Characterization of Human Liver α-D-Mannosidase Purified by Affinity Chromatography

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Human liver acidic α-D-mannosidase was purified 1400-fold by a relatively short procedure incorporating chromatography on concanavalin A–Sepharose and affinity chromatography on Sepharose 4B–ε-aminohexanoylmannosylamine. In contrast with the acidic enzymic activity the neutral α-mannosidase did not bind to the concanavalin A–Sepharose so the two types of α-mannosidase could be separated at an early stage in the purification. The only significant glycosidase contaminant after affinity chromatography on the mannosylamine ligand was α-L-fucosidase, which was selectively removed by affinity chromatography on the corresponding fucosylamine ligand. The final preparation was free of other glycosidase activities. The pI of the purified enzyme was increased from 6.0 to 6.45 on treatment with neuraminidase. Although the pI and the mol.wt. (220000) suggested that α-mannosidase A had been purified selectively, ion-exchange chromatography on DEAE-cellulose indicated that the preparation consisted predominantly of α-mannosidase B. This discrepancy is discussed in relation to the basis of the multiple forms of human α-mannosidase. The purified enzyme completely removed the α-linked non-reducing terminal mannose from a trisaccharide isolated from the urine of a patient with mannosidosis. A comparison of the activity of the pure enzyme towards the natural substrate and synthetic substrates suggests that the same enzymic activity is responsible for hydrolysing all the substrates. These results validate the use of synthetic substrates for determining the mannosidosis genotype. They are also further evidence that mannosidosis is a lysosomal storage disease resulting from a deficiency of acidic α-mannosidase.

The widespread occurrence of α-linked D-mannose in mammalian glycoproteins and the recognition that the lysosomal storage disease, mannosidosis, results from a deficiency of acidic α-D-mannosidase (EC 3.2.1.24) have created interest in the characterization and purification of mammalian acidic α-D-mannosidase. Purification procedures for the enzyme from several mammalian sources have been described (Snaith & Levvy, 1969; Bosmann & Hemsworth, 1971; Okumura & Yamashina, 1973; Dewald & Touster, 1973). However, particular interest lies in the activity from humans and cattle, because mannosidosis has only been described in these two species (Öckerman, 1967; Hocking *et al.*, 1972). Previously we have described the general properties of the enzyme in these two species (Phillips *et al.*, 1974a,b) and have shown that the two forms, A and B, of human acidic α-mannosidase are immunologically identical (Phillips *et al.*, 1975). A preparation which had been purified 200-fold by conventional techniques was used as the antigen in these experiments, but it was still contaminated with α-L-fucosidase (EC 3.2.1.51) and to a lesser extent with β-D-N-acetylhexosaminidase (EC 3.2.1.30). A different strategy is necessary to remove these

closely related glycosidases. The observation that lysosomal hydrolases bind to concanavalin A–Sepharose (Bishayee & Bachawat, 1974; J. D. Hocking, personal communication) has been exploited to isolate and concentrate the lysosomal enzymes from a crude human liver extract. The development of affinity chromatography media for α-L-fucosidase (Robinson & Thorpe, 1974a) and α-D-mannosidase (Robinson *et al.*, 1975) has provided the means for the fractionation of this mixture of lysosomal enzymes. The present paper describes a procedure incorporating chromatography on concanavalin A–Sepharose followed by affinity chromatography by which human liver α-mannosidase can be purified free of other glycosidases.

Synthetic substrates have been very useful for detecting inborn errors of metabolism due to enzymic deficiencies. For unequivocal diagnosis it is necessary to demonstrate that the enzymic activity detected with such a substrate is capable of transforming the natural substrate. The activity of the purified α-mannosidase towards synthetic substrates has therefore been compared with its activity towards the presumed natural substrate, a trisac-

charide isolated from the urine of a patient with mannosidosis. The validity of using synthetic substrates to detect persons heterozygous or homozygous for the mannosidosis genotype is discussed.

Materials and Methods

Enzyme assays

Acidic and neutral α-D-mannosidase were assayed by using the fluorigenic substrate 4-methylumbelliferyl α-D-mannopyranoside (Koch–Light Laboratories, Colnbrook, Bucks., U.K.) as described previously (Phillips et al., 1974a). One unit of activity is that amount of enzyme that transforms $1 \mu mol$ of substrate/min under these conditions. The effect of Zn^{2+} and EDTA on the α-mannosidase activity was investigated by including 1 mM-$ZnSO_4$ and 1 mM-EDTA in the substrate solutions, giving a final concentration of 0.5 mM in the incubation mixture. The McIlvaine (1921) phosphate/citrate buffer system was used to investigate the pH-dependence of the activity. The purified enzyme was also assayed by using the chromogenic substrate, p-nitrophenyl α-D-mannopyranoside (Koch–Light) by a modification of the procedure of Conchie et al. (1959). β-D-Mannosidase (EC 3.2.1.25) was assayed by using p-nitrophenyl β-D-mannopyranoside (Koch–Light) (Phillips et al., 1975). Other glycosidases were assayed during the purification procedure by using the appropriate fluorigenic substrate (Koch–Light) and the following procedures; α-L-fucosidase (Robinson & Thorpe, 1974b), N-acetyl-β-D-glucosaminidase (Leaback & Walker, 1961) and β-D-galactosidase (EC 3.2.1.23) and β-D-glucosidase (EC 3.2.1.21) (Price & Robinson, 1966).

Determination of mannose

Mannose was measured by a modification of the coupled enzymic procedure of Tarentino et al. (1970). Instead of measuring the total change in E_{340}, the initial rate of oxidation of NADH was measured by using an SP.1800 spectrophotometer thermostatically controlled at 37°C. To validate this procedure the rate of reaction was measured when known quantities of D-mannose dissolved in $20 \mu l$ of 0.2 M-sodium phosphate adjusted to pH 4.25 with 0.1 M-citric acid were added to the coupling mixture: 0.3 M-KCl, 0.3 ml; 0.1 M-$MgCl_2$, 0.1 ml; 0.002 M-ATP [Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.], 0.05 ml; 0.01 M-phosphoenolpyruvate (Sigma), 0.05 ml; 0.012 M-NADH (Sigma) in 1% $NaHCO_3$, 0.01 ml; yeast hexokinase [Boehringer Corp. (London) Ltd., London W5, U.K.], 3.8 units; rabbit muscle lactate dehydrogenase (Boehringer), 5.0 units; rabbit muscle

pyruvate kinase (Boehringer), 1.0 unit; 0.1 M-Tris/ HCl buffer, pH 8.0, 0.1 ml; water to produce a final volume of 1 ml. The rate of the reaction was found to be linear for at least 1 min and directly proportional to the amount of mannose in the $20 \mu l$ sample over the range 5–50 nmol. A standard curve relating the rate of decrease in E_{340} to the amount of mannose was constructed and standards were run every time the assay was used. The rates obtained when $20 \mu l$ of buffer alone or $20 \mu l$ of buffer containing $1 \mu mol$ of the trisaccharide storage product were added to the coupling mixture were very low.

Purification of acidic α-mannosidase

(1) *Initial fractionation.* Post-mortem human liver (1 kg), which had been stored at −20°C until required, was homogenized in 1.5 litres of 10 mM-sodium phosphate buffer, pH 6.0, in an MSE Ato-Mix at 4°C. The homogenate was centrifuged in the $6 \times 250 ml$ angle rotor of an MSE High-Speed 18 centrifuge for 30 min at 4°C and 25000g ($r_{av.}$ 14.2 cm). The material precipitating from the supernatant between 30 and 55% saturation with $(NH_4)_2SO_4$ was recovered by centrifugation at 37000g for 15 min and redissolved in 400 ml of 10 mM-sodium phosphate buffer, pH 6.8, containing 0.5 M-NaCl and 0.1 mM-$MgCl_2$, -$MnCl_2$ and -$CaCl_2$ and dialysed against 10 litres of the same buffer for 24 h at 4°C.

(2) *Chromatography on concanavalin A–Sepharose.* The dialysed material was chromatographed on a column (8 cm × 5 cm diam.) of concanavalin A–Sepharose [Pharmacia (G.B.) Ltd., London W.5, U.K.] which had been equilibrated in the same buffer. The column was eluted with the equilibration buffer at room temperature (18°C) at a flow rate of 200 ml/h until the E_{280} of the effluent became zero, when the material that had bound to the column was eluted with 500 ml of 0.5 M-methyl α-D-mannopyranoside in the equilibration buffer. The fractions (100 ml) were assayed for acidic and neutral α-mannosidase activity at pH 4.0 and 6.5 respectively. Those fractions containing the acidic α-mannosidase were pooled and adjusted to 60% saturation with respect to $(NH_4)_2SO_4$ by the addition of solid $(NH_4)_2SO_4$ at 4°C. The precipitate was collected by centrifugation at 37000g for 15 min., redissolved in 150 ml of 50 mM-sodium phosphate buffer, pH 6.0, and then dialysed against 20 litres of the same buffer for 24 h at 4°C.

(3) *Affinity chromatography on mannosylamine-substituted CH-Sepharose.* The affinity medium, D-mannosylamine linked to Sepharose 4B–ε-aminohexanoic acid (CH-Sepharose 4B; Pharmacia) by a carbodi-imide reaction, was prepared by the procedure of Robinson et al. (1975). It was equilibrated in 50 mM-sodium phosphate buffer, pH 6.0, and packed into columns (5 cm × 2.5 cm diam.). Dialysed material (50 ml) from chromatography

on concanavalin A–Sepharose was loaded on to each column. Elution was started with the equilibration buffer, and fractions (25 ml) were collected at a flow rate of 20 ml/h. When the E_{280} of the effluent fell below 0.1 elution was continued with a solution of 0.5 M-mannose in the same buffer. The fractions were assayed for acidic α-mannosidase and those containing enzymic activity specifically eluted by the mannose were pooled. The protein in these fractions was precipitated by $(NH_4)_2SO_4$, final saturation 60%, and redissolved in 10 ml of 50 mM-sodium phosphate buffer, pH 6.0. After dialysis against 10 litres of the same buffer at 4°C the preparation was re-assayed for α-mannosidase and several other glycosidases.

(4) *Affinity chromatography on agarose–ε-amino hexanoylfucosylamine.* As α-L-fucosidase was the only detectable glycosidase contaminant, this enzyme was removed from the preparation by chromatography on a column (5 cm × 2.5 cm diam.) of the corresponding fucosylamine affinity ligand, which had been prepared and provided by R. Thorpe (Robinson & Thorpe, 1974a). The column was equilibrated and elution started with 50 mM-sodium phosphate buffer, pH 6.0. Fractions (5 ml) were collected at a flow rate of 20 ml/h. All of the α-mannosidase but none of the α-L-fucosidase was recovered in the material that was unretarded by the column. Those fractions containing α-mannosidase were pooled and stored at 4°C.

Polyacrylamide-gel electrophoresis

Electrophoresis of samples from the various stages in the purification was carried out in 5% (w/v) polyacrylamide gels by using the discontinuous buffer system of Davis (1964). α-Mannosidase activity and proteins were detected on the gel surface as described previously (Phillips *et al.*, 1975).

Isoelectric focusing

The purified α-mannosidase was analysed by isoelectric focusing in an Ampholine type 8100 column (LKB Instruments, London S.E.20, U.K.) at 5°C (Vesterberg & Svensson, 1966; Phillips *et al.*, 1974a). Purified α-mannosidase (0.3 unit), which had been incubated with 10 units of neuraminidase from *Vibrio cholerae* (B grade, Calbiochem, Hereford, U.K.) in 2 ml of 50 mM-sodium phosphate buffer, pH 6.0, for 3 h at 37°C, was also analysed. The neuraminidase, which had been dialysed previously for 18 h against the buffer at 4°C to remove any low-molecular-weight contaminants, did not contain any α-mannosidase activity.

Ion-exchange chromatography

α-Mannosidase peaks A and B were prepared from an homogenate (20%, w/v) of human liver by

chromatography on DEAE-cellulose (Whatman DE 23; Whatman Biochemicals, Maidstone, Kent, U.K.) in 10 mM-sodium phosphate buffer, pH 6.0 (Phillips *et al.*, 1974a).

Determination of molecular weight by sucrose-gradient centrifugation

The molecular weights of various preparations of α-mannosidase were determined by sucrose-gradient centrifugation essentially by the method of Martin & Ames (1961). Samples (100 μl) in 10 mM-sodium phosphate buffer, pH 6.0, were layered over a linear 5–25% (w/v) sucrose gradient in 4.65 ml of the same buffer. Bovine liver catalase and yeast alcohol dehydrogenase (Sigma) were run as mol.wt. standards (244000 and 150000 respectively) in separate tubes in each experiment. Urease (type IV, Sigma), mol.wt. 492000, was run in one experiment. After centrifugation for 16 h at 4°C and 120000g ($r_{av.}$ 86.1 mm) in the 6 × 5 ml swing-out rotor of an MSE High-Speed 65 centrifuge, the gradients were fractionated by using an MSE Tube Piercer. The fractions (0.1 ml) were assayed for α-mannosidase or catalase and alcohol dehydrogenase as described by Martin & Ames (1961). The sucrose gradients were measured by using an Abbe 60 refractometer (Bellingham and Stanley, London N.15, U.K.). Estimates of the molecular weights were obtained by using the relationship:

$$\log\frac{d_1}{d_2} = \frac{2}{3}\log\frac{M_1}{M_2}$$

where d_1 and d_2 are the distances travelled from the meniscus by the unknown of mol.wt. M_1 and the standard of mol.wt. M_2 respectively.

Hydrolysis of natural substrate

A sample of the trisaccharide α-D-Man*p*(1→3)-β-D-Man*p*(1→4)-β-D-GlcNAc*p* isolated from the urine of a patient with mannosidosis (Nordén *et al.*, 1973) was provided by Dr. A. Lundblad (Department of Clinical Chemistry, University Hospital, Lund, Sweden). Purified α-mannosidase (0.1 ml, containing 0.03 unit) was diluted to 0.5 ml with 0.2 M-sodium phosphate adjusted to pH 4.25 with 0.1 M-citric acid and incubated at 37°C with 1 μmol of the trisaccharide dissolved in 0.5 ml of water. At suitable time-intervals the amount of mannose in 20 μl of the incubation mixture was measured by the modification of the procedure of Tarentino *et al.* (1970) described above. The pH-dependence of the hydrolysis of the trisaccharide was measured over the range 3.5–5.5 in the McIlvaine (1921) buffer system. The initial velocity of the reaction was measured at pH 4.25 at a series of trisaccharide concentrations and the kinetic parameters, K_m and V,

Table 1. *Purification of human liver acidic α-D-mannosidase activity*

Step	Specific activity (units/mg of protein)	Purification	Yield (%)
Homogenate	0.0019	1	100 (161 units)
Supernatant (25000g, 30min)	0.0033	1.74	86
Precipitate 30–55%-satd. (NH₄)₂SO₄	0.02	10.5	31
Chromatography on concanavalin A–Sepharose	0.136	72	27
Chromatography on mannosylamine-substituted CH-Sepharose 4B	1.08	570	3.6
Chromatography on agarose–ε-aminohexanoyl-fucosylamine	2.6	1370	3.6 (5.9 units)

calculated by the graphical method of Lineweaver & Burk (1934). K_m and V were also determined for the two synthetic substrates, 4-methylumbelliferyl α-D-mannopyranoside and p-nitrophenyl α-D-mannopyranoside, at pH 4.25.

Protein determination

Protein was measured by the Folin method (Lowry *et al.*, 1951) with bovine serum albumin as a standard.

Results

Purification of human liver acidic α-mannosidase

The acidic α-mannosidase from human liver was purified 1370-fold by the procedure shown in Table 1. The neutral α-mannosidase was separated from the acidic activity at an early stage in the purification by chromatography on concanavalin A–Sepharose (Fig. 1). The only detectable glycosidase contaminant after affinity chromatography on the mannosylamine ligand was α-L-fucosidase (0.25 unit/mg of protein). Analysis of the preparation at this stage by polyacrylamide-gel electrophoresis (Fig. 2a) showed that it contained several protein bands, two of which possessed α-L-fucosidase activity and another α-mannosidase activity. The α-L-fucosidase components and two other protein bands were removed by passing the preparation through the fucosylamine ligand affinity column (Fig. 2b and 2c). The final preparation consisted of a major protein band with α-mannosidase activity, and two minor protein bands which were enzymically inactive. It was stored at 4°C for 8 weeks without loss of activity.

Characterization of preparation

(1) *Isoelectric focusing.* The pI of the purified α-mannosidase was pH 6.0 (Fig. 3a). The acidic
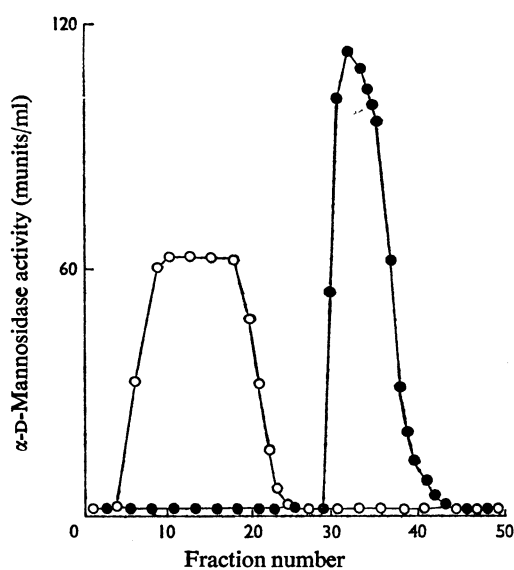

Fig. 1. *Chromatography of human liver α-mannosidase on concanavalin A–Sepharose*

The material precipitated from a crude liver extract between 30 and 55% saturation with (NH₄)₂SO₄ was applied to the column in 10mM-sodium phosphate buffer, pH 6.8, containing 0.5M-NaCl, 0.1mM-MgCl₂, 0.1mM-CaCl₂ and 0.1mM-MnCl₂. The neutral activity (○) was unretarded and the acidic activity (●) was eluted with the same buffer containing in addition 0.5M-methyl α-D-mannopyranoside: 87% of the acidic activity was recovered.

activity in the predominant peak in α-mannosidase A (separated by chromatography on DEAE-cellulose) and in unfractionated human liver had the same pI value (Phillips *et al.*, 1974a; Carroll *et al.*, 1972). No activity with a pI of 5.45, which is characteristic of the activity in α-mannosidase B separated on
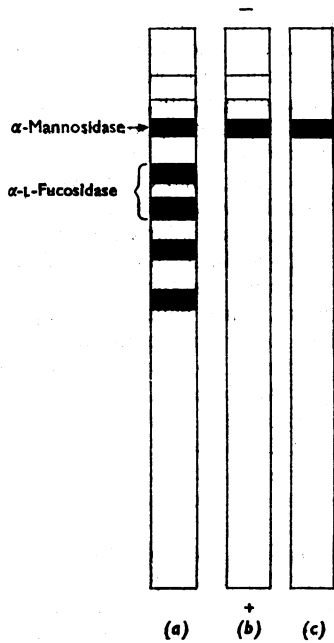
Fig. 2. *Polyacrylamide-gel electrophoresis of the α-mannosidase preparation before and after chromatography on the fucosylamine ligand affinity column*

(a) Before chromatography; stained for protein; (b) after chromatography; stained for protein; (c) after chromatography; stained for α-mannosidase. Details of the electrophoresis and staining methods are described in the Materials and Methods section. The α-L-fucosidase bands were identified by comparison with purified human liver α-L-fucosidase.

Fig. 3. *Isoelectric focusing of the purified α-mannosidase before and after treatment with neuraminidase*

(a) Purified α-mannosidase (0.3 unit), pI 6.0; (b) purified α-mannosidase (0.3 unit) which had been preincubated with neuraminidase, pI, 6.45. Fraction size, 2.2 ml. ●, Acidic α-mannosidase; ----, pH.

DEAE-cellulose, was present. Treatment of the preparation with neuraminidase raised the pI from 6.0 to 6.45 (Fig. 3b). This is consistent with the removal of sialic acid residues.

(2) *Ion-exchange chromatography on DEAE-cellulose.* α-Mannosidase B was the major component in the elution profile when the preparation was analysed on DEAE-cellulose at pH 6.0. The ratio of the activities in peaks A and B was 1:7, compared with a ratio of 1:1.5 in a crude liver extract (Phillips et al., 1974a). This result appeared at variance with the isoelectric-focusing analysis, which had indicated that the preparation consisted predominantly of α-mannosidase A. A sample from each stage in the purification was therefore analysed on DEAE-cellulose. It was found that after chromatography on concanavalin A–Sepharose the vast majority of the activity in the preparation was obtained as α-mannosidase B on DEAE-cellulose. α-Mannosidase A separated on DEAE-cellulose is known to change
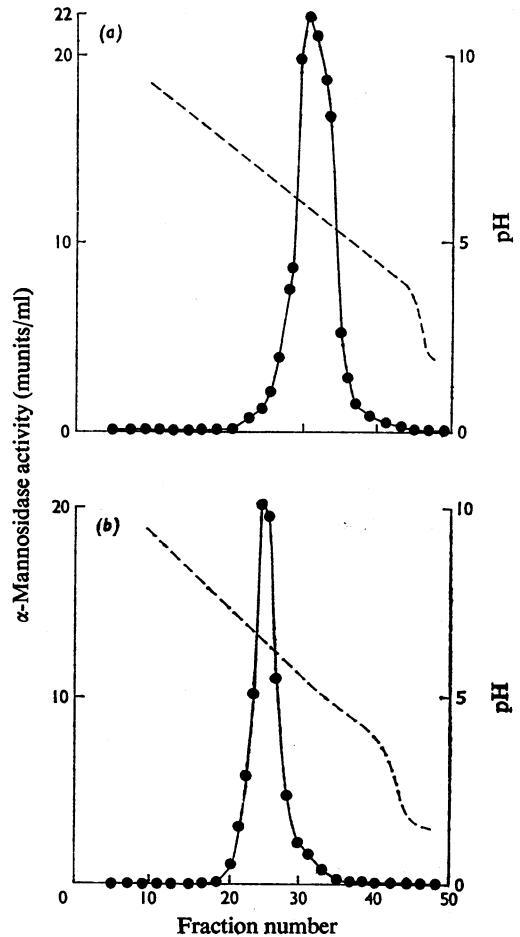
partially and spontaneously into α-mannosidase peak B with a possible increase in molecular weight (Phillips et al. 1974a).

(3) *Molecular weight.* The preparation had a mol.wt. of 220000 ± 10000 by sucrose-density-gradient centrifugation (Fig. 4a). The mol.wt. of the acidic activity in a crude liver extract was 230000 ± 7000 when determined by the same method (Fig. 4b). The corresponding mol.wt. for the acidic activity in a crude extract as determined by gel filtration was 250000–300000. More detailed analysis of the gel-filtration data had suggested that α-mannosidase B has a higher mol.wt. than α-mannosidase A (Phillips

et al. 1974*a*). Because of this the mol.wts. of α-mannosidases A and B separated on DEAE-cellulose were also determined by sucrose-density-gradient centrifugation (Fig. 4*c*). The activity in peak A appeared to be homogeneous with a mol.wt. of
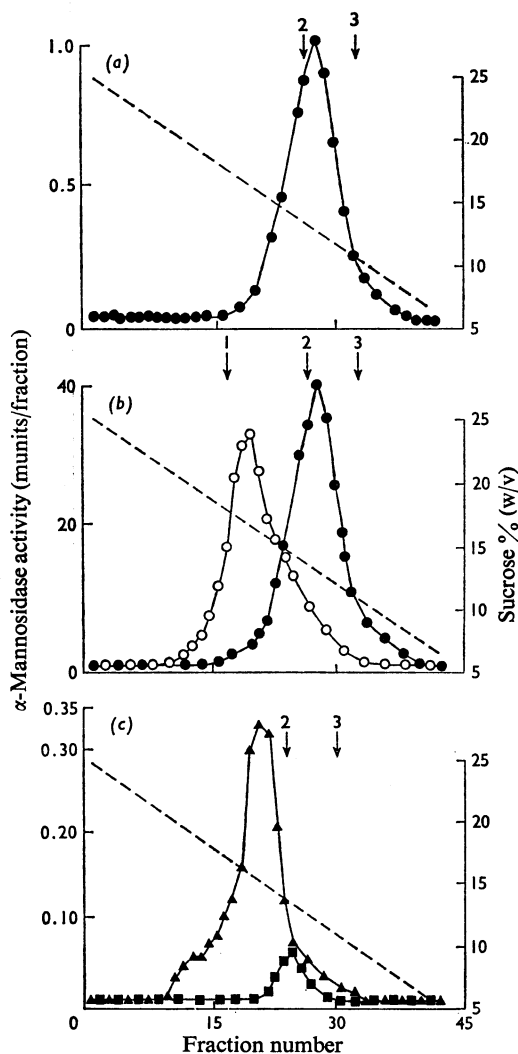
220000, which is the same value as for the purified α-mannosidase and the acidic activity in the crude extract. In contrast, the activity from peak B appeared to be heterogeneous, with a major peak and a distinct shoulder corresponding to mol.wts. of 300000 ± 20000 and 460000 ± 20000 respectively. These results confirm that the activity in peak B has a higher molecular weight than the activity in peak A. To determine the molecular weight of an enzyme by sucrose-density-gradient ultracentrifugation it is necessary to assume that the enzyme under investigation and the standards have the same partial specific volume. This assumption may not be valid if the enzyme is composed of several subunits (Martin & Ames, 1961). Acidic α-mannosidase is probably a complex of subunits. However, the similar values obtained for the molecular weight of the enzyme by gel filtration and sucrose-density-gradient centrigation suggest that the assumption is valid in this case.

(4) *Effect of Zn^{2+} and EDTA on activity.* The pH optimum for the hydrolysis of 4-methylumbelliferyl α-D-mannopyranoside by the purified enzyme was 4.25, the same value as for the acidic activity in a crude extract. Zn^{2+} lowered the pH optimum to 3.75, but the increase in activity at this pH was only 8% compared with an increase of typically 100% for the acidic activity in a crude extract. Simi¹arly the inhibitory effect of EDTA on the acidic activity was abolished during purification. Loss of sensitivity
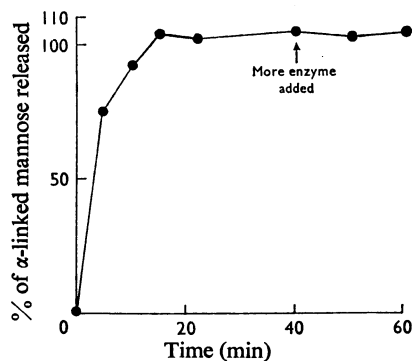

Fig. 4. *Sucrose-density-gradient centrifugation of α-mannosidase*

(*a*) Purified α-mannosidase, (*b*) crude liver extract, (*c*) peaks A (■) and B (▲) separated on DEAE-cellulose. ●, Acidic α-mannosidase; ○, neutral α-mannosidase; ----, sucrose gradient. The molecular weight standards are: 1, urease, 2, catalase, 3, alcohol dehydrogenase. Details of the centrifugation procedure and analysis of the gradients are given in the Materials and Methods section.

Fig. 5. *Hydrolysis by purified acidic α-mannosidase of a trisaccharide isolated from the urine of a patient with mannosidosis*

Purified acidic α-mannosidase (0.03 unit) was incubated with 1 μmol of the trisaccharide at pH 4.25 and 37°C. At suitable time-intervals the amount of mannose released was measured as described in the Materials and Methods section. After 40 min further α-mannosidase (0.03 unit) was added to the incubation mixture as indicated by the arrow. ●, Percentage of α-linked mannose in trisaccharide released.
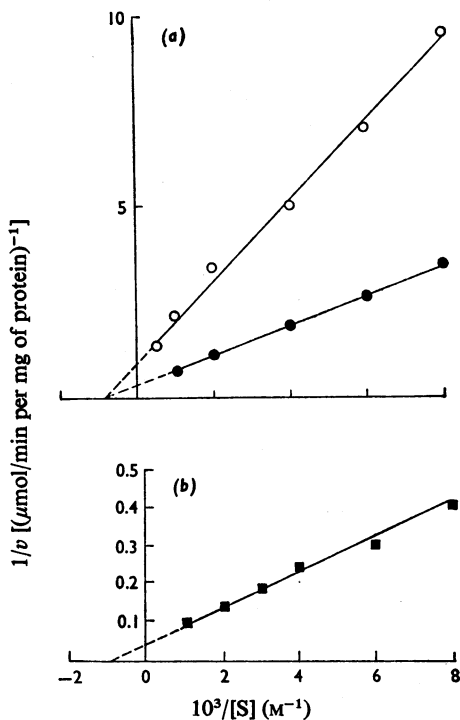
Fig. 6. *Determination of kinetic parameters, K_m and V, for natural and synthetic substrates for purified acidic α-mannosidase*

All the assays were performed at the common pH optimum, 4.25, and K_m and V were calculated graphically. (*a*) O, p-Nitrophenyl α-D-mannopyranoside; ●, 4-methylumbelliferyl α-D-mannopyranoside; (*b*), α-D-Manp(1→3)-β-D-Manp(1→4)-β-D-GlcNAcp.

reducing terminal had been released. The K_m for the hydrolysis of the trisaccharide, 1.0mM, was comparable with the values for 4-methylumbelliferyl and p-nitrophenyl α-D-mannopyranoside, 1.1 and 1.2mM respectively (Fig. 6). However, the theoretical V towards the natural substrate, the trisaccharide, was 20μmol/min per mg of protein, compared with values of 2.85 and 1.25μmol/min per mg of protein for the two synthetic substrates.

Discussion

Chromatography on concanavalin A–Sepharose followed by chromatography on a specific affinity ligand is a rapid and simple procedure for the purification of glycosidases. A similar strategy may be applicable to all lysosomal hydrolases, as the results of Bishayee & Bachawat (1974) and J. D. Hocking (personal communication) suggest that most if not all lysosomal hydrolases bind to concanavalin A–Sepharose. In this respect human liver acidic α-mannosidase is a typical lysosomal hydrolase. The failure of the neutral activity to bind to concanavalin A–Sepharose suggests either that it is not a glycoprotein or that it does not possess carbohydrate prosthetic groups specific for concanavalin A. This difference between the acidic and neutral α-mannosidases is further evidence that they are structurally and genetically distinct. The neutral forms of β-galactosidase (Norden & O'Brien, 1974) and β-N-acetylhexosaminidase (I. L. Braidman, personal communication) also do not bind to concanavalin A–Sepharose, whereas their corresponding acidic forms do.

The removal of the neutral activity from the preparation before affinity chromatography on the mannosylamine ligand is essential because of the very high affinity between the ligand and the neutral activity, which can prevent binding of the acidic activity (Robinson *et al.*, 1975). Although it was not possible to separate α mannosidase and α-L-fucosidase completely by conventional chromatographic techniques (Phillips *et al.*, 1975), their co-purification on the mannosylamine affinity-chromatography column needs explanation. Model building showed that D-mannose in the C1 conformation and L-fucose in the 1C conformation have the same configuration at C-2, C-3 and C-4 in the pyranose ring. This could be the structural feature that the two enzymes recognize, and steric hindrance by the hexanoyl arm on C-1 could make other structural features unimportant. This configuration is exclusive to these two sugars and D-rhamnose and L-galactose, but to our knowledge L-galactosidase and D-rhamnosidase have not been reported in human tissues. The involvement of only a part of the pyranose ring could explain the relatively low affinity of the enzymes for the mannosylamine ligand. In contrast, the fucosylamine ligand

to Zn²⁺ and EDTA had been observed previously on purification by conventional techniques (Phillips *et al.*, 1975).

(5) *Activity towards trisaccharide isolated from urine of patient with mannosidosis*. Initial experiments showed that the pH optimum for the hydrolysis of the trisaccharide α-D-Manp(1→3)-β-D-Manp(1→4)-D-GlcNAcp was 4.25, the same value as for the hydrolysis of the synthetic substrates, 4-methyl-umbelliferyl and p-nitrophenyl α-D-mannopyranoside. The progress curve for the hydrolysis of the trisaccharide at the optimum pH shows that the reaction stops when 1.04mol of mannose have been released from each mol of the trisaccharide (Fig. 5). Addition of more enzyme did not result in the release of further mannose. As there was no β-D-mannosidase in the purified α-mannosidase, it was assumed that all of the α-linked mannose at the non-

is highly specific for α-L-fucosidase (Robinson & Thorpe, 1974a) and was used as a final step to remove the contaminating α-L-fucosidase. The C-6 methyl group is presumably important in the recognition of fucose.

Analysis of the preparation by isoelectric focusing and sucrose-density-gradient centrifugation suggested that α-mannosidase A had been purified selectively. In contrast, ion-exchange chromatography indicated that the preparation consisted predominantly of α-mannosidase B. The probable explanation for this apparent discrepancy is the known partial conversion of α-mannosidase A into α-mannosidase B on ion-exchange chromatography (Phillips et al., 1974a). The results in the present paper suggest that this process takes place more readily with the purified enzyme. Prolonged storage of α-mannosidase peak B from DEAE-cellulose leads to the re-formation of some peak-A enzyme as judged by ion-exchange chromatography (N. C. Phillips, unpublished work). Therefore forms A and B are interconvertible at least on ion-exchange chromatography and post-translational events may be responsible for the multiple forms of the enzyme. This would be consistent with the immunological identity of α-mannosidases A and B (Phillips et al., 1975).

Sucrose-density-gradient centrifugation and isoelectric focusing suggest that α-mannosidase A is the predominant acidic form in crude liver extracts. Although activity with a pI of 5.45, which is characteristic of α-mannosidase B, is observed in crude extracts, its concentration is variable and may depend on the previous treatment of the sample. Therefore we suggest that α-mannosidase A is the predominant native form of human acidic α-mannosidase and is the form that is purified by affinity chromatography. However, as soon as the purified α-mannosidase A is subjected to ion-exchange chromatography or possibly other treatments, it undergoes partial conversion into form B, which has a higher molecular weight and a lower pI. A possible explanation for this phenomenon is that the ion-exchanger binds ions or molecules that stabilize α-mannosidase A. Removal of these moieties could enable presumed subunits to reassemble into larger aggregates.

Its affinity for concanavalin A and its increase in pI on treatment with neuraminidase indicate that acidic α-mannosidase is a glycoprotein. Human α-mannosidases A and B are both converted by neuraminidase into a form that binds to CM-cellulose more strongly than the untreated forms (Chester et al., 1975). This new form is presumably the same as the form with a pI of 6.45 described by us. These results and the interconversion of forms A and B preclude α-mannosidase A being asialo-α-mannosidase B. Needleman & Koenig (1974) have shown by isoelectric focusing that the pI values of several rat kidney lysosomal isoenzymes are increased after treatment with neuraminidase.

Acidic α-mannosidase appears to lose its sensitivity to Zn^{2+} and EDTA during purification. These observations support our previous suggestion that Zn^{2+}- and EDTA-insensitive α-mannosidase activity occurs normally in human liver (Phillips et al., 1974a). Carlsen & Pierce (1972) have also shown that partially purified rat epididymal α-mannosidase is not inhibited by EDTA. These results suggest that the enzyme is activated by Zn^{2+} rather than being a Zn^{2+}-containing metalloenzyme. There could be a correlation between the loss of sensitivity to Zn^{2+} and the ease of conversion from α-mannosidase A into α-mannosidase B on purification. However, partially purified α-mannosidases A and B, separated on DEAE-cellulose, are both activated by Zn^{2+} and inhibited by EDTA. The role of Zn^{2+} in the structure and/or the mechanism of action of mammalian acidic α-mannosidase remains to be investigated. Snaith (1975) has reported that jack-bean α-mannosidase is a true zinc metalloenzyme.

A comparison of the activity of the purified enzyme towards the presumed natural substrate and synthetic substrates suggests the same enzymic activity is responsible for hydrolysing all these substrates. This validates the use of assays with synthetic substrates for determining the genotype of persons suspected of being heterozygous or homozygous for mannosidosis. The demonstration that the acidic α-D-mannosidase, which is absent in mannosidosis, completely hydrolyses the terminal α-linked mannose in the storage product is strong evidence that mannosidosis is a lysosomal storage disease resulting from a deficiency of acidic α-mannosidase. Hultberg (1970) showed that the residual activity in the liver of a patient with mannosidosis had very low activity towards the storage product compared with the activity in a normal liver. Acidic α-mannosidase peaks A and B, separated on DEAE-cellulose, can hydrolyse the storage products from the urine of a patient with mannosidosis, whereas the neutral peak C cannot (Hultberg et al., 1975).

The data of Hultberg et al. (1975) indicate that human liver acidic α-mannosidase has a very low aglycone specificity. Purified pig acidic α-mannosidase is capable of hydrolysing $\alpha(1\rightarrow2)$, $\alpha(1\rightarrow3)$, $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$ linkages of oligomannosides but at a rate much lower than for p-nitrophenyl α-D-mannopyranoside (Okumura & Yamashina, 1973). In contrast the enzyme from rat brain releases mannose from glycoproteins at a rate comparable with the rate of hydrolysis of the synthetic substrate (Bosmann & Hemsworth, 1971). The results in the present paper show that although the pH optimum and K_m for the synthetic and natural substrates were similar, the theoretical V for the hydrolysis of the natural substrate was greater than the values for the synthetic substrates. All these

observations suggest that the aglycone moiety is not important in specificity determination but affects the rate of hydrolysis.

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