balston, Maidstone, Kent, U.K.), NaB^3H_4 and Aquasol (New England Nuclear, Boston, Mass., U.S.A.), Dowex 50 (Serva Feinbiochemica, Heidelberg, West Germany), Bio-Gel AG 3-X4A (BioRad Laboratories, Richmond, Calif., U.S.A.), human serum albumin and neuraminidase from *Clostridium perfringens* Type VI (Sigma Chemical Co., Saint Louis, Mo., U.S.A.), and 4-methylumbelliferyl-α-D-Man*p* (Koch-Light Laboratories, Colnbrook, Bucks., U.K.) were obtained commercially. The natural substrates α-D-Man*p*-(1 → 3)-β-D-Man*p*-(1 → 4)-D-GlcNAc (M_2 G), α-D-Man*p*-(1 → 2)-α-D-Man*p*-(1 → 3)-β-D-Man*p*-(1 → 4)-D-GlcNAc (M_3 G), and α-D-Man*p*-(1 → 2)-α-D-Man*p*-(1 → 2)-α-D-Man*p*-(1 → 3)-β-D-Man*p*-(1 → 4)-D-GlcNAc (M_4 G) were isolated from human mannosidosis urine [3,4]. α-D-Man*p*-(1 → 6)-β-D-Man*p*-(1 → 4)-β-D-GlcNAc*p*-(1 → 4)-β-D-GlcNAc*p*-(1 → 4)-β-D-GlcNAc (M_2 G_3) was isolated from calf mannosidosis urine [6].

Methods

Enzyme assays

For the determination of α-mannosidase activity during purification 100 μl of the enzyme solution was incubated at 37° C for 30 min with 100 μl of a 1.6 mM solution of 4-methylumbelliferyl-α-D-Man*p* in citrate/phosphate buffer (100 mM citric acid and 200 mM $NaH_2PO_4 \cdot 2H_2O$) at pH 4.0 or 6.0. The reaction was stopped by the addition of 3 ml of 250 mM glycine/NaOH buffer, pH 10.4, and the fluorescence measured using an Aminco-Bowmann spectrofluorimeter (excitation wavelength 348 nm, emission wavelength 450 nm). The protein in the column fractions was measured by reading the absorbance at 280 nm, and by the method of Lowry et al. [8] for pooled fractions using human serum albumin as standard.

Partial purification of the acidic α-mannosidases

Procedure I. The details given here apply to the purification of α-mannosidases A and B_2 from human liver. All enzyme purification work was carried out at 4° C unless otherwise stated. Human liver (150 g) was diced and homogenized in 1 l of redistilled water. The homogenate was stirred for 30 min before centrifugation at 43 000 × *g* for 1 h. The supernatant (1010 ml) was adjusted to pH 4.0 by dropwise addition of 53.0 ml of 500 mM citrate buffer, warmed at 37° C for 1 h and centrifuged at 43 000 × *g* for 30 min. The

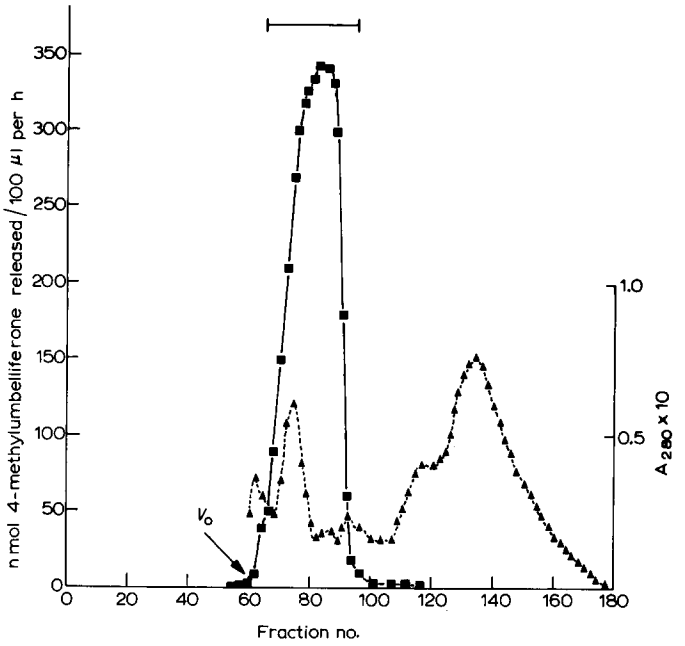

Fig. 1. Sephadex G-200 chromatography of human liver acidic α-mannosidase. 55 ml of the concentrated enzyme fraction from the citrate buffer step were applied to a column of Sephadex G-200 (5 × 100 cm, V_0 = 590 ml) previously equilibrated with 50 mM $NaH_2PO_4/NaHPO_4$ buffer, pH 6.0, containing 150 mM NaCl. Fractions (10 ml) were eluted with the same buffer at a flow rate of 55 ml/h. The fractions indicated by the horizontal bar were pooled. ■———■, α-mannosidase activity at pH 4.0; ▲- - - - -▲, protein (A_{280nm}).

supernatant (1040 ml) was concentrated to 55 ml using an Amicon ultrafiltration unit fitted with a UM-10 membrane filter and then applied to a column of Sephadex G-200. Eluted fractions were assayed for α-mannosidase activity (Fig. 1). The fractions containing the enzyme activity were pooled (306 ml)

TABLE I

PURIFICATION OF ACIDIC α-MANNOSIDASES FROM HUMAN LIVER (PROCEDURE I)

Fraction	Volume (ml)	Total activity (nmol/h × 10^5)	Total protein (mg)	Specific activity (nmol/mg per h)	Purification-fold	Recovery (%)
Homogenate	1150	218.5	28 750	760	1	100
Supernatant	1040	205.9	13 520	1523	2	94
pH 4.0 supernatant	55	207.9	9130	2277	3	94
Sephadex G-200	22	147.5	462	31 927	42	67.5
DEAE-cellulose, pH 6.0						
(a) α-Mannosidase A	5	18	40.5	44 444	58.5	8.2
(b) α-Mannosidase B_2	7.5	36	63	57 143	75.2	16.5
CM-cellulose, pH 5.0						
α-Mannosidase A	4	9.1	6	151 667	200	4.2
DEAE-cellulose, pH 7.0						
α-Mannosidase B_2	7	32.9	23.1	142 424	187	15.1

and concentrated to 23 ml. This was dialysed against 10 mM NaH_2PO_4/NaOH buffer, pH 6.0, and applied to a DEAE-cellulose column (2.5 × 40 cm), pre-equilibrated with the same buffer. Fractions (10 ml) were eluted with this buffer for 8 h and then with a linear salt gradient (0—150 mM NaCl in buffer) at a flow rate of 55 ml/h. Fractions containing α-mannosidase A activity (192 ml) and B_2 activity (411 ml) were pooled and concentrated to 5 and 7.5 ml, respectively. For further purification, α-mannosidase A was dialysed against 10 mM NaH_2PO_4/NaOH buffer, pH 5.0, and applied to a column (1.6 × 20 cm) of CM-cellulose. Fractions (7 ml) were collected at a flow rate of 55 ml/h for 4 h with the same buffer and then with a salt gradient as before and the pooled enzyme activity was concentrated to 4 ml. α-Mannosidase B_2 was dialysed against the same buffer at pH 7.0 before chromatography on DEAE-cellulose at this pH. Samples were eluted as for enzyme A. The pooled activity was concentrated to 7 ml. The final enzyme preparations were dialysed against the 10 mM NaH_2PO_4/NaOH buffer at pH 6.0. The purification is shown schematically in Table I. The same schedule was also followed for the acidic α-mannosidases A and B_2 from calf liver which were purified 300- and 240-fold, respectively.

Partial purification of acidic and neutral α-mannosidases

Procedure II. Approx. 70% of the neutral α-mannosidase activity found in human liver is very labile [7]. A greater loss of the neutral activity was observed during the citrate buffer precipitation stage used in the purification of the acidic enzymes (procedure I). In order to isolate the residual form of α-mannosidase C in a higher yield, 25 g of human liver were homogenized in 60 ml of 10 mM NaH_2PO_4/NaOH buffer, pH 6.0, and 45 ml of the supernatant obtained after centrifugation at 43 000 × *g* for 1 h were applied to a column of DEAE-cellulose at pH 6.0. The subsequent procedure was as described before. The fractions containing enzymes A, B_2 and C were pooled separately and dialysed against the same buffer. In addition, fractions X (between α-mannosidases A and B_1) and Y (between B_2 and C), which had very little or no activity against the synthetic substrate [7] were pooled and concentrated.

Incubation of α-mannosidase forms with oligosaccharides isolated from mannosidosis urine

The enzymes were stable at 4°C for up to 6 weeks when stored in 10 mM NaH_2PO_4/NaOH buffer, pH 6.0. In order to compare the different enzymes, the preparations were diluted appropriately to achieve similar enzyme activities (100 µl of the human enzymes A and B_2 released approx. 27 000 nmol 4-methylumbelliferone/h and the bovine enzymes A and B_2 released approx. 40 000 nmol/h).

25 µl of a 10 mM oligosaccharide solution were incubated at 37°C for various times with 175 µl of 100 mM sodium acetate buffer, pH 4.5, and 100 µl of the purified acidic enzymes. After incubation, α-mannosidase A was removed from the reaction mixture by treatment with CM-52 at pH 5.0. To remove α-mannosidase B_2 or C, the pH was adjusted to 6.0 with 1 M NaOH before treatment with DE-52 at pH 6.0. The enzyme-free sample was evaporated to dryness using a rotatory evaporator (water bath temperature 40°C) and the residue was redissolved in 200 µl of 500 mM NaOH. 100 µl of a 5

mCi/nmol solution of NaB^3H_4 in 500 mM NaOH were added to the sample, which was left overnight at room temperature. Excess NaB^3H_4 was destroyed by addition of glacial acetic acid and the sample was desalted by passage through Dowex 50 (H^+) and Bio-Gel AG3-X4A (OH^-), and finally evaporated to dryness. Boric acid present in the mixture was removed by adding methanol (3 × 2 ml) and evaporating to dryness. The reduced product was then redissolved in 150 µl of water. Two 50-µl samples were chromatographed in ethyl acetate/acetic acid/water (3 : 1 : 1, v/v) for 18 h on Whatman No. 1 paper. The sugars in one sample were visualized using a silver dip reagent [9] and the corresponding areas from the other sample were cut out. The products were eluted from the papers with water and evaporated to dryness. The residue was dissolved in 2 ml of water and 1 ml transferred to a vial containing 10 ml of Aquasol. The radioactivity in the sample was counted on a Nuclear Chicago Mark II liquid scintillation counter. The recoveries following this procedure were of the order of 90—95%. In the case of residual α-mannosidase C and enzymes A and B_2 obtained in procedure II from human liver, the enzyme activities were lower than those for the more purified fractions (3600 nmol of 4-methylumbelliferone released/100 µl per h). Consequently, the incubation times were increased to 48 h and the enzyme solution was added in two portions (200 µl/24 h). Bacterial growth in these incubations was prevented by adding NaN_3 to a final concentration of 0.01%.

Results

Specificity studies on human acidic α-mannosidases

The pH optimum for both enzymes was at pH 4.5 (Fig. 2). When α-mannosidases A and B_2 were incubated with the mannose-containing oligosaccharides for 24 h at 37°C, both enzymes were found to hydrolyse all the oligosaccharides (Table II). However, α-mannosidase B_2 was more active towards the substrates than enzyme A.

For the determination of apparent K_m, the enzymes were incubated for 10 h with M_2G and M_4G. Both enzymes had the same K_m of 5.5 mM using either substrate. However, the V values were different. The ratios of V for α-mannosidase B_2 to that of A were 1.7 for M_2G and 3.4 for M_4G (Table II).

Effect of Zn^{2+} and neuraminidase treatment on human acidic α-mannosidases

To test for the effect of Zn^{2+}, M_2G and sodium acetate buffer at pH 4.0 were used, since a shift in the pH optimum in the presence of this metal ion has earlier been reported [10]. A 6-fold increase in the activities of the enzymes was found when the Zn^{2+} concentration was 5 mM. Higher concentrations of this metal were found to inhibit the enzyme activities.

Human α-mannosidases A and B_2 were also incubated with M_2G after treatment with neuraminidase as described previously [7]. In each case the asialo enzyme was equally active towards the natural substrate as the sialylated form.

Specificity studies on bovine liver acidic α-mannosidases

α-Mannosidases A and B_2, purified from calf liver, were incubated for

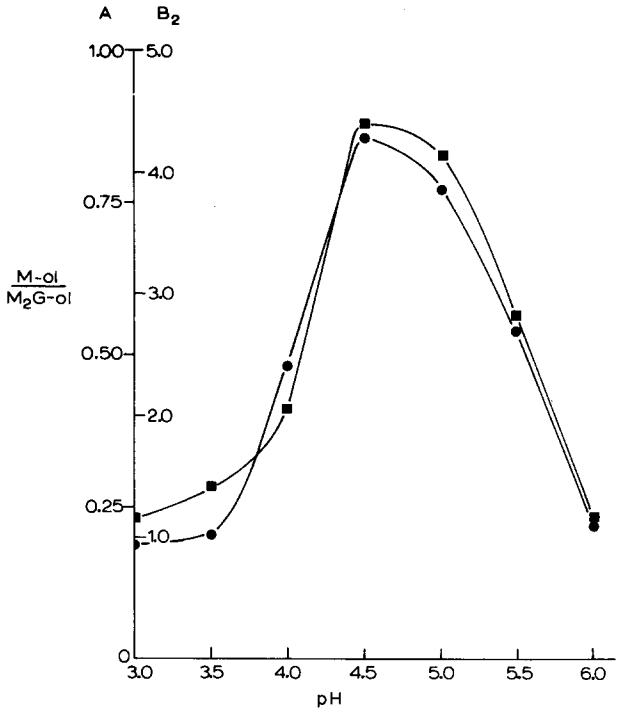

Fig. 2. Effect of pH on the activity of human liver acidic α-mannosidases. 25 μl of a 10 mM solution of M_2G were incubated at 37°C for 10 h in the presence of 175 μl of 100 mM sodium acetate buffer (ranging in pH from 3.0 to 6.0) and 100 μl of purified α-mannosidases A and B_2 (each enzyme liberated approx. 27 000 nmol of 4-methylumbelliferone/100 μl per h). The rest of the procedure was as described in Methods. ■———■, α-mannosidase A (scale under A); ●———●, α-mannosidase B_2 (scale under B_2). M-ol, α-D-mannitol; M_2G-ol, α-D-Man*p*-(1 → 3)-β-D-Man*p*-(1 → 4)-*N*-acetyl-D-glucosaminitol.

TABLE II

SPECIFICITY STUDIES ON HUMAN AND BOVINE ACIDIC α-MANNOSIDASES

Substrate	Human acidic α-mannosidases					Bovine acidic α-mannosidases				
	% hydrolysis		K_m* (mM)	VB_2/VA	M-ol/MG-ol**	% hydrolysis		K_m* (mM)	VB_2/VA	M-ol/MG-ol**
	A	B_2				A	B_2			
M_2G	55	95	5.5	1.7	1.0	37	64	5.2	1.7	1.0
M_3G	35	90	—	—	2.0	28	53	—	—	2.0
M_4G	27	90	5.5	3.4	3.0	20	37	4.9	1.7	3.0
M_2G_3	10	35	—	—	1.0***	—	—	—	—	—
4-MU-α-D-Man*p*†	—	—	2.5	2.2	—	—	—	2.8	1.7	—

* The K_m values given are apparent K_m values calculated by the method of Lineweaver and Burk [11].

** M-ol, α-D-mannitol; MG-ol, β-D-Man*p*-(1 → 4)-*N*-acetyl-D-glucosaminitol.

*** The ratio expressed is that of M-ol to MG_3-ol(β-D-Man*p*-(1 → 4)-β-D-GlcNAc*p*-(1 → 4)-β-D-GlcNAc*p*-(1 → 4)-*N*-acetyl-D-glucosaminitol).

† 4-MU-α-D-Man*p*, 4-methylumbelliferyl-α-D-mannopyranoside.

24 h with the mannose-containing oligosaccharides obtained from human mannosidosis urine. Both enzymes were found to hydrolyse all these substrates but to different extents (Table II).

Enzyme B_2 was found to be somewhat more active than enzyme A. Furthermore, both enzymes were most active towards the smallest oligosaccharide. The apparent K_m for M_2G and M_4G with each enzyme was about 5 mM and is therefore apparently identical to that with the human enzymes. However, the ratio for the V of α-mannosidase B_2 to that of A was 1.7 for both the substrates examined.

Specificity studies on human liver α-mannosidase C

This enzyme has a pH optimum of 6.0 when 4-methylumbelliferyl-α-D-Man*p* is used as the substrate [7] but it had very little activity on any of the mannosidosis oligosaccharides at pH 6.0 (2—3% hydrolysis). In contrast, the enzyme was found to be more active on these substrates when incubated at pH 4.5 (M_2G, M_3G, M_4G, and M_2G_3 were hydrolysed to 20, 15, 15, and 25% respectively). Under the same conditions α-mannosidases A and B_2 from the same liver preparation hydrolysed M_2G to 55 and 92%, respectively. No hydrolysis of M_2G was obtained when fractions X and Y, taken from the intermediate regions between the three α-mannosidase peaks, were incubated either at pH 4.5 or 6.0.

The neutral enzyme was also treated with neuraminidase and separated from any contaminating acidic α-mannosidase by chromatography on DE-52. Even after this treatment the neutral enzyme was found to hydrolyse 15% of M_2G at pH 4.5.

Discussion

The simultaneous loss of two types of acidic α-mannosidase in human mannosidosis [1] results in the urinary excretion of oligosaccharides with terminal α(1 → 2)- and α(1 → 3)-linked mannose residues [3,4]. However, both enzymes appear to hydrolyse each type of linkage. The two enzymes can also cleave the terminal α(1 → 6)-linked mannose residue found in the oligosaccharide isolated from bovine mannosidosis urine. The findings that the ratios of reduced M to MG in the human oligosaccharides M_2G, M_3G, and M_4G and reduced M to MG_3 in the bovine oligosaccharide M_2G_3 were 1,2,3 and 1, respectively, indicated that no β-mannosidase activity was present in the purified enzymes. Thus the α-mannosidases, though non-specific with respect to the mannosidic linkages studied, are stereospecific.

The possibility that α-mannosidases of the A and B types are under the same genetic control has been discussed before [5,7]. Our findings support this hypothesis. However, the need for two types of enzymes to accomplish the same purpose is not clear. The only difference observed between the two types of enzymes was that α-mannosidase A appeared to be less effective against the natural substrates than enzyme B_2 and that its effectiveness decreased when the molecular size of the substrate increased, even though the affinity of the enzyme for each substrate was unaltered. When the acidic enzymes were treated separately with neuraminidase, the asialo derivative of each enzyme was

found to be as active towards M_2 G as its sialylated form. This suggests that the active native enzyme may undergo sialylation or conformational changes in order to facilitate transportation rather than to achieve a specific enzymatic function.

Both the human acidic enzymes were found to cleave the terminal $\alpha(1 \rightarrow 6)$-linked mannose residue from the bovine mannosidosis urine pentasaccharide (M_2 G_3) but to a lesser extent than the $\alpha(1 \rightarrow 2)$-linked mannose from the human mannosidosis urine pentasaccharide (M_4 G). Similarly, the hydrolysis of the human oligosaccharides by the bovine α-mannosidases was less than that expected from their activities with the synthetic substrate, even though the K_m values for the bovine enzymes were very similar to those obtained for the human enzymes. It is probable that these differences are due to slight differences in the active sites of the enzymes from the two species.

The presence of α-mannosidase C in mannosidosis tissue [1] and its cytoplasmic localization [12] would suggest that this enzyme is not involved in the degradation of the mannose-containing oligosaccharides. However, our findings show that the residual α-mannosidase C is active against the oligosaccharides at pH 4.5 but not at pH 6.0, which is the pH optimum of this enzyme with the synthetic substrate [7]. One possible explanation of this observation is that an acidic α-mannosidase of the B type co-elutes during ion-exchange chromatography in the same region as α-mannosidase C and is responsible for the hydrolysis of the oligosaccharides. α-Mannosidase B, after neuraminidase treatment has been shown to be excluded from DE-52, whereas the neutral enzyme is unaffected [7]. Consequently, the neutral enzyme was treated by neuraminidase to remove any contaminating acidic α-mannosidase, but was still found to have activity against M_2 G at pH 4.5. However, it is unlikely that the major function of this enzyme in vivo is the cytoplasmic degradation of mannose-containing oligosaccharides.

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