Purification and Properties of Acid β-Galactosidase from Feline Liver†

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ABSTRACT: Acid β-galactosidase (EC 3.2.1.23) was purified 19 000-fold from feline liver with a 13% recovery using a four-step procedure that involved (1) lectin chromatography on concanavalin A–Sepharose 4B, (2) ion-exchange chromatography on DEAE-cellulose, (3) affinity chromatography on Sepharose 4B–6-aminohexyl 1-thio-β-D-galactopyranoside, and (4) gel filtration on Sepharose 6B. The purified protein eluted from Sepharose 6B as two symmetrical peaks of protein coincident with two peaks of enzyme activity. The two forms of the enzyme had apparent molecular weights of 700 000 and 130 000. Molecular weight estimation by sucrose gradient centrifugation revealed a single 115 000 molecular weight form. Reduced and denatured β-galactosidase migrated as a single major band with an apparent molecular weight of 62 000 during polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The purified protein demonstrated a single protein precipitin arc that coincided with a single arc of enzyme activity when examined by immunoelectrophoresis, using an antiserum to partially purified feline liver acid β-galactosidase. Over 95% of the acid β-galactosidase activity from liver supernatants and the purified enzyme preparation was precipitated in immunotitration experiments. The amount of antiserum needed to precipitate a given quantity of activity was the same in both cases. Purified β-galactosidase hydrolyzed synthetic β-D-galactosides, α-L-arabinosides, and β-D-fucosides, as well as the β-linked galactose moieties of ganglioside G_{M1} and asialofetuin. The enzyme also catalyzed the transfer of galactose from *p*-nitrophenyl β-D-galactoside and ganglioside G_{M1} to various carbohydrate acceptors.

β-D-Galactosidases (EC 3.2.1.23) from a number of different sources have been investigated (Wallenfels & Weil, 1972). One of several mammalian β-D-galactosidases is a lysosomal enzyme (Sellinger et al., 1960) with a broad substrate specificity that includes the terminal β-linked galactose moieties of synthetic glycosides (Ho & O'Brien, 1971), disaccharides and glycosaminoglycans (Distler & Jourdian, 1973), glycoproteins (Sato & Yamashina, 1974) and various glycolipids including ganglioside G_{M1} (Norden et al., 1974), asialoganglioside G_{M1} (Tanaka et al., 1975), and lactosylceramide (Miller et al., 1977). This enzyme, known as acid β-galactoside or ganglioside G_{M1} β-galactosidase, has been the subject of intense investigation both because there is interest in exploring its chemical, physical, and enzymatic properties (Robinson et al., 1967; Chytil, 1965; Tomino & Meisler, 1975, Frost et al., 1978) and because an inborn deficiency of acid β-galactosidase is the primary enzyme defect in the human lysosomal storage disorder, G_{M1} gangliosidosis (O'Brien, 1978).

The description of at least four separate cases of G_{M1} gangliosidosis in cats (Handa & Yamakawa, 1971; Blakemore, 1972; Cheetham et al., 1974; Baker et al., 1971) has generated considerable interest in the cat as a model system for the study of the disease and for the evaluation of various strategies of therapeutic intervention in the lysosomal storage disorders (Baker et al., 1976). Recent studies of the biochemical pathology (Holmes & O'Brien, 1978a) and enzymology (Holmes & O'Brien 1978b) in a case of feline G_{M1} gangliosidosis have further demonstrated the basic similarities between the human and feline disorders and the suitability of the feline model for the study of experimental enzyme replacement.

In order to obtain β-galactosidase for enzyme replacement studies and provide a basis for comparing the properties of the enzyme from normal and mutant animals, we have purified and characterized acid β-galactosidase from normal feline liver. Although the purification of feline β-galactosidase has recently been reported by Anderson et al. (1978), the extreme instability of their purified enzyme and the small amount of protein in the final preparation limited the scope of their investigation. The method we now describe provided a highly purified enzyme in increased quantities and with a dramatically increased stability compared to those reported previously (Anderson et al., 1978). These important advantages permitted a more extensive characterization of the purified protein.

Experimental Procedures

Materials

Feline liver tissue was obtained fresh from male and female cats of mixed breeds and stored at −20 °C until use. All of the 4MU[1] substrates were obtained from Koch-Light Laboratories Ltd. as was pure sodium taurocholate. The PNP substrates, bovine brain gangliosides, sugars, and protein standards were from Sigma Chemical Co. D-Galactose dehydrogenase was a product of Boehringer-Mannheim Biochemicals. Fetuin was purified and desialylated by the method of Spiro (1960) and tritiated by a modification of the method of Morrell et al. (1971). Ganglioside G_{M1} was purified from bovine brain gangliosides (Penick et al., 1966) after neuraminidase treatment of the crude ganglioside fraction (Wenger & Wardell, 1973). The purified compound was tritium labeled (Radin et al., 1969).

Sepharose 4B and 6B, Con A–Sepharose 4B, and Blue Dextran 2000 were obtained from Pharmacia. DEAE-cellulose (De-52) was a product of Whatman. The affinity resin 4B–6-aminohexyl-1-thio-β-D-galactopyranoside was synthesized and kindly provided by Dr. R. G. Frost (Miller et al., 1976).

All gel electrophoresis reagents were obtained from Ames Co. with the exception of $NaDodSO_4$ (BDH) and Coomassie blue G-250 (Bio-Rad Laboratories). Carrier ampholytes were

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[1] Abbreviations used: 4MU, 4-methylumbelliferyl; PNP, *p*-nitrophenyl; Con A–Sepharose, concanavalin A bound covalently to Sepharose 4B; ganglioside G_{M1}, Galβ1–3GalNAcβ1–4(NeuAcα2–3)Galβ1–4 Glcβ1–1′-ceramide; $NaDodSO_4$, sodium dodecyl sulfate.

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from LKB. Purified agar was obtained from Colab. Precoated cellulose and silica gel 60 analytical thin-layer chromatography plates were a product of E. M. Laboratories. Solvent systems 1 and 2 were butanol/pyridine/0.1 N HCl (5:3:2, v/v) and butanol/acetic acid/water (3:1:1, v/v), respectively.

The following buffers were used in various purification steps and analytical procedures: buffer 1, 2 mM $NaPO_4$, 10 mM NaCl, 0.02% NaN_3, pH 7.0; buffer 2, 10 mM $NaPO_4$, 25 mM NaCl, 0.02% NaN_3, pH 7.0; buffer 3, 10 mM $NaPO_4$, 500 mM NaCl, 0.02% NaN_3, pH 7.0; buffer 4, 10 mM $NaPO_4$, 10 mM NaCl, 1 mM EDTA, pH 7.0; buffer 5, 10 mM CH_3COONa, 25 mM NaCl, 1 mM EDTA, pH 5.4; buffer 6, 5 mM $NaPO_4$, 150 mM NaCl, 0.02% NaN_3, pH 7.2; buffer 7, 10 mM $NaPO_4$, 100 mM NaCl, 0.02% NaN_3, pH 7.0.

Methods

Purification of β-Galactosidase. (1) Tissue Extraction. Minced liver tissue (1.2 kg) was homogenized at 4 °C in a Waring blender (1 min at low speed and 1 min at high speed) with 6 L of buffer 1. The homogenate was centrifuged for 60 min at 20000*g*. The supernatant (5 L) was filtered through cheesecloth and used for Con A–Sepharose chromatography. Unless otherwise specified all operations were carried out at 4 °C.

(2) Con A–Sepharose Chromatography. Portions (1.5 L) of liver supernatant were applied to 50-mL beds of Con A–Sepharose at a flow rate of 30–60 mL/h. The columns were washed with buffer 3, and acid β-galactosidase was eluted with α-methylmannoside as described by Norden & O'Brien (1974). The α-methyl mannoside effluents were combined and concentrated over a PM-10 membrane in an Amicon pressure cell.

(3) DEAE Chromatography. Con A–Sepharose purified β-galactosidase (35 mL) was dialyzed against three 2-L portions of buffer 4, centrifuged (40000*g* for 45 min), and applied to a 2.5 × 39 cm bed of DE-52 that was equilibrated with the same buffer. The column was washed with 1.5 L of buffer 4 and then eluted with 1.0 L of a concave NaCl gradient (10–250 mM). This was followed by elution with 400 mL of a linear NaCl gradient (250–750 mM). The salt-eluted β-galactosidase activity was divided into four pools. The pools were individually concentrated as described above.

(4) Affinity Chromatography. Pool 1 from the ion-exchange step (11.5 mL) was dialyzed against three 1-L changes of buffer 5, centrifuged (40000*g* for 45 min), and applied to a 1.9 × 23 cm bed of Sepharose 4B–6-aminohexyl 1-thio-D-galactopyranoside that had been equilibrated with buffer 5. The flow rate during application was 2 mL/h. The affinity column was washed with buffer 5 until the OD_{280} of the effluent reached a stable minimum value and the bound β-galactosidase was eluted with 300 mL of buffer 5 made 50 mM in D-galactose. During sugar elution, the outflow of the affinity column was pased through a 1.5-mL bed of Con A–Sepharose which bound 100% of the eluted β-galactosidase activity. The small lectin column was eluted with 10 mL of 750 mM α-methylmannoside in buffer 3. This technique allowed the affinity-purified enzyme to be recovered in a small volume that was further concentrated to 1.5 mL using a collodian bag apparatus.

(5) Sepharose 6B Chromatography. The affinity-purified enzyme was applied to a 0.9 × 100 cm column of Sepharose 6B and eluted with buffer 3 at a flow rate of 7.5 mL/h. The fractions containing enzyme activity were pooled and concentrated in a collodian bag apparatus.

Enzyme Assays. 4MU-β-D-galactosidase activity was assayed in 50 mM sodium acetate, 100 mM sodium chloride, 0.5 mM 4MU-β-D-galactoside, pH 4.1. 4MU-α-L-arabinosidase and PNP-β-D-fucosidase activities were assayed under the same conditions using substrate concentrations of 5 and 40 mM, respectively. Activity toward PNP-β-D-galactoside was assayed as previously described (Distler & Jourdian, 1973). The hydrolysis of [^{3}H]galactose from labeled ganglioside G_{M1} and asialofetuin was assayed as described by Norden & O'Brien (1973, 1974). 4MU-β-D-glucosidase was assayed by the method of Ho & O'Brien (1971). The purified enzyme was assayed for potential contaminating hydrolase activities using 4MU substrates as described by Miller et al. (1976). A unit of enzyme activity transforms 1 nmol of substrate per minute at 37 °C. Protein determinations were done by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Kinetic Studies. Kinetic studies were done using the standard assay systems and varying the substrate concentrations. Only initial rates were measured. Data were plotted by the method of Lineweaver & Burk (1934). Final values for K_m and V_{max} were determined using the computer program of Cleland (1967).

Glycosyltransferase Assays. The glycosyltransferase activity of the highly purified enzyme was studied using PNP-galactoside and ganglioside G_{M1} as the galactose donors. Potential sugar and alcohol acceptors were added to the standard assays. When the PNP substrate was used, total substrate hydrolysis was calculated as usual and free galactose was quantitated enzymatically with galactose dehydrogenase as described by Norden et al. (1974), except NADH was estimated spectrophotometrically at 340 nm. Products of these assays and sugar standards were examined directly by thin-layer chromatography. Solvent system 1 was used for cellulose plates; sugars were detected with the silver nitrate reagent (Trevelyan et al., 1950). Solvent system 2 was used for silica gel plates, where sugars were detected by spraying with 0.5% orcinol in 50% sulfuric acid and heating at 100 °C for 10–20 min. Tritiated products formed by the transfer of [^{3}H]-galactose from ganglioside G_{M1} to an acceptor were examined by thin-layer chromatography in solvent system 1. Sample zones were scraped from the plates and counted in a scintillation counter, and the mobilities were compared with those of known standards.

Polyacrylamide Gel Electrophoresis. Analytical gel electrophoresis was done in the presence of sodium dodecyl sulfate ($NaDodSO_4$) as described by Weber & Osborn (1969). Protein samples (20 μg) were made 1% (w/v) in $NaDodSO_4$ and 5% (v/v) in 2-mercaptoethanol and heated at 80 °C for 5 min. Electrophoresis was carried out in 5, 7.5, 10, and 12.5% gel rods at 8 mA/tube for 6 h at 22 °C. The gels were fixed and stained with Coomassie brilliant blue G-250 and destained by diffusion (Weber & Osborn, 1969). Stained gels were scanned for absorbance at 570 nm using an E.C. 910 transmission densitometer. The molecular weight of the β-galactosidase subunit was determined from a plot of log molecular weight vs. mobility of the marker proteins: bovine serum albumin (68 000), ovalbumin (45 000), soybean agglutinin (30 000), and myoglobin (17 500).

Native β-galactosidase was examined by disc gel electrophoresis (7.5% gel) at pH 9.5, using the system of Davis (1964), and by isoelectric focusing in 7.5% polyacrylamide gels over the pH range 3.5–10 as previously described (Frost et al., 1978). In both cases, enzyme activity was located by cutting gels into 1.7-mm slices and incubating the slices with the 4MU substrate (0.5 mM in 0.3 M citrate, pH 4.1).

Table I: Purification of Acid β-Galactosidase from Feline Liver[a]

fraction	vol (mL)	act. (units)	protein (mg)	sp act. (units/mg)	purifcn (x-fold)	recov. (%)
homogenate	5940	150 744[b]	171 666	0.88	1.0	100
supernatant	5090	134 692[b]	91 925	1.47	1.7	89
Con A-Sepharose adsorbed	37	124 641	1 073	116.0	132	83
DEAE pool 1	12	41 748	99	422.0	479	28
affinity adsorbed	1.5	22 557	1.90	11 872	13 490	15
Sepharose 6B	1.0	19 624	1.15	17 053	19 378	13

[a] Enzyme activity was assayed with 4MU-β-D-galactopyranoside. [b] These activities have been corrected for the contribution of neutral β-galactosidase to the acid 4MU-β-galactosidase assay. The neutral enzyme was removed from the preparation in the Con A-Sepharose step.

Gel Filtration. As a criterion of purity, a sample (0.11 mL) of the purified enzyme (125 μg of protein, 2100 units of 4MU activity) was chromatographed on a 0.9 × 28 column of Sepharose 6B using buffer 7 as the eluant. The flow rate (1.45 mL/h) and fraction size (0.19 mL) were precisely maintained with a peristaltic pump (LKB 2120, varioperpex II). Fractions were assayed for enzyme activity and protein (Lowry et al., 1951). To provide estimation of the molecular weight of β-galactosidase, gel filtration columns were calibrated with protein standards (Andrews, 1970). The standard proteins were ovalbumin (45 000), bovine serum albumin (68 000), bovine γ-globulins (165 000), catalase (245 000), and thyroglobulin (670 000).

Sucrose Gradients. Linear gradients of 5–20% (w/v) sucrose in buffer 2 (gradient volume, 4.56 mL) were prepared, run, and fractionated as described by Martin & Ames (1961). Samples (0.10 mL) containing 7 μg of purified enzyme (120 units) and 100 μg of bovine serum albumin were applied to the gradients. The gradients were centrifuged at 39 000 rpm in a Beckman SW41 rotor at 4 °C for 12 h. The sedimentation behavior of β-galactosidase was followed by enzyme activity (4MU substrate). Bovine serum albumin served as the internal standard and was located by determining total protein using the method of Bradford (1976).

Immunological Techniques. Rabbit antibodies to feline liver β-galactosidase were raised to an 8000-fold purified enzyme preparation (sp act. 7000 units/mg) isolated by Con A–Sepharose chromatography and affinity chromatography. The antigen was injected intradermally at multiple sites on the back using the following schedule: day 1, 64 μg of protein in Freunds complete adjuvant; day 11, 31 μg in Freunds incomplete adjuvant; day 18, 16 μg in incomplete adjuvant. The rabbit was bled through the dorsal artery of the ear at 1-week intervals, and the relative titers of the sera were determined by immunotitration.

A portion of the antiserum was purified on an immunoadsorbent column prepared by linking unadsorbed proteins from the affinity chromatography step to Sepharose 4B (Porath et al., 1973).

Immunotitrations were performed by mixing 0.02 mL of a serial twofold dilution of antiserum (diluted in buffer 6) with an equal volume of antigen. After a 2-h incubation at 23 °C and 48 h at 4 °C, the mixture was centrifuged at 20000*g* for 30 min. Nonprecipitated enzyme activity was assayed with the 4MU substrate on 0.01-mL aliquots of the supernatant. When liver supernatants were examined, the assays were carried out at pH 3.5 to minimize interference by neutral β-galactosidases.

Immunoelectrophoresis was done on microscope slides in 1% agar layers. $NaPO_4$ (40 mM), pH 7.4, was used in the gel and in the electrode chambers. Electrophoresis was carried out at 80 V for 2.5 h. After immunodiffusion at 0–4 °C, unprecipitated protein was removed from the gels by soaking them at 23 °C in buffer 6. Precipitin arcs were stained for enzyme activity using the 4MU substrate (0.5 mM in 0.3M citrate, pH 4.1). After soaking for 2 h in distilled water, the gels were air-dried and stained for protein with Coomassie blue (Weber & Osborn, 1969).

Results

Purification. The purification of acid β-galactosidase is summarized in Table I. The four-step procedure resulted in a 19 000-fold purification and an overall recovery of 13%. While affinity chromatography of preparations purified to the Con A–Sepharose step resulted in a substantial purification (8000-fold), the addition of an ion-exchange step was necessary to achieve maximum purification. The apparent loss of activity during DEAE-cellulose chromatography reflects our selectivity in pooling the most purified fractions, rather than an instability of the enzyme under the conditions of fractionation. The total recovery during this step was 80%.

Maximum binding of feline β-galactosidase to the thiogalactoside column was achieved by decreasing the NaCl concentration from 100 mM (Miller et al., 1976) to 25 mM. We also found that, when the affinity-purified enzyme was concentrated, using a small bed of Con A–Sepharose and a collodion bag instead of a conventional pressure cell (Amicon UM-10 or PM-10 membranes), the recovery during the affinity step increased from 20 to 50% of the activity applied. β-Galactosidase activity eluted as a broad peak in the 120 000 to 700 000 molecular weight range during the final purification step on Sepharose 6B.

Criteria of Purity. Purified feline liver acid β-galactosidase migrated as a single major band on 5, 7.5, 10, and 12% polyacrylamide gels under denaturing conditions in the presence of $NaDodSO_4$ (Figure 1). Several minor bands were also detected in the purified preparation. Electrophoresis of the native enzyme at pH 9.5 in the Davis system resolved four bands of protein; however, the complete loss of enzyme activity during electrophoresis made it impossible to correlate activity and protein. Prerunning the gels did not prevent inactivation.

When the purified enzyme was chromatographed on Sepharose 6B (pH 7.0, 100 mM NaCl), a strict coelution of protein and enzymatic activity was observed (Figure 2). Acid β-galactosidase eluted as two symmetrical peaks each containing about 50% of the total activity. Each activity peak was coincident with a protein peak. The specific activity was constant across both peaks, and the mean value was within 5% of the specific activity of the starting material. The approximate molecular weights of the two peaks were 700 000 and 130 000.

The purified preparation contained low levels of contaminating hydrolase activities. Those detected and their relative amounts in units/unit of acid β-galactosidase (4MU substrate) were as follows: β-D-hexosaminidase, 0.01; α-L-fucosidase, 0.007; α-D-galactosidase, 0.003; α-D-mannosidase, 0.003;

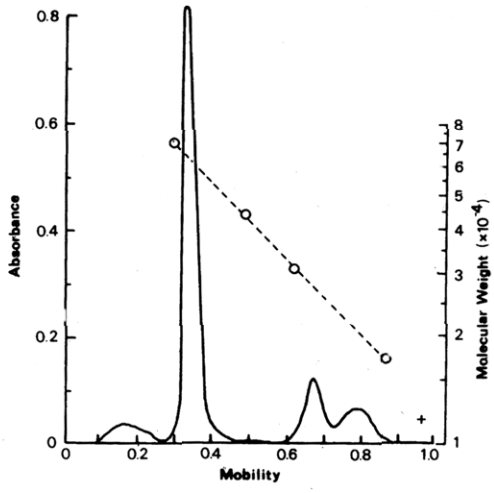

FIGURE 1: Polyacrylamide gel electrophoresis of purified feline β-galactosidase. Feline liver β-galactosidase and standard proteins were electrophoresed in 7.5% gels in the presence of sodium dodecyl sulfate and stained with Coomassie blue as described in the text. (—) Plot of absorbance at 570 nm vs. mobility for a stained gel containing 20 μg of 19000-fold purified β-galactosidase. The profile was obtained with a scanning densitometer. (O–O) Semilogarithmic plot of mobility vs. molecular weight for standard proteins run in the same system. In order of increasing mobility the four standards were bovine serum albumin, ovalbumin, soybean agglutinin, and myoglobin. Mobilities are relative to the bromophenol blue tracking dye. The origin and cathode are at the left of the figure.

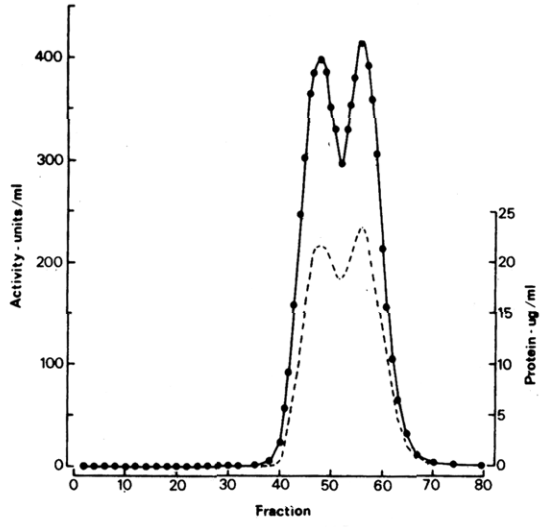

FIGURE 2: Analytical Sepharose 6B column chromatography of purified β-galactosidase. A 0.20-mL sample of the purified enzyme (125 μg of protein, 2100 units of 4MU activity) was fractioned on a small column of Sepharose 6B using 10 mM $NaPO_4$, 100 mM NaCl, 0.2% NaN_3, pH 7.0, as the eluant. The fractions were assayed for enzyme activity using the 4MU substrate and for protein by the method of Lowry et al. (1951). Additional details appear in the text. The specific activity of the starting material was 17053 units/mg of protein. The mean specific activity of the fractions comprising peak 1 (fractions 45–51) was 18373 ± 860, while the value for peak 2 (fractions 53–59) was 17600 ± 340.

β-D-glucuronidase, 0.003; β-D-glucosidase, 0.001.

As a a final criterion of purity, the preparation was examined by immunoelectrophoresis using an antiserum to partially purifed acid β-galactosidase. Enyzme purified to the DEAE stage (480-fold) gave multiple protein precipitin arcs using this antiserum (Figure 3a). In contrast, highly purified acid β-galactosidase gave a single protein precipitin arc coincident with a single arc of enzyme activity (Figure 3b).

Molecular Weight Determinations. As described above, feline liver acid β-galactosidase eluted from Sepharose 6B as a single broad peak of activity when chromatographed at pH

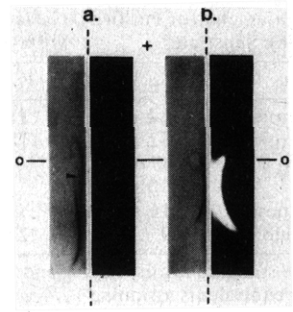

FIGURE 3: Immunoelectrophoresis of feline β-galactosidase. Feline liver acid β-galactosidase purified to the DEAE stage (480-fold) and the final purified preparation (19000-fold) were examined by immunoelectrophoresis in 1% agar gels, using antiserum to partially purified (8000-fold) feline liver β-galactosidase as described in the text. (a) Both sample wells contained 40 μg of DEAE purified acid β-galactosidase. The arrow identifies the faint β-galactosidase protein precipitin arc. (b) Both sample wells contained 3 μg of the final purified enzyme preparation. The gels were stained for protein (left half) and for enzyme activity (right half). (O) Position of sample wells; (---) the plane of the antiserum trough.

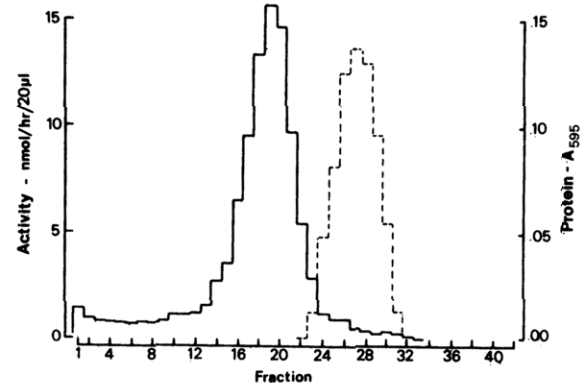

FIGURE 4: Sucrose gradient centrifugation of purified β-galactosidase. Seven micrograms of purified β-galactosidase and 100 μg of bovine serum albumin were centrifuged in 5–20% (w/v) linear gradients of sucrose as described in the text. β-Galactosidase (—) was located by assaying the fractions (4MU substrate). The bovine serum albumin standard (---) was detected by protein determination using the method of Bradford (1976). Fractions are numbered consecutively from 20% sucrose (fraction 1) to 5%.

7.0 in 0.5 M NaCl (in the final purification step) and eluted as two symmetrical peaks of molecular weights 700000 and 130000 at pH 7.0 in 0.1 M NaCl. An additional estimation of molecular weight was made by sucrose gradient centrifugation. On sucrose gradients the enzyme sedimented as a single symmetrical peak of activity (Figure 4). Based on the sedimentation rate of the bovine serum albumin standard, the molecular weight of feline liver acid β-galactosidase was calculated to be 115000.

Subunit Analysis. The major protein band observed after $NaDodSO_4$–gel electrophoresis of the reduced enzyme had an apparent molecular weight of 62000 relative to protein standards (Figure 1). The minor protein bands gave values of 29000 and 21000, respectively.

Isoelectric focusing in polyacrylamide gels demonstrated a single peak of enzyme activity (4MU substrate). The peak was centered around a pH value of 5.4 and ranged from 4.9–5.7.

General Enzymatic Properties. The purified acid β-galactosidase was stable when stored at 4 °C in buffer 3 at a protein concentration of 1.2 mg/mL; no loss of enzyme activity (4MU or ganglioside G_{M1} substrates) was observed over an 8-month period under these conditions. The pH optima of the enzyme for 4MU, ganglioside G_{M1}, and asia-

Table II: Kinetic Parameters of Purified β-Galactosidase with Natural and Synthetic Substrates

substrate	K_m (mM)	V_{max} (units/mg)
4MU-β-D-galactoside	0.14 ± 0.02[a]	18875 ± 9.16
PNP-β-D-galactoside	0.62 ± 0.05	18431 ± 318
ganglioside G_{M1}	0.17 ± 0.02	1213 ± 79
asialofetuin[b]	1.50 ± 0.20	1989 ± 426
4MU-α-L-arabinoside	2.16 ± 0.18	2111 ± 86
PNP-β-D-fucoside[c]	56.9 ± 7.9	27462 ± 2675

[a] Errors shown are standard errors. [b] K_m and V_{max} are expressed as galactose equivalents assuming 12.4 mol of Gal/mol of ASF. [c] Because of the limited solubility of this substrate, all of the data were collected at sub-K_m concentrations; the highest concentration examined was 35 mM.

lofetuin substrates were 4.1, 4.5, and 4.1, respectively. The detergent and NaCl requirements for ganglioside G_{M1} hydrolysis were the same as those reported for the human liver enzyme (Norden & O'Brien, 1973). Activity with 4MU substrate was mildly stimulated by NaCl; hydrolysis increased 13% as NaCl concentration was increased from 7 to 100 mM. Hydrolysis of the 4MU substrate was not affected by Mg^{2+} or Ca^{2+} (0–50 mM), PO_4 or SO_4 (0–100 mM), EDTA (0–2 mM), Triton X-100 (0–1%, w/v), or Zn^{2+} (0–1 mM). Enzyme activity was completely inhibited by 0.2 mM Hg^{2+}.

Kinetic Properties. The kinetic parameters of the purified enzyme for some natural and synthetic substrates are shown in Table II. Double reciprocal plots of the data were linear in all cases.

Glycosyltransferase Activity. The purified enzyme catalyzed the transfer of galactose from PNP-β-D-galactoside and ganglioside G_{M1} to various carbohydrate acceptors that were added to the standard assay systems. The rate of hydrolysis (*p*-nitrophenol production) of the PNP substrate was stimulated by most of the acceptors tested, and in all cases galactose dehydrogenase assays showed that a portion of the liberated galactose was reincorporated into glycosidic bonds (Table III). When the products of assays containing exogenous *N*-acetylgalactosamine and glucose (with and without enzyme) were examined by thin-layer chromatography on silica gel using solvent system 2, the assays containing enzyme had orcinol-positive spots that were absent in the controls. In the case when glucose was added, the new compound (spot) had the same mobility as lactose. The products of these reactions were not analyzed further.

Studies of the glycosyltransferase activity with the PNP substrate as the donor and glucose as the acceptor revealed that the rate of new glycoside formation was linear for at least 30 min and was a function of acceptor concentration. The pH optimum for hydrolysis was 4.2 in the presence and absence of acceptors but maximum glycoside formation occurred at pH 4.8.

Stimulation of PNP-β-D-galactoside hydrolysis was also observed when methanol and 2-mercaptoethanol were added to the assay. In each case the stimulation was accompanied by a decrease in the release of free galactose.

Ganglioside G_{M1} was also an effective donor for the glycosyltransferase reaction. [^{3}H]G_{M1} hydrolysis was inhibited by all of the sugars tested; however, the analysis of the assay products by thin-layer chromatography demonstrated that a substantial portion of the liberated [^{3}H]galactose was associated with compounds that migrated slower than authentic galactose. For example, when glucose was the acceptor, 57% of the total [^{3}H]galactose appeared in the galactose spot, while 38% of the total migrated with the same mobility as authentic lactose.

Table III: Glycosyltransferase Activity of Purified Feline Liver Acid β-Galactosidase

substrate[a]	acceptor	concn (mM)	% control act.	% incorpn into glycosides
PNP-β-D-galactoside[b]	glucose	10	112	1
		21	117	9
		42	129	21
		84	147	36
		168	171	54
		210	177	59
	mannose	210	111	24
	α-methyl glucoside	210	87	65
	α-methyl mannoside	210	111	21
	N-acetylgalactosamine	210	141	
	N-acetylglucosamine	210	84	56
	maltose	210	153	68
	raffinose	53	107	16
	stachyose	21	105	15
	xylose	210	161	38
	methanol	176	109	5
		352	123	11
		705	140	21
		1410	165	38
		1760	174	46
	β-mercaptoethanol	20	113	0
		41	120	0.2
		82	129	9
		123	138	16
		163	141	20
		200	147	24
ganglioside G_{M1}[c]	glucose	250	72	38
	N-acetylgalactosamine	250	85	27
	inositol	250	70	30
	maltose	250	86	35

[a] The standard assay systems described in Methods were used. [b] The percent incorporation figures represent the proportion of the total hydrolyzed galactose that did not react with galactose dehydrogenase. [c] Assay products were examined by thin-layer chromatography on cellulose using solvent system 1. The percent incorporation figures represent the proportion of the total [^{3}H]galactose released from ganglioside G_{M1} that had a chromatographic mobility lower than that of authentic galactose.

The enzyme did not incorporate free [^{14}C]galactose into glycosides in the presence of the most effective sugar acceptor from Table III.

Immunological Properties. Precipitating antibodies to the feline liver enzyme were obtained after immunizing a rabbit with an 8000-fold purified preparation of β-galactosidase. Immune sera precipitated 95–100% of the acid β-galactosidase activity from crude or purified enzyme preparations in immunotitration experiments. (The neutral β-galactosidase activity was not precipitated.) A given quantity of antiserum precipitated an equivalent amount of enzyme activity from liver supernatants and the 19000-fold purified preparation (Figure 5).

When liver supernatants or partially purified enzyme preparations were examined by immunoelectrophoresis using unadsorbed antiserum, several protein precipitin arcs were observed. In all cases, only one arc had β-galactosidase activity (Figure 3a). The enzymatically inactive arcs showed reactions of nonidentity with the active one. After purification of the antiserum by adsorption, a single precipitin arc of coincident protein and activity was observed on immunoelectrophoresis of both crude and purified enzymes. The results of immu-

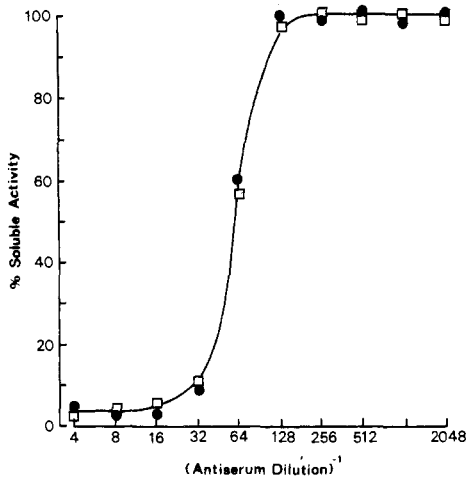

FIGURE 5: Immunotitration of crude and purified β-galactosidase. 0.20 units of β-galactosidase activity (4MU substrate) from feline liver supernatant (●) or the purified preparation (□) were mixed with serial twofold dilutions of antiserum. Nonprecipitated β-galactosidase was detected by enzyme assay (4MU substrate) after removal of the immunoprecipitates by centrifugation. Additional details appear in the text.

notitration experiments were not changed by antiserum adsorption.

Discussion

The purification of feline liver acid β-galactosidase by the method reported here was both rapid and efficient, resulting in a 19 000-fold purification and a 13% recovery. The final preparation was highly purified on the basis of analytical gel chromatography, $NaDodSO_4$–polyacrylamide, gel electrophoresis and immunoelectrophoresis.

Affinity chromatography had been widely used in the purification of β-galactosidases from a variety of sources (Miller et al., 1976). The aminohexyl-1-thio-β-D-galactoside–Sepharose 4B conjugate was chosen for affinity chromatography in the present study because this ligand offered the advantage of specific elution of the bound enzyme under mild conditions using competitive sugar inhibitors (Miller et al., 1976). Thus, the present method differs from those using the *p*-aminophenylthiogalactoside conjugate and nonspecific elution conditions in the affinity chromatography step (Distler & Jourdian, 1973; Anderson et al., 1978).

The affinity column used in the present study has also been used to purify acid β-galactosidase from human liver (Miller et al., 1976; Frost et al., 1978). However, the binding properties of the feline and human enzymes were quite different. While 97% of the human liver enzyme was bound at pH 5.0–5.5 in 100 mM NaCl (Miller et al., 1976), only 30% of the feline enzyme bound under these conditions. This difference may be related to the marked stimulation (80%) of the activity of human liver β-galactosidase by NaCl (Ho & O'Brien, 1971) compared to the modest effect of NaCl on the activity of the feline liver enzyme.

$NaDodSO_4$–gel electrophoresis of feline acid β-galactosidase demonstrated a major subunit with an apparent molecular weight of 62 000. The occurrence of a single 60 000–70 000 molecular weight subunit in purified acid β-galactosidase from other mammalian species (Distler & Jourdian, 1973; Norden et al., 1974; Tomino & Meisler, 1975) and genetic evidence that the mouse enzyme is coded for by a single structural gene (Breen et al., 1977) suggest that the minor bands in our purified preparation are either contaminants that associate with the native enzyme (Cheetham & Dance, 1976) or proteolytic fragments of the 62 000 molecular weight subunit generated intralysosomally or during purification (Brot et al., 1978). The strict coelution of activity and protein during gel filtration is consistent with either hypothesis. It is not known if the multiple electrophoretic forms observed on basic polyacrylamide gels represent isomers that differ in molecular weight or charge.

Two molecular weight species of feline acid β-galactosidase (130 000 and 700 000) were observed on gel filtration of the native protein at pH 7.0 in 100 mM NaCl. The failure to resolve two distinct species by gel filtration at pH 7.0 in 500 mM NaCl (during purification) was apparently due to a salt-dependent aggregation of the feline enzyme as previously reported for acid β-galactosidase from human liver (Cheetham & Dance, 1976). Changes in the aggregation state of mammalian β-galactosidases have also been shown to occur spontaneously (Norden et al., 1974) and in response to changes to pH (Tomino & Meisler, 1975; Kuo & Wells, 1978) and protein concentration (Frost et al., 1978).

The apparent disaggregation of the feline enzyme to a single 115 000 molecular weight species under the conditions of sucrose gradient centrifugation suggests that the two species observed on gel filtration are related as aggregates. The evidence for a 62 000 molecular weight subunit under denaturing conditions further implies that at neutral pH the basic structural component of the enzyme is a dimer of 62 000 molecular weight polypeptides. Acid β-galactosidase with a molecular weight of 120 000 has also been observed at neutral pH in purified preparations from human liver (Frost et al., 1978), mouse liver (Tomino & Meisler, 1975), and rat mammary gland (Kuo & Wells, 1978), although fully active 60 000–70 000 molecular weight forms of the enzyme have been isolated (Norden et al., 1974; Distler & Jourdian, 1973).

The near-total precipitation of crude or purified enzymes by anti-β-galactosidase antiserum and the presence of the same single antigenic form in all preparations analyzed by immunoelectrophoresis indicate that the different molecular weight species of feline liver acid β-galactosidase possess common polypeptide sequences and are, most likely, related as aggregates of a single antigenically and enzymatically active component.

The general enzymatic properties of the feline liver enzyme were similar to those of acid β-galactosidase from other sources (Norden et al., 1974; Distler & Jourdian, 1973). Feline acid β-galactosidase hydrolyzed both the synthetic and natural β-D-galactosidases, as well as 4MU-α-L-arabinoside and PNP-β-D-fucoside. The pathological storage of galactose-containing glycolipids and oligosaccharides in cats with a congenital deficiency of acid β-galactosidase (Holmes & O'Brien, 1978b) is an additional indication that the enzyme plays an important role in hepatic glycolipid and glycoprotein catabolism in vivo.

The purified enzyme catalyzed the transfer of galactose from the PNP substrate to exogenous acceptors with a high efficiency. The acceptor specificity for carbohydrates was similar to that of β-galactosidase from bovine testes (Distler & Jourdian, 1973) although the acceptor activity of alcohols illustrated the generality of the transglycosidation reaction (Wallenfels & Weil, 1972). We found that the natural substrate ganglioside G_{M1} could also serve as a galactose donor in this reaction. The physiological importance of β-galactosidase-catalyzed transglycosidation reaction in mammals has yet to be fully investigated; however, the rat mammary gland enzyme appears to function in the synthesis of a naturally occurring milk oligosaccharide (6β-galactinol) by the direct

transfer of galactose from lactose to myoinositol (Kuo & Wells, 1978).

The purification of acid β-galactosidase from feline liver was recently reported by Anderson et al. (1978). The specific activity of their purified enzyme was comparable to ours, but the activity of their preparation had a half-life of only 6 h. In addition, their recovery of enzyme protein was one-fourth the amount (per gram of liver tissue) reported here. This combination of negative features hindered their characterization of the purified protein (Anderson et al., 1978) and they did not investigate the enzyme's substrate specificity, glycosyltransferase activity, subunit structure, or immunological properties.

Our method of purification is rapid and efficient and should be useful in the preparation of feline acid β-galactosidase for enzyme replacement studies and related investigations of the feline G_{M1} gangliosidosis mutants. The feline model will be of value in assessing various strategies of therapeutic intervention in human G_{M1} gangliosidosis.

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