Characterization of Purified Human Liver Acid β -D-Galactosidases A₂ and A₃

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1. Human liver acid β -galactosidase A₂ and A₃ were isolated by chromatography on concanavalin A–Sephadex 4B, Sephadex 6B, and Sephadex 4B–6-aminohexyl 1-thio- β -D-galactopyranoside. β -Galactosidase A₂ and A₃ were purified to final specific activities of 45.5 and 20.6 μ mol/min per mg respectively with 4-methylumbelliferyl β -D-galactopyranoside as substrate. 2. Form A₂ had a mol.wt. of $150\,000 \pm 15\,000$ (gel filtration) and appeared as a single band of protein (mol.wt. $65\,000 \pm 1000$) on electrophoresis in the presence of sodium dodecyl sulphate. 3. Form A₃ had a mol.wt. (gel filtration) of $660\,000 \pm 66\,000$. On electrophoresis in the presence of sodium dodecyl sulphate, form A₃ appeared as a major band of protein (72% of total) of mol.wt. $65\,000 \pm 1000$ and minor protein bands of mol.wt. $44\,000 \pm 1000$ and $26\,000 \pm 1000$ and $22\,000 \pm 1000$. 4. Gel-filtration chromatography of purified β -galactosidase A₃ generated approximately equal amounts of forms A₃ and A₂. β -Galactosidase A₁ was not detected by gel-filtration chromatography of partially or highly purified preparations of forms A₂ and A₃. 5. Both forms A₂ and A₃ had identical isoelectric points of 4.42 ± 0.02 . The data suggest that forms A₂ and A₃ are dimeric and multimeric forms of β -galactosidase A₁. 6. Amino acid analysis of β -galactosidase A₂ gave a ratio of acidic to basic amino acids of 2.6:1. 7. β -Galactosidase A₂ contained 7.5% carbohydrate by weight and sialic acid, D-galactose, D-glucosamine and D-mannose were present in the molar proportions 1.1:1.0:1.7:2.7.

The distribution of mammalian β -galactosidases among various subcellular fractions, the existence of multiple forms of these enzymes, and their kinetic properties with respect to model substrates have been studied in crude enzyme preparations (Wallenfels & Malhotra, 1960; Wallenfels & Weil, 1972; Robinson, 1974). Two forms of human liver 'acid' β -galactosidase able to hydrolyse the terminal β -galactoside residue from ganglioside G_{M1} (Norden & O'Brien, 1973; Ho *et al.*, 1973), believed to be lysosomal (Alpers, 1969), are present at less than 2% of control values in the autosomal recessive inherited disease G_{M1} gangliosidosis (Okada & O'Brien, 1968; Ho *et al.*, 1973). A major form of G_{M1} β -galactosidase (form A₁) has been isolated as an apparently homogeneous protein (Norden *et al.*, 1974). β -Galactosidase A₁ had a mol.wt. of 72000 on sodium dodecyl

sulphate/polyacrylamide-gel electrophoresis and 65000 to 75000 by gel filtration under non-denaturing conditions. The minor form (A₃) of G_{M1} β -galactosidase had an estimated mol.wt. of 600000–800000 from gel-filtration chromatography (Norden & O'Brien, 1973; Ho *et al.*, 1973), but has not been isolated in a highly purified state. However, it was found that anti-(β -galactosidase A₁) antiserum quantitatively precipitated both β -galactosidase A₁ and A₃ (Norden *et al.*, 1974), suggesting that they have polypeptide in common and are coded in part by the same locus. O'Brien (1975) postulated that form A₃ is a multimeric aggregate of form A₁. In addition to activity towards G_{M1} ganglioside, both purified β -galactosidase A₁ and A₃ cleave lactosylceramide (Miller *et al.*, 1977). Both forms A₁ and A₃ are distinct from the 'neutral' forms of β -galactosidase (designated B₁ and B₂), which have an apparent mol.wt. of 45000 and a different substrate specificity (Norden & O'Brien, 1973; Ho *et al.*, 1973; Norden *et al.*, 1974).

The existence of a third form of acid β -galactosidase (form A₂) in crude enzyme preparations from human liver has been reported (Cheetham & Dance, 1976). Form A₂ had an estimated mol.wt. by gel-filtration chromatography of 160000 to 170000. Form A₁ aggregated to a higher-molecular-weight

Abbreviations used: The abbreviation G_{M1} stands for ganglioside G_{M1} [by the nomenclature of Svennerholm (1963)]: Gal β 1–3GalNAc β 1–4 (NeuAc α 2–3) Gal β 1–4Glu β 1–1'ceramide. The G_{M1} β -galactosidases are referred to as forms A₁, A₂, A₃, as proposed by Cheetham & Dance (1976). G_{M1} β -galactosidases A₁ and A₃ were referred to as G_{M1} β -galactosidases A and B respectively in previous publications (Norden & O'Brien, 1973; Ho *et al.*, 1973; Norden & O'Brien, 1974; Norden *et al.*, 1974; O'Brien, 1975; Miller *et al.*, 1976, 1977).

form (presumably form A₃) in the presence of increasing NaCl concentrations. In addition, a number of treatments of form A₃ resulted in the production of forms A₁ and A₂ at the expense of A₃. Cheetham & Dance (1976) and Ho *et al.* (1973) postulated that form A₃ may be composed of forms A₁ and A₂ bound to fragments of lysosomal membrane. The present study was undertaken to isolate sufficient quantities of these acid β -galactosidases and to compare some of their physical and chemical properties.

Experimental

Materials

Sephacrose 6B, DEAE-Sephadex A-25 and concanavalin A-Sephacrose 4B were from Pharmacia, Uppsala, Sweden; D-galactono- γ -lactone and 4-methylumbelliferyl β -D-galactopyranoside were from Koch-Light Laboratories, Colnbrook, Bucks., U.K.; and D-galactose was from Sigma Chemical Co., St. Louis, MO, U.S.A. Chemicals for polyacrylamide-gel electrophoresis were from Miles Laboratories, Elkhart, IN, U.S.A. Trimethylchlorosilane and hexamethyldisilazane were from Pierce Chemical, Rockford, IL, U.S.A., and 3% SE-30 on 100/120 Gas-Chrom Q was from Applied Science Laboratories, State College, PA, U.S.A. Ampholines (pH range 3–10) were from LKB, Bromma 1, Sweden. Amicon ultrafiltration devices and Diaflo UM-10 membranes were obtained from Amicon Corp., Lexington, MA, U.S.A. 6-Aminohexyl 1-thio- β -D-galactopyranoside was prepared as previously described (Chipowsky & Lee, 1973), and coupled to CNBr-activated Sepharose 4B (Blumberg *et al.*, 1970). The gel derivative contained 1.4 μ mol of 6-aminohexyl 1-thio- β -D-galactopyranoside/ml of Sepharose.

Methods

Purification of acid β -galactosidase. All procedures were carried out at 0–4°C unless otherwise specified. Acid β -D-galactosidase was assayed as described with 4-methylumbelliferyl β -D-galactopyranoside as substrate (Norden & O'Brien, 1974), and protein was determined by the method of Lowry *et al.* (1951). Human livers were obtained at autopsy and appeared normal on gross inspection; they were stored at –20°C until used. Portions (500g total wet wt.) of the liver from four autopsied individuals were cut into small pieces and homogenized at 4°C in a Waring blender for 1 min at low speed and 1 min at high speed with 2.5 litres of 5 mM-sodium phosphate buffer, pH 7.0, containing 10 mM-NaCl and 0.04% (w/v) NaN₃. The homogenate was centrifuged for 90 min at 24000g and the resulting supernatant fluid (2600 ml) was chromatographed on a column (2.5 cm $\times$ 72 cm) of concanavalin A-Sephacrose as previously described (Norden & O'Brien, 1974;

Miller *et al.*, 1976). The α -methyl D-mannoside effluent was concentrated to a protein concentration of 18 mg/ml by ultrafiltration at 280–350 kPa (40–50 lb/in²), using an Amicon concentrator equipped with a UM-10 Diaflo membrane. The initial isolation procedure was repeated five times with a total of 2.6 kg wet wt. of liver tissue. Samples of the concentrated G_{M1} β -galactosidase (22 ml) were dialysed for 16 h against 2 $\times$ 4 litres of 10 mM-sodium phosphate, pH 7.0, containing 100 mM-NaCl, 0.1 mM-EDTA and 0.04% (w/v) NaN₃. The dialysed acid β -galactosidase was concentrated over a UM-10 Diaflo membrane to 35 ml (11 mg of protein/ml) and chromatographed at a flow rate of 20 ml/h on a column (5.5 cm $\times$ 98 cm) of Sepharose 6B, which was equilibrated with 10 mM-sodium phosphate, pH 7.0, containing 100 mM-NaCl, 0.1 mM-EDTA and 0.04% (w/v) NaN₃. Fractions (5 ml) were collected and assayed for β -galactosidase. The column was calibrated by the method of Andrews (1970), with Blue Dextran 2000 (mol. wt. 2×10^6 ; V_0 665 ml), bovine thyroglobulin type I (Sigma Chemical; 6.7×10^6 ; V_e 1128 ml), bovine serum albumin dimer (1.3×10^5 ; V_e 1398 ml) and bovine serum albumin (6.8×10^4 ; V_e 1530 ml). Two distinct peaks of β -galactosidase were detected in the effluent, form A₃ (V_e 1075 ml) and form A₂ (V_e 1386 ml). The fractions containing β -galactosidase A₃ and A₂ were combined and concentrated over a Diaflo UM-10 membrane to a protein concentration of 2.5 mg/ml and 10 mg/ml respectively. The concentrated samples of β -galactosidase A₂ and A₃ from several gel-filtration runs were combined respectively for purification by affinity chromatography.

β -Galactosidase forms A₂ and A₃ were each chromatographed on Sepharose 4B–6-aminohexyl 1-thio- β -D-galactopyranoside as previously described (Miller *et al.*, 1976), except for the following change. Fractions containing 250 units (nmol of substrate cleaved/min) of β -galactosidase A₂ were diluted with 10 mM-sodium phosphate, pH 7.0, containing 100 mM-NaCl, 0.1 mM-EDTA and 0.04% (w/v) NaN₃ to a final protein concentration of 3.0 mg/ml, and dialysed for 20 h against 2 $\times$ 15 litres of 10 mM-sodium acetate, pH 5.4, containing 100 mM-NaCl, 0.1 mM-EDTA and 0.04% (w/v) NaN₃. The dialysed sample was centrifuged for 30 min at 48000g and the resulting supernatant fluid was applied to a column (2 cm $\times$ 20 cm) of Sepharose 4B–6-aminohexyl 1-thio- β -D-galactopyranoside. The elution and concentrations of β -galactosidase A₂ and A₃ were as previously described (Miller *et al.*, 1976).

Molecular-weight determination by gel-filtration chromatography. The molecular weight of β -galactosidase A₂ and A₃, obtained after purification on Sepharose 4B–6-aminohexyl 1-thio- β -D-galactopyranoside, was determined by gel-filtration chromato-

graphy on a column (0.9 cm $\times$ 100 cm) of Sepharose 6B. The gel was equilibrated with 10 mM-sodium phosphate, pH 7.0, containing 100 mM-NaCl, 1 mM-EDTA and 0.04% (w/v) NaN_3 , and calibrated by the method of Andrews (1970) using Blue Dextran 2000 (mol.wt. 2×10^6 ; V_0 26.2 ml), bovine thyroglobulin type I (6.7×10^5 , K_{av} 0.37), catalase (2.3×10^5 , K_{av} 0.56), γ -globulin (1.6×10^5 , K_{av} 0.65), bovine serum albumin (6.8×10^4 , K_{av} 0.70), ovalbumin (4.3×10^4 , K_{av} 0.77). Each protein standard was chromatographed at a protein concentration of 10 mg/ml, with a column flow rate of 4.3 ml/h; fractions (0.5 ml) were collected. Samples of purified β -galactosidase A_2 and A_3 (125 and 260 μ g respectively) were applied to the column at protein concentrations of 2.5 and 2.6 mg/ml respectively.

Ion-exchange chromatography. Samples of β -galactosidase A_2 (1.1 mg, 1.96 mg/ml) were dialysed for 16 h against 1 litre of 10 mM-sodium phosphate, pH 7.0, containing 10 mM-NaCl, 0.1 mM-EDTA and 0.04% (w/v) NaN_3 , and applied to a column (0.9 cm $\times$ 9.0 cm) of DEAE-Sephadex A-25 equilibrated with the same buffer. The column was then developed with a linear gradient of NaCl generated by mixing 125 ml each of column buffer and column buffer containing 300 mM-NaCl. The tubes comprising the peak of β -galactosidase were combined (61 ml) and concentrated by ultrafiltration over a Diaflo UM-10 membrane to 0.5 ml (0.69 mg/ml).

Polyacrylamide-gel electrophoresis. Samples of purified β -galactosidase A_2 and A_3 were subjected to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate as described by Weber & Osborn (1969). The following proteins (mol.wt.) were selected as reference standards: phosphorylase A (9.4×10^4), bovine serum albumin (6.8×10^4), ovalbumin (4.3×10^4), and soya bean agglutinin (3.0×10^4). A portion (10 μ g) of each protein standard was made 1% (w/v) in sodium dodecyl sulphate and 5% (v/v) in 2-mercaptoethanol and these were heated at 80°C for 5 min. Samples of β -galactosidase A_2 and A_3 (38 μ g of each) were made 1% (w/v) in sodium dodecyl sulphate and either: (a) subjected directly to electrophoresis, (b) made 5% (v/v) in 2-mercaptoethanol and subjected to electrophoresis or (c) made 5% (v/v) in 2-mercaptoethanol and heated at 80°C for 5 min. Electrophoresis was in 5% (w/v) polyacrylamide gels at 8 mA/tube for 6 h at 22°C. Gels were fixed and stained for 2 h in 0.2% (w/v) Coomassie Brilliant Blue G-250 and destained by diffusion in 7% (v/v) acetic acid/4% (v/v) methanol as described by Weber & Osborn (1969). Gels were scanned by using an EC910 Transmission Densitometer (E.C. Apparatus Corp., St. Petersburg, FL, U.S.A.) coupled to an Omniscribe recorder (Houston Instrument, Austin, TX, U.S.A.).

Isoelectric focusing. Isoelectric focusing of purified

β -galactosidase A_2 and A_3 was performed in 7.5% (w/v) polyacrylamide gels. Isoelectric focusing gels were prepared as for normal gels, except for replacement of buffer by 2% (w/v) Ampholine, pH range 3–10, and were focused for 5–6 h at 250 V (Wrigley, 1968). Samples containing 20 μ g of protein in 100 μ l of 40% (w/v) sucrose made 2% (w/v) in Ampholine were applied to the tops of the gels and layered with 50 μ l of 20% (w/v) sucrose made 2% (w/v) in Ampholine. The cathode (top) solution was 4% (w/v) ethanolamine and the anode (bottom) solution was 2% (w/v) H_2SO_4 . After focusing, the gels were immediately sliced into 0.17 cm sections. β -Galactosidase activity in gel slices was determined by incubation of each section of gel with 100 μ l of 0.5 mM-4-methylumbelliferyl β -D-galactopyranoside containing 500 mM-sodium citrate, pH 4.35. The fractions were incubated for 60 min at 37°C and terminated by the addition of 1 ml of 85 mM-glycine/carbonate buffer, pH 10.0, measuring the production of 4-methylumbelliferone fluorimetrically (Ho *et al.*, 1973). The pH profile was obtained by slicing parallel gels; each slice was eluted for 2 h at room temperature (22°C) with 1 ml of water before pH measurement. Isoelectric points were determined by direct comparison of the enzymic activity in gel slices with the pH of the fraction.

Amino acid analysis. Samples (200 μ g) of purified β -galactosidase A_2 were prepared and quantitatively analysed in triplicate for amino acids, essentially as described by Spackman *et al.* (1958). Samples of the enzyme were put in microcarius tubes and sufficient conc. HCl was added to give a final concentration of 6 M-HCl. The ampules were evacuated, sealed and heated at 110°C for 24 h. The hydrolysates were evaporated to dryness and dissolved in 0.2 ml of 200 mM-citrate buffer, pH 2.2. These solutions were analysed on a single-column Beckman 116 amino acid analyser equipped with an Autolab System AA computing integrator.

Carbohydrate analysis. The carbohydrate composition of purified β -galactosidase A_2 (130 and 260 μ g samples) was determined by g.l.c. of trimethylsilyl-monosaccharides, which were prepared and chromatographed as described by Clamp *et al.* (1971), with the following modifications. (a) Methanolysis was carried out in methanolic 1.5 M-HCl (0.5 ml) at 80°C for 12 h in vials with Teflon-lined caps; (b) the methanolysates were neutralized with 0.15 ml of anhydrous pyridine and acetylated for 1 h at 23°C by adding 0.100 ml of acetic anhydride; and (c) the samples were then thoroughly dried in a vacuum desiccator over phosphoric oxide and silylated by using 'silylating agent a' (Clamp *et al.*, 1971). Analyses were done on a Nuclear-Chicago Selecta System 5000 gas-liquid chromatograph equipped with a flame-ionization detector and a column (2 m $\times$ 2 mm) of 3% SE-30 on 100/120 Gas-Chrom Q.

Table 1. *Purification of acid β -galactosidase*

Stepwise purification of human liver acid β -galactosidases A_2 and A_3 was accomplished as described under 'Methods'.

Purification step	Protein (mg)	Activity (μ mol/min)	Specific activity (μ mol/min per mg)	Purification (fold)	Recovery (%)
Homogenate	307000	2740	0.0009	1.00	100
Supernatant	130800	2040	0.016	1.75	75
Concanavalin A-Sepharose	3370	1140	0.339	38.1	42
Sepharose 6B					
Form A_2	1380	750	0.544	61.1	27
Form A_3	296	240	0.812	91.1	8.8
Sepharose 4B-6-aminoethyl 1-thio- β -D-galactopyranoside					
Form A_2	8.5	387	45.5	5110	14
Form A_3	1.8	37	20.6	2310	1.4

Fig. 1. Polyacrylamide-gel electrophoresis of purified acid β -galactosidases A_2 and A_3

Samples of purified acid β -galactosidase A_2 and A_3 were subjected to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate as described by Weber & Osborn (1969). The direction of electrophoresis was from the top to the bottom of the gels, as shown, with the anode at the bottom. Gel 1, 38 μ g of β -galactosidase A_2 purified as in Table 1; gel 2, 38 μ g of purified β -galactosidase A_2 obtained after further purification by DEAE-Sephadex chromatography as described in the text; gel 3, 38 μ g of purified β -galactosidase A_3 ; gel 4, 10 μ g of standard proteins in order of increasing mobility: phosphorylase A, bovine serum albumin, ovalbumin and soya bean agglutinin.

Results

Purification of human liver acid β -galactosidase

Acid β -galactosidase was isolated from human liver supernatant liquid by column chromatography on concanavalin A-Sepharose, followed by chromatography on a column of Sepharose 6B to separate the different molecular-weight forms. Two distinct peaks of β -galactosidase activity were detected: form A_3 , eluted between 1030 and 1200 ml (apparent mol.wt. 830000 ± 83000), and form A_2 , eluted between 1330 and 1500 ml (apparent mol.wt. 148000 ± 15000). Of the total β -galactosidase activity applied to the column, 21% was recovered as form A_3 and 65% as form A_2 . Form A_1 was only occasionally observed as a small shoulder eluted after the majority of the A_2 peak (between 1530 and 1580 ml). At most, 8% of the applied activity was eluted in the region expected for form A_1 . Fractions containing form A_2 were combined from each chromatography, excluding the small shoulder of form A_1 . Forms A_3 and A_2 were further purified by affinity chromatography on Sepharose 4B-6-aminoethyl 1-thio- β -D-galactopyranoside. Form A_3 was purified to a final specific activity of 20.6μ mol/min per mg, and form A_2 to 45.5μ mol/min per mg, with 4-methylumbelliferyl β -D-galactopyranoside as substrate. The purification of acid β -galactosidase A_2 and A_3 is summarized in Table 1.

Characterization of purified acid β -galactosidases A_2 and A_3

Polyacrylamide-gel electrophoresis. The purity of the isolated acid β -galactosidases A_2 and A_3 was determined by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Fig. 1). A single band of protein with a mobility corresponding to a subunit mol.wt. of 65000 ± 1000 was observed

on electrophoresis of 38 μ g of β -galactosidase A_2 . Electrophoresis of the same amount of β -galactosidase A_3 revealed a major protein band with the same mobility as that for β -galactosidase A_2 , and three minor protein bands with mobilities corresponding to mol.wts. of 44000 ± 1000 , 26000 ± 1000 and 22000 ± 1000 . When forms A_2 and A_3 were prepared for electrophoresis by (a) heating at 80°C for 6 min in the presence of 1% (w/v) sodium dodecyl sulphate and 5% (v/v) 2-mercaptoethanol, (b) incubation with the same concentrations of sodium dodecyl sulphate and 2-mercaptoethanol for 10 min at 22°C , and (c) incubation with 1% (w/v) sodium dodecyl sulphate for 10 min at 22°C , the patterns observed were identical with those in Fig. 1. After scanning, the major peak of β -galactosidase A_2 comprised 94% of the total stained area of the gel. For β -galactosidase A_3 , the area under the major peak represented 72% of the total area (Fig. 2). The lightest staining minor band in form A_2 was not detected on scanning, owing to its low concentration.

Ion-exchange chromatography on DEAE-Sephadex. The purity of acid β -galactosidase A_2 was also assessed on ion-exchange chromatography on a column of DEAE-Sephadex (Fig. 3). A single unimodal peak of β -galactosidase activity was eluted with

a linear gradient of NaCl. The effluent comprising the majority of the peak (tubes 60–100) was combined; this had a specific activity of 44620 (0.35 mg of protein), identical with that of the sample applied. A single band of protein of mol.wt. 65000 ± 1000 was observed on electrophoresis of this further purified sample in the presence of sodium dodecyl sulphate (Fig. 1). Noteworthy was the absence of microheterogeneity of highly purified form A_2 .

Molecular-weight determination by gel-filtration chromatography. On gel chromatography homogeneous β -galactosidase A_2 was eluted as a single peak with a mol.wt. of 150000 ± 15000 , very close to the value determined for the partially purified protein (148000 ± 15000). β -Galactosidase A_3 was eluted as two peaks, each with nearly equivalent activity (Fig. 4). The small form had a molecular weight identical with that observed for β -galactosidase A_2 , 150000 ± 15000 . The larger form had a mol.wt. of 660000 ± 66000 , a value somewhat lower than that observed during the purification of β -galactosidase A_3 (830000 ± 83000).

Isoelectric focusing. When samples of purified acid β -galactosidase A_2 and A_3 were electrofocused in polyacrylamide gels (Fig. 5) the two forms of β -galactosidase had nearly identical isoelectric points:

Fig. 2. Densitometric scan of sodium dodecyl sulphate polyacrylamide gels of acid β -galactosidase A_2 and A_3 . Gels 1 and 3 shown in Fig. 1 were scanned using an EC910 Transmission densitometer coupled to an Omniscrite recorder. The gels were illuminated with a high-intensity incandescent lamp of wavelength 480 to 650 nm. ●, Gel 1, 38 μ g of purified acid β -galactosidase A_2 ; ○, gel 3, 38 μ g of purified acid β -galactosidase A_3 .

Fig. 3. DEAE-Sephadex A-25 chromatography of purified acid β -galactosidase A_2 .

Acid β -galactosidase A_2 (1.1 mg, 0.5 ml) in 10 mM-sodium phosphate, pH 7.0, containing 10 mM-NaCl, 0.1 M-EDTA and 0.04% (w/v) NaN_3 was applied to a column (0.9 cm $\times$ 9.0 cm) of DEAE-Sephadex A-25 equilibrated with the same buffer. At fraction 28, the column was developed with a linear gradient of NaCl generated by mixing 125 ml each of column buffer with column buffer containing 300 mM-NaCl. Portions (10 μ l) of each fraction were assayed: ●, 4-methylumbelliferyl β -D-galactopyranosidase activity (nmol/5 min); ○, NaCl concentration (mM). The fractions included by the bar were combined and concentrated for protein determination and polyacrylamide-gel electrophoresis.

Fig. 4. *Sepharose 6B gel-filtration chromatography purified acid β -galactosidase A_2 and A_3*

A column (0.9cm $\times$ 100cm) of Sepharose 6B was equilibrated with 10mM-sodium phosphate, pH7.0, containing 100mM-NaCl, 1mM-EDTA and 0.04% (w/v) NaN₃, and calibrated with protein standards as described in the text (Andrews, 1970). Samples of purified acid β -galactosidase A_2 and A_3 (125 and 260 μ g respectively) were chromatographed at a flow rate of 4.3ml/h. Portions (10 μ l) of each fraction were assayed: ●, β -galactosidase A_2 (nmol/5min); ○, β -galactosidase A_3 (nmol/5min).

the pI of form A_2 was 4.40 and that of form A_3 was 4.45.

Amino acid analysis. Quantitative amino acid analysis of purified acid β -galactosidase A_2 revealed the most abundant amino acid to be leucine, with aspartic acid and glutamic acid as additional major constituents, together comprising 20.3% of the total amino acids (Table 2). Lysine and arginine comprised only 7.65% of the total amino acids.

Carbohydrate analysis. Form A_2 contained 7.5% carbohydrate per mg of protein. Mannose, glucosamine, galactose and sialic acid were the major constituents, and these sugars were present in the proportions 2.7:1.7:1.0:1.1 respectively (Table 3). Trace amounts of fucose and glucose were also detected.

Discussion

In previous studies of crude human liver supernatants, most of the acid β -galactosidase migrated as form A_1 on gel-filtration chromatography (Norden & O'Brien, 1973; Ho *et al.*, 1974; Cheetham & Dance, 1976). In our study only a minor peak of acid β -galactosidase A_1 was observed on gel filtration of a concanavalin A-Sepharose-purified preparation; the majority was eluted as form A_2 . The apparent molecular weight of our highly purified β -galactosidase A_2 was 150000, similar to the value reported for less-purified preparations (Cheetham & Dance, 1976).

Fig. 5. *Polyacrylamide-gel isoelectric focusing of purified acid β -galactosidase A_2 and A_3*

Acid β -galactosidase A_2 and A_3 (15 μ g of A_2 and 20 μ g of A_3) were subjected to isoelectric focusing in 7.5% polyacrylamide gels as described in the text (Wrigley, 1968). The cathode (top) solution was 4% (w/v) ethanolamine and the anode (bottom) solution was 2% H₂SO₄. After focusing, the gels were sliced into 0.17cm sections and assayed for β -galactosidase as described in the text: (a) β -galactosidase A_2 ; (b) β -galactosidase A_3 . ●, 4-Methylumbelliferyl β -D-galactopyranosidase activity (nmol/60min); ○, pH gradient.

The specific activity of our most highly purified preparations of form A_2 (45.5 μ mol/min per mg) was nearly identical with those reported for homogeneous form A_1 (Norden & O'Brien, 1973). Highly purified A_2 gave only a single band of protein of 65000 mol.wt. on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, in confirmation of the hypothesis that form A_2 is a dimer of form A_1 (Cheetham & Dance, 1976). It appears that form A_1 aggregates to form A_2 and that the aggregation is especially favoured after purification and concentration of form A_1 . Once formed, form A_2 is stable even in dilute solutions. Whether form A_2 exists *in vivo* is an unresolved question.

Acid β -galactosidase A_3 represented approx. 20% of the total acid β -galactosidase activity recovered from gel-filtration chromatography and had an

Table 2. *Amino acid composition of purified acid β -galactosidase A₂*

Amino acid	Composition (mol/100mol)
Aspartic acid	10.7
Threonine	5.9
Serine	6.6
Glutamic acid	9.6
Proline	7.2
Cysteine	1.0
Glycine	9.5
Alanine	6.4
Valine	5.0
Methionine	1.1
Isoleucine	4.0
Leucine	11.0
Tyrosine	5.9
Phenylalanine	5.8
Histidine	2.0
Lysine	5.0
Arginine	3.6

Table 3. *Carbohydrate composition of purified acid β -galactosidase A₂*

Total carbohydrate	
Monosaccharide	(mol/100mol)
D-Mannose	40.9
D-Glucosamine	25.7
D-Galactose	15.5
Sialic acid	16.3
L-Fucose	0.01

apparent mol.wt. of $830\,000 \pm 83\,000$ in crude preparations and $660\,000 \pm 66\,000$ in the purest preparation. These values are within the molecular-weight range (600 000–800 000) previously reported for β -galactosidase A₃ in unfractionated human liver supernatants (Norden & O'Brien, 1973). Our most highly purified preparations of β -galactosidase A₃ had a specific activity of $20.6 \mu\text{mol/min per mg}$, a value that is 45% of the final specific activity of purified β -galactosidase A₂. Purified β -galactosidase A₃ contained one major band of protein (72% of the total), of mol.wt. $65\,000 \pm 1000$, and three minor bands of protein. The major band of protein co-migrated with form A₁, in keeping with the proposal that β -galactosidase A₃ is an aggregated form of β -galactosidase A₁ (O'Brien, 1975). We attempted to determine if the minor bands of protein present in form A₃ had β -galactosidase activity, but were unsuccessful, owing to the small amounts of form A₃ available. The recovery of form A₃ was very low (1.4%) apparently because of its tendency to disaggregate. Thus the question whether form A₃ is a multimer of form A₁ *in vivo* or is composed of forms A₁ and A₂ bound to fragments of lysosomal membrane (Cheetham & Dance, 1976) remains unresolved. The data suggest

that the majority of our purified form A₃ comprises form A₁ monomers, in keeping with the multimer proposal. We consider it likely that the three minor bands are contaminants rather than structural constituents of form A₃, consonant with the lower specific activity of form A₃. However, homogeneous form A₃ must be isolated in larger quantities to settle the question.

The amino acid analysis of the purified acid β -galactosidase A₂ revealed a high content of neutral (alanine, valine, isoleucine, and leucine, 26.3 mol/100mol) and acidic amino acids (20.3 mol/100mol), and a low content of basic (7.65 mol/100mol) and sulphur-containing amino acids (2.10 mol/100mol). The amino acid composition determined for β -galactosidase A₂ is consistent with the isoelectric point of 4.40 determined for the purified protein and similar to that obtained (A. G. W. Norden & J. S. O'Brien, unpublished work) on analysis of apparently homogeneous acid β -galactosidase A₁.

Acid β -galactosidase A₂ is similar to other purified mammalian lysosomal hydrolases in carbohydrate content. Human (Alhadeff & Freeze, 1977) and rat (Opheim & Touster, 1977) liver α -L-fucosidase, rat liver (Tomino *et al.*, 1975) and preputial gland (Himeno *et al.*, 1975) β -glucuronidase, and bovine testis β -galactosidase (Hieber *et al.*, 1976) contain 5–8% carbohydrate. The major monosaccharides in these hydrolases are mannose and N-acetylglucosamine, present in molar ratios ranging from 1:1 to 2:1. The amounts of the other sugars vary from one enzyme to the other. Our preparation of β -galactosidase A₂ has a higher ratio of galactose/mannose than the other hydrolases. More detailed studies of the structure of the carbohydrate chains of acid β -galactosidase A₂ are necessary to achieve an understanding of the role played by its oligosaccharide chains in binding phenomena.

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