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PURIFICATION OF G_{M1}-GANGLIOSIDE AND CERAMIDE LACTOSIDE β-GALACTOSIDASE FROM RABBIT BRAIN

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Summary

The major β-galactosidase of rabbit brain has been purified over 400-fold. The enzyme converts G_{M1}-ganglioside; Gal β1 → 3 GalNAc β1 → 4 (NANα2 → 3) Gal β1 → 4 Glc → ceramide (G_{M1}) into Tay Sachs ganglioside GalNAc β1 → 4 (NANα2 → 3) Gal β1 → 4 Glc → ceramide (G_{M2}-ganglioside) and ceramide lactoside, Gal β1 → 4 Glc → ceramide (Gal-Glc-Cer) into glucocerebroside, Glc → ceramide (Glc-Cer). The enzyme also hydrolyzes the synthetic substrates NPh-Gal and MeUmb-Gal. It is eluted as a single peak from Sephadex G-200 columns when natural and synthetic substrates were used and has an isoelectric point of 6.3. We were unable to resolve activity towards G_{M1}-ganglioside and Gal-Glc-Cer by polyacrylamide electrophoresis in two buffer systems. With G_{M1} the pH optimum was 4.3 in acetate buffer and the K_m value 78 μM while with Gal-Glc-Cer, a pH optimum of 4.5 and a K_m of 17μM were found. Hydrolysis of both natural and synthetic substrates was inhibited by γ-D-galactonolactone, D-galactose and lactose. The data strongly suggest that a single β-galactosidase hydrolyzes all the substrates tested.

Introduction

β-Galactosidases (β-D-galactoside galactohydrolase, EC 3.2.1.23) have been partially purified from a variety of mammalian sources including brain, liver, intestine and testes [1—4]. All of these enzymes have been characterized largely with the synthetic substrates NPh-Gal and MeUmb-Gal. In the brain, three major glycosphingolipids, G_{M1}-ganglioside, Gal-Glc-Cer and ceramide galacto-

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Abbreviations and trivial names: MeUmb-Gal, 4-methylumbelliferyl-β-D-galactoside; NPh-Gal, *p*-Nitrophenyl-β-D-galactoside; G_{M1}-ganglioside Gal β1 → 3 GalNAc β1 → 4 ($NAN_{\alpha}2$ → 3) Gal β1 → 4 Glc → ceramide; G_{M2}-ganglioside, Gal NAc β1 → 4 ($NAN_{\alpha}2$ → 3) Glc → ceramide; Gal-Glc-Cer, Gal β1 → 4 Glc → ceramide; CMH, Glc → ceramide; DEAE-cellulose, diethylaminoethyl cellulose.

side are catabolized by β-galactosidases. The enzyme which hydrolyzes the latter glycolipid has been purified from rat brain and shown to be capable of hydrolyzing both ceramide galactoside and Gal-Glc-Cer [5]. However, it is likely distinct from enzymes which hydrolyze G_{M1}-ganglioside [6—9].

In the human, two distinct heritable abnormalities related to deficiencies in specific β-galactosidases have been described. The first, Krabbe Leukodystrophy, results from a deficiency in ceramide galactose β-galactosidase [10]. In the second, G_{M1}-gangliosidosis, hydrolysis of G_{M1} its asialo derivative and ceramide lactose is impaired [11,12]. Affected patients also exhibit a generalized deficiency in β-galactosidase activity when assayed with synthetic substrates [11—13].

There have been few reports on extensive purification of the enzymes which hydrolyze G_{M1}-ganglioside and Gal-Glc-Cer. Jungalwala and Robins [1] developed a method for purification of two β-galactosidases from rabbit brain. However, they found that these enzymes progressively lost their ability to degrade Gal-Glc-Cer during purification. G_{M1}-ganglioside was not tested as a substrate. In the present report, the procedure developed by these workers has been re-investigated. Our results indicate that both G_{M1}-ganglioside and Gal-Glc-Cer are substrates for the brain enzymes and note further that both are likely hydrolyzed by the same enzyme.

Materials and Methods

Frozen brains of young rabbits were obtained from Pel-Freez Biologicals Inc., Rogers, Arkansas and used within one week of delivery. *p*-Nitrophenyl-β-galactoside, galactose oxidase and crystalline bovine serum albumin were obtained from Sigma and 4-methylumbelliferyl β-galactoside from Koch-Light Labs., Colnbrook, U.K. Sephadex G-200 was obtained from Pharmacia. Carrier ampholines were a product of LKB Instruments. *N*-lignoceroyldihydrolactosylceramide (CDH) was obtained from Miles-Yeda Labs., Kankakee, Illinois. G_{M1}-ganglioside was isolated from human brain according to the method of Suzuki [14] and purified by column and thin layer chromatography [15]. G_{M2}-ganglioside was used as a crude preparation isolated from the brain of a patient who died from G_{M2}-gangliosidosis. Glc-Cer isolated from the spleen of a patient with Gaucher's Disease was a gift from Dr J.A. Lowden of this hospital.

Anion exchange resin CGA-540 (100—200 mesh) was a product of the J.T. Baker Chem. Co. The resin was obtained in the chloride form and converted to the acetate form by exhaustive washing with 1 M acetic acid. The material was then dried overnight on a Buchner funnel and stored at 6 °C until used. All other chemicals were reagent grade.

Preparation of tritium-labelled glycolipids

Gal-Glc-Cer (10 mg) was oxidized in the terminal galactose by galactose oxidase (250 units) and the resulting aldehyde reduced with sodium boro[^{3}H]-hydride (25 Ci/mol) according to the method of Radin et al. [8]. The final product was re-purified on silica gel G thin layers (250 μm with the solvent $CHCl_3/CH_3OH/H_2O$ (70/30/4). Final specific activity was 8.5 Ci/mol. G_{M1}-

ganglioside (63.6 mg) was tritiated by the same method using 500 units galactose oxidase, except that after reduction of the sodium boro[^{3}H]hydride with acetic acid (1 M) the extract was dialysed against distilled water, lyophilized and purified on silica gel G layers as above with the solvent $CHCl_3$/CH_3 OH/ 2.5 M NH_4 OH (55/35/8, v/v/v). Final specific activity was 7.2 Ci/mol.

Enzyme assays. Assay mixtures for hydrolysis of NPh-Gal were prepared according to Jungalwala and Robins [1]. The release of *p*-nitrophenol was quantitated as outlined by Callahan et al. [16]. Absorbances were compared to a *p*-nitrophenol standard prepared under the same conditions and read at 400 nm. With the fluorogenic substrate, the assay mixtures contained in 0.25 ml final volume, 25 μmol citrate buffer, pH 4.3, 0.125 μmol MeUmb-Gal and enzyme. The reaction was stopped by adding 2 ml of 0.1 M 2-amino-2-methyl-1-propanol/HCl buffer, pH 10.4 and fluorescence was measured according to Robins et al. [17].

Gal-Glc-Cer β-galactosidase was measured with [^{3}H] Gal-Glc-Cer [8] but the pH of the reaction mixture was 4.6. Each assay contained 21.5 nmol of [^{3}H] Gal-Glc-Cer (15 000 cpm), 0.25 mg Triton X-100, 1 mg sodium taurocholate (Sigma), 200 μmol acetate buffer (pH 4.6) and enzyme in a final volume of 0.5 ml. Blank values averaged 100 cpm. The substrate was stable several weeks when stored at 6°C in chloroform/methanol (2/1, v/v) at a concentration of 1 mg/ml.

G_{M1}-ganglioside β-galactosidase activity was determined by a method developed in this laboratory. G_{M1}-ganglioside, dissolved in chloroform/methanol (2/1, v/v) was dried in test tubes with a stream of nitrogen. Each assay contained 40 μg (262 μM) [^{3}H] Gal G_{M1}-ganglioside (20 000 cpm) 0.25 mg sodium taurocholate, 0.1 mg Cutscum (Fisher Scientific), 1 μmol sodium chloride, 12.5 μmol sodium acetate buffer (pH 4.3) water and enzyme. The final volume was 0.1 ml. Incubations were carried out for 1 h at 37°C. To terminate the reactions, 0.4 ml of an ice cold solution containing 1 mg galactose and 1 mg bovine serum albumin was added followed by boiling for 3 min.

The tubes were chilled and centrifuged for 20 min at 2000 rev./min at room temperature. The resulting clear supernatant fluid was collected into a calibrated conical centrifuge tube containing 0.5 g of CGA-540 anion exchange resin in the acetate form. After mixing, the volume was adjusted to 3 ml with water and centrifuged as above. The clear supernatant fluid (2 ml) was counted directly. Blank values ranged from 100—200 cpm. For both the above assays, the [^{3}H] galactose released was determined with 10 ml Bray's scintillant in a liquid scintillation spectrometer. Counting efficiency was 45—48% in all instances. Units of activity are defined as nmol of substrate hydrolyzed (G_{M1}-ganglioside and Gal-Glc-Cer) or of product released (nitrophenol or 4-methylumbelliferone) per hour at 37°C. Specific activity is units per mg protein. Protein was determined according to Lowry et al. [18] with a crystalline bovine serum albumin standard.

Enzyme purification. The procedure followed is that of Jungalwala and Robins [1]. The major steps include homogenization of the tissue in 0.1 M potassium phosphate buffer (pH 7.0) followed by preparation of an acetone powder. The enzyme is released from the powder by re-homogenization as above and concentrated by ammonium sulfate fractionation.

The 25—50% $(NH_4)_2SO_4$ pellet dissolved in 5 mM Tris/phosphate (pH 7.3) was dialyzed and chromatographed on DEAE-cellulose. Two enzyme species (unadsorbed and adsorbed) were resolved at this stage. The adsorbed major enzyme was further purified by Sephadex G-200 gel filtration and by isoelectric focusing.

Isoelectric focusing. For isoelectric focusing we used a Uniphor 7900 column electrophoresis system (LKB Instruments). The pooled adsorbed enzyme from Sephadex G-200 was concentrated in an Amicon ultrafiltration cell fitted with PM-10 membrane (25 lb/inch^2) and subsequently dialyzed 20 h against 2 × 1 l 0.05 M sucrose at 2°C. The clear, colourless solution obtained after centrifugation (31 500 × g_{av} at 2°C and 30 min) was focused for 40 h at 520 V with 3% carrier ampholytes, pH range 5—7. The column temperature was thermostatically controlled to 3—4°C with a Haake FK 10 circulating bath. After focusing the column was emptied from below and the contents collected in 1.2 ml fractions. The pH of every other fraction was determined in an ice-water bath. The appropriate fractions were pooled, dialyzed 65 h against 5 l 0.1 M potassium phosphate, pH 7.0 (4 changes) and stored frozen at −10°C. The final enzyme obtained was stable for at least 6 months.

Discontinuous polyacrylamide gel electrophoresis. Electrophoresis was carried out in 7.5% gels at pH 8.3 according to Davis [19] and at pH 4.3 according to Reisfeld et al. [20]. To detect enzyme activity the gels were immersed in test tubes containing 0.5 mM MeUmb-Gal in 0.1 M acetate buffer, pH 4.3 and incubated at 37°C for 1 h. The substrate-buffer solution was then decanted and replaced with 0.1 M 2-amino-2-methyl-1-propanol/HCl buffer, pH 10.4. The enzyme was located by viewing the gels under ultraviolet light. The gels were then cut into 1 mm slices and collected into a small volume (0.1 ml) of 0.1 M phosphate buffer (pH 7.0) and let stand overnight at 6°C. At this time the gel slice was removed and β-galactosidase activity eluted into the buffer was measured with both synthetic and natural substrates.

Results

Enzymatic hydrolysis of G_{M1}-ganglioside

Anionic (sodium taurocholate) and neutral (Cutscum) detergents were required for cleavage of G_{M1}-ganglioside. Maximum hydrolysis was obtained with 0.08—0.2 mg Cutscum and 0.2—0.3 mg sodium taurocholate. These results are similar to those reported by Sloan et al. [21]. Sodium chloride (10 mM) was found to facilitate linearity of the reactions. This has also been observed by other workers in studies on human liver β-galactosidase [2,13].

Most of the unreacted G_{M1}-ganglioside co-precipitated with heated denatured albumin. Any residual substrate was effectively removed by the anion exchange resin. The resin was capable of removing up to 95% of the total substrate in the absence of the albumin. The amount of albumin used for termination of the reaction (1 mg) gave the best results. Recovery of labelled galactose carried through the assay procedure was 95%. Except for the crude homogenate (Fraction 1, Table I), the reaction was linear for up to 90 min and with protein concentrations (Fig. 1). In general, all assays were carried out with at least four protein concentrations and incubated for 1 h.

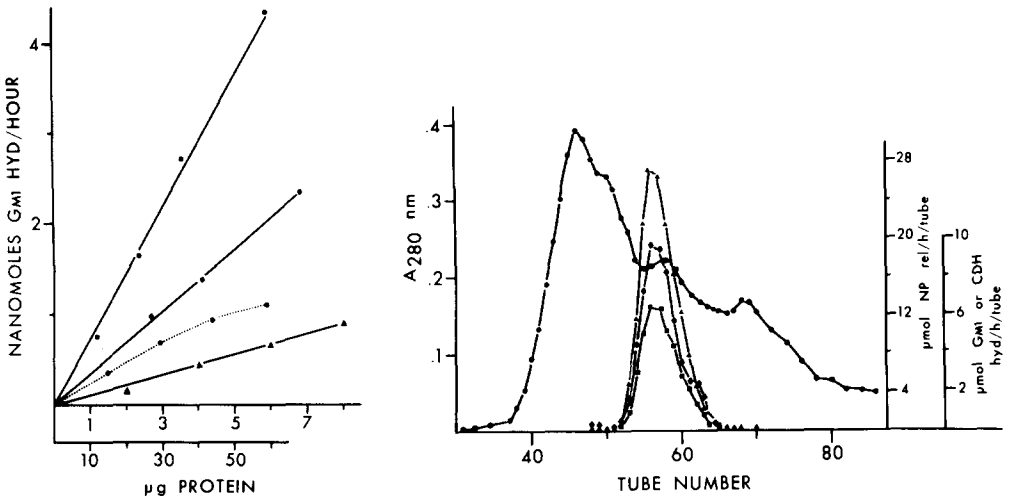

Fig. 1. Effect of protein concentration on [^{3}H] Gal G_{M1}-ganglioside hydrolysis. Enzyme fractions (see Table I) were assayed at four protein concentrations. All fractions were diluted 10-fold in 100 mM phosphate (pH 7.0) prior to assay. G_{M1}-β-galactosidase was measured as described in the text. β-Galactosidase in Fraction I was measured with 0—59 μg protein (●- - - - -●). Activity in Fraction II (▲———▲), Fraction IV B (◆———◆) and Fraction V (■———■) was measured with 0—8 μg protein. 1 nmol of G_{M1}-ganglioside hydrolyzed is equivalent to 819 cpm released.

Fig. 2. Chromatography of adsorbed β-galactosidase on Sephadex G-200. The enzyme from Fraction IV B was filtered through the column (2.5 × 100 cm) with 0.1 M phosphate buffer (pH 7.0) as eluant. Flow rate was 4.5 ml/h and each fraction was 4.1—4.5 ml. Fractions were analyzed for β-galactosidase activity using NPh-Gal (▲———▲), G_{M1}-ganglioside (◆———◆) and Gal-Glc-Cer (CDH) (■———■) as substrates. Protein is shown as A_{280nm} (●———●). Recovery of enzyme activity was 89%.

Purification of $G_{M\,1}$-ganglioside and Gal-Glc-Cer β-galactosidase

The major steps in the purification have been outlined in Methods and are included in Table I. Our data on hydrolysis of the synthetic substrate, NPh-Gal agree with those of Jungalwala and Robins [1]. However, in contrast to their report the hydrolytic activity with both natural and synthetic substrates increased at each step in the procedure.

The two species of β-galactosidase were separated by DEAE-cellulose chromatography. Both the unadsorbed and adsorbed enzymes hydrolyzed both natural substrates and NPh-Gal (Table I). The unadsorbed enzyme was not purified further. The adsorbed enzyme eluted from Sephadex G-200 as a single peak (Fig. 2). Activity with NPh-Gal, Gal-Glc-Cer and $G_{M\,1}$-ganglioside was coincident suggesting that a single hydrolyzed all three substrates. At this stage the enzyme had been purified over 400-fold with respect to hydrolysis of NPh-Gal and $G_{M\,1}$-ganglioside and about 220-fold for hydrolysis of Gal-Glc-Cer. The ratio of the specific activities for NPh-Gal and $G_{M\,1}$-ganglioside hydrolysis was fairly constant (about 2.2/1) at all stages of purification. Comparison of the specific activities of NPh-Gal and $G_{M\,1}$-ganglioside to Gal-Glc-Cer were more erratic however, but averaged 7.9/1 and 3.7/1 respectively.

The yields of activities at this stage were 5.7, 2.9 and 3.9% for NPh-Gal, Gal-Glc-Cer and $G_{M\,1}$-ganglioside β-galactosidase respectively. This is comparable to the yield (6.1%) obtained by Jungalwala and Robins [1].

TABLE I

PURIFICATION OF β-GALACTOSIDASE FROM 400 g RABBIT BRAIN

Fraction	Total protein (mg)	Volume (ml)	NPβ-Gal			Gal-Glc-Cer			GMl-ganglioside		
			Total units ($\times 10^5$)	Specific activity	Purif. (-fold)	Total units ($\times 10^4$)	Specific activity	Purif. (-fold)	Total units ($\times 10^4$)	Specific activity	Purif. (-fold)
Crude	53 100	1 800	26.3	49.5	1.0	45.0	8.5	1.0	117	22.0	1.0
Soluble extract	6 930	1 760	13.0	187.9	3.8	16.9	24.5	2.9	78.7	113.6	5.2
$(NH_4)_2SO_4$ precipitation											
0—0.25	540	128	0.2	38.8	0.8	0.3	5.0	0.6			
0.25—0.50	581	83	5.1	871.6	17.6	7.0	120.1	14.1	19.6	337.4	15.3
DEAE-cellulose chromatography											
A unadsorbed	38	2	0.06	143.9	2.9	0.02	43.7	5.1	0.05	130.1	5.9
B adsorbed	73	46	3.12	3 398.0	80.8	4.36	559.0	65.8	15.0	2 052.7	93.3
Sephadex G-200	7.1	12	1.51	21 268.0	429.7	1.32	1 859	218.7	6.4	9 049.1	411.3

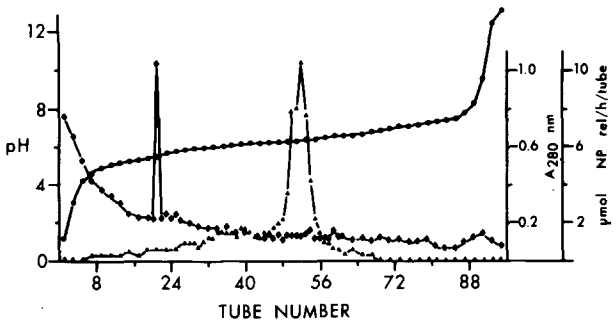

Fig. 3. Isoelectric focusing of β-galactosidase. The adsorbed enzyme from Sephadex G-200 (Fraction V, Table I) was focused as described. The column was eluted at a flow rate of 1 ml/min. Each fraction (1.2 ml) was analyzed for β-galactosidase with NPh-galactoside (▲———▲). Protein is expressed as A_{280nm} (◆———◆) and the pH gradient is (●———●). Recovery of enzyme activity was 74%. The contents of tubes 49—54 were pooled, dialyzed as described against 100 mM phosphate (pH 7.0) and concentrated by ultrafiltration.

The enzyme obtained after Sephadex G-200 chromatography had an isoelectric point of 6.3 at 3—4°C. (Fig. 3). The enzyme was again found as a single sharp peak of activity. The final enzyme obtained after prolonged dialysis and Amicon concentration was then examined by polyacrylamide electrophoresis. The enzyme solution contained one major and at least two minor protein bands. Enzyme activity with MeUmb-Gal, Gal-Glc-Cer and G_{M1}-ganglioside was not separated after polyacrylamide electrophoresis (Fig. 4) and in all instances

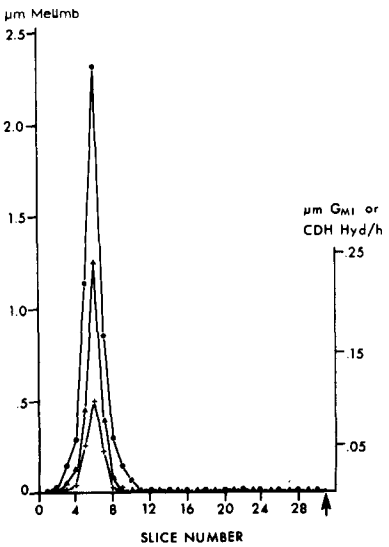

Fig. 4. Polyacrylamide electrophoresis of rabbit brain β-galactosidase. The protein solution (Fraction V, Table I) was exposed for 90 min at 2°C and 1.5 mA per gel. The enzyme was localized and the gels cut into 1 mm slices as described. Enzyme activity expressed as units per 10.1 ml elution fluid was measured with MeUmb-Gal (●———●), G_{M1}-ganglioside (▲———▲) and Gal-Glc-Cer (CDH) (+———+). The arrow on the abscissa indicates the distance travelled by a bromphenol blue marker.

was found in the protein staining region. The Rf of the enzyme at pH 8.3 was 0.22 with respect to the bromphenol blue marker. Electrophoresis at pH 8.3 was found to be more satisfactory than pH 4.3. The ratio of G_{M1}-ganglioside to Gal-Glc-Cer activity was the same after electrophoresis as before.

The major contaminant of the enzyme after isoelectric focusing was the carrier ampholytes which were not completely removed by prolonged dialysis or by ultrafiltration. Refiltration of the focused enzyme through an additional Sephadex G-200 column effectively removed the remaining ampholytes but with a resultant loss in specific activity. This effect is likely due to excess dilution of the enzyme. The presence of the contaminating ampholines had no noticeable effect on enzyme activities measured in any of the assays used in this study.

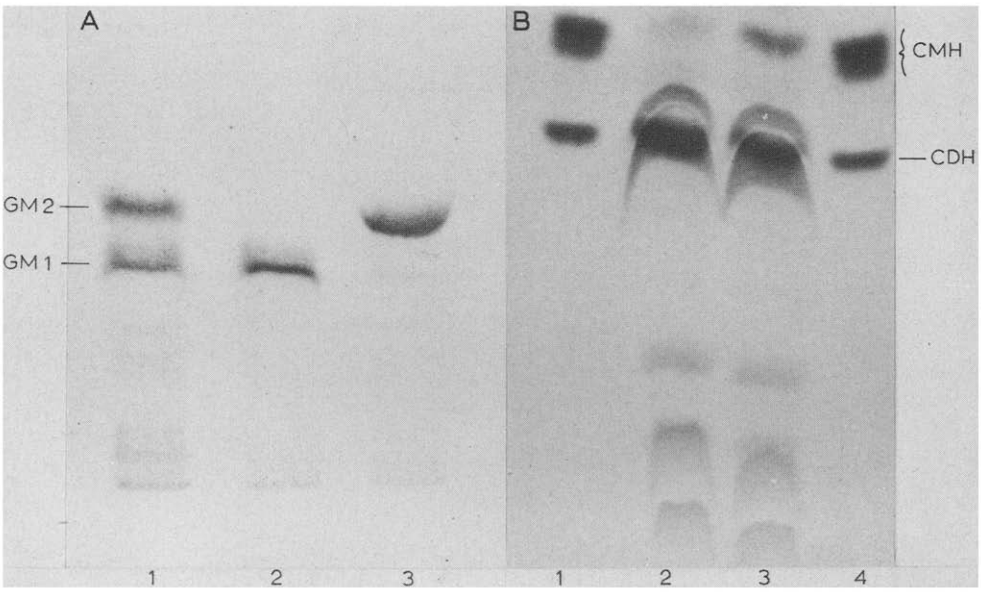

Fig. 5. Hydrolysis of G_{M1}-ganglioside and Gal-Glc-Cer (CDH) by β-galactosidase. A, Unlabelled G_{M1}-ganglioside was incubated in the standard assay mixture for 1 h at 37°C. The reaction was stopped by the addition 1 ml of solvent (chloroform/methanol, 2/1, v/v) and the contents were dried with a stream of nitrogen. The entire residue was dissolved in the above solvent (50 μl) and spotted on silica gel G layers (250 μm). The chromatogram was developed with chloroform/methanol/2.5 N NH_4OH (55/35/8, v/v/v) and the gangliosides were visualized with a resorcinol spray reagent. Lane 1 is standard G_{M1}-ganglioside and G_{M2}-ganglioside (20 μg each) incubated in the complete assay system without enzyme; Lane 2, G_{M1}-ganglioside (40 μg, 262 μM) incubated in the complete assay mixture without enzyme; Lane 3, as lane 2, except 5.9 μg enzyme (53.4 units, Fraction V) was included. B, Unlabelled Gal-Glc-Cer (30 μg) was incubated 2 h at 37°C in a final volume of 0.5 ml. The reaction was stopped with 2.5 ml of the above solvent. After thorough mixing, the lower phase was recovered, dried and the entire residue, dissolved in 50 μl chloroform/methanol (2/1, v/v) was spotted on silica gel G layers (250 μm). The layer was developed with chloroform/methanol/water (70/30/4, v/v/v) and glycolipids were visualized with 50% sulfuric acid spray at 120°C. Lane 1, standard Glc-Cer (30 μg) and Gal-Glc-Cer (30 μg); Lane 2 complete assay with 30 μg Gal-Glc-Cer and enzyme added just before reaction was stopped; Lane 3, as for lane 2, except 10.8 μg (107 units) enzyme added; Lane 4, standard as lane 1. The glycolipids Glc-Cer and Gal-Glc-Cer were identified by the purple colours developed with the spray reagent. The other visible materials stained yellow and were contributed by the detergent mixture used in the assay.

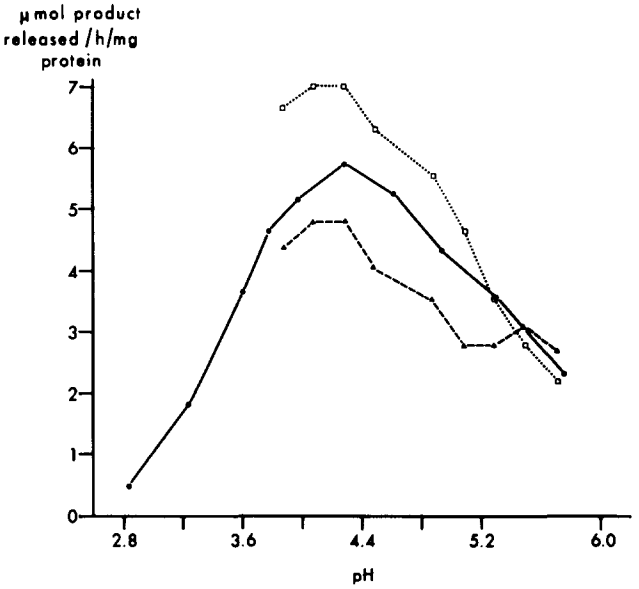

Fig. 6. The pH-dependence of synthetic substrate hydrolysis. The adsorbed enzyme (0.12 μg and 1.2 μg) was incubated in the presence of MeUmb-Gal and NPh-Gal respectively for 1 h at 37°C. The fluorogenic substrate was tested with acetate (▲- - - - -▲) and citrate (●———●) buffers while the NPh-Gal was tested with acetate buffers (□- - - - -□). All points are average of duplicate measurements.

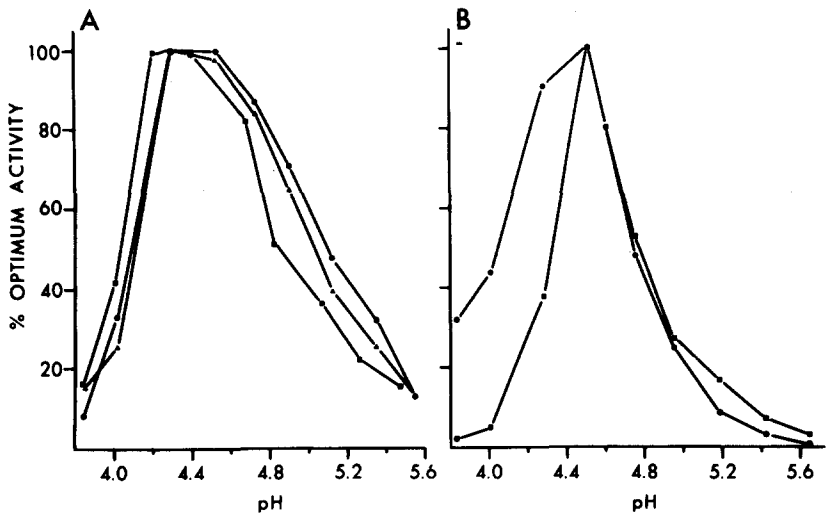

Fig. 7. pH dependence of the hydrolysis of G_{M1}-ganglioside and CDH. All incubations were run in duplicate for 1 h at 37°C and all enzyme fractions were diluted 10-fold in 100 mM phosphate (pH 7.0). In A, G_{M1}-ganglioside was incubated with enzyme (10 μl) from Fraction IV (1.6 μg ▲———▲), Fraction V (0.6 μg ●———●) and after electrofocusing (0.6 μg ■———■) in acetate buffers of varying pH. In B, Gal-Glc-Cer was incubated with enzyme (20 μl from Fraction V (1.2 μg, ●———●) and pure enzyme (1.2 μg, ■———■) in acetate buffers of varying pH. The specific activities were expressed as percent of the optimum value.

TABLE II

KINETIC CONSTANTS OF ADSORBED β-GALACTOSIDASE

Substrate	Buffer	pH opt.	K_m (mM)	V (nm/min)
NPh-Gal	acetate	4.2	0.17	0.051
MeUmb-Gal	citrate	4.3	0.40	0.30
G_{M1}-ganglioside	acetate	4.3	0.078	0.22
Gal-Glc-Cer	acetate	4.5	0.017	0.035

Properties of the adsorbed β-galactosidase

The enzyme converted Gal-Glc-Cer to Glc-Cer and G_{M2}-ganglioside was formed by hydrolysis of G_{M1}-ganglioside (Fig. 5). The conversion of G_{M1}-ganglioside to G_{M2}-ganglioside was complete within 1 h while the same amount of enzyme was incapable of completely hydrolysing Gal-Glc-Cer in the same time. About 50% conversion of Gal-Glc-Cer was achieved when the enzyme concentration was increased to 10.8 μg protein and the incubation time doubled (Fig. 5B).

With the synthetic substrates NPh-Gal and MeUmb-Gal we found the pH optimum was 4.0—4.3 and the action was similar in both acetate and citrate buffers (Fig. 6). The hydrolysis of G_{M1}-ganglioside and Gal-Glc-Cer showed pH optimum at 4.3 and 4.5 respectively (Fig. 7). The pH optimum obtained with ceramide lactoside was 4.5—4.6 at all stages of purification. In citrate buffers, the action of the enzyme on both natural substrates was reduced to about 10% of the activity in acetate buffers.

The effect of varying substrate concentration on the hydrolysis of all substrates tested is summarized in Table II. The affinity of the enzyme for natural substrates is greater than that found with either synthetic substrate.

TABLE III

INHIBITION OF β-GALACTOSIDASE BY CARBOHYDRATES

All inhibitor solutions were prepared in water immediately before use. Enzyme Fraction V was used in these experiments. All assays were carried out in duplicate as described.

Substrate	Inhibitor (mM)	Inhibition (%) *		
		γ-D-Galactono-lactone	Galactose	Lactose
MeUmb-Gal	0.4	58.7	21.6	11.9
	2.0	86.2	51.8	21.6
	5.0	100.0	73.9	21.6
G_{M1}-ganglioside	0.4	49.6	12.9	− 6.8
	2.0	76.9	23.9	− 7.3
	5.0	88.4	46.0	9.3
Gal-Glc-Cer	0.8	79.4	24.1	− 2.4
	4.0	96.6	72.9	17.7
	8.0	100.0	82.6	31.3

* The initial specific activities were 15 571, 6776 and 3186 nmol product released. (MeUmb-Gal) and substrate hydrolyzed (G_{M1} and Gal-Glc-Cer respectively) per h per mg protein. The negative numbers represent increased activity over the control values.

With G_{M1}-ganglioside, the K_m value was the same (78 μM) at both pH 4.3 and at pH 5.0. The enzyme was inactive when tritium-labelled galactocerebroside (251 μM) replaced ceramide lactoside or G_{M1}-ganglioside in their respective assay mixtures. Nor was there any action on this substrate in either assay in the presence of pure sodium taurocholate.

Galactose and γ-D-galactonolactone were effective inhibitors of the hydrolysis of MeUmb-Gal, G_{M1}-ganglioside and Gal-Glc-Cer, whereas lactose was not as effective an inhibitor when G_{M1}-ganglioside was the substrate (Table III). Neither gluconolactone nor γ-L-galactonolactone (at 5 mM) inhibit more than 10% the hydrolysis of any substrate. Further studies on the nature of the inhibition by these sugars are under investigation. G_{M1}-ganglioside and Gal-Glc-Cer were replaced in their respective assay mixtures by 0.5 mM MeUmb—Gal. Under these conditions hydrolysis was stimulated about 2-fold. Taurocholate alone completely inhibited the hydrolysis whereas either Cutscum alone or triton X-100 alone also stimulated the reaction 2-fold.

Discussion

We have purified a β-galactosidase from rabbit brain. The isolated enzyme is adsorbed by DEAE-cellulose at pH 7.3. It migrates as a single peak on Sephadex G-200 and on isoelectric focusing. The isoelectric point was 6.3 at 3°C. The enzyme hydrolyses NPh-Gal, MeUmb-Gal, Gal-Glc-Cer and G_{M1}-ganglioside at all stages of purification. Enzyme activity towards MeUmb-Gal, Gal-Glc-Cer and G_{M1}-ganglioside was not resolved by any method utilized including polyacrylamide electrophoresis in two buffer systems.

The procedure followed for isolation of β-galactosidase was that of Jungalwala and Robins [1] and we have achieved a similar (420-fold) purification. In their procedure however, there was a decrease in Gal-Glc-Cer β-galactosidase activity with increasing purity while we found a consistent increase. This difference may result from the relatively insensitive assay procedure they employed coupled with a decreased yield of enzyme at each step in the method.

A method for measurement of G_{M1}-ganglioside catabolism was developed during this investigation. The validity of the assay employed seems best expressed by Figs 1 and 5. The reaction was linear with incubation time, protein concentration and the enzyme as predicted converted G_{M1}-ganglioside to G_{M2}-ganglioside when checked by an independent method (Fig. 5). This assay has recently been used successfully by Miyatake and Suzuki in their studies on rat brain β-galactosidase [9].

It is unlikely that the final enzyme preparation obtained in this work contains a mixture of two or more distinct β-galactosidases. Rather, it is probable that the single enzyme can hydrolyze several substrates at one or more active sites. Further work on the detailed mechanism of action is required to answer this question. Other workers have isolated β-galactosidases which act on glycolipid and artificial substrates simultaneously. Distler and Jourdian [4] purified about 600-fold from bovine testes a β-galactosidase which catalyzed the breakdown of Gal-Glc-Cer, G_{M1}-ganglioside and other natural and synthetic substrates. Sloan [22] isolated a β-galactosidase from human liver which acted on G_{M1}-ganglioside and MeUmb-Gal while Meisler [23] isolated from the

same source an enzyme which acted on labelled Gal-Glc-Cer and NPh-Gal. In none of these reports, however, were both Gal-Glc-Cer and G_{M1}-ganglioside used simultaneously with synthetic substrates to follow the purification. This is an important aspect of the present study because our data suggest that, at least in rabbit brain, both can be hydrolyzed by the same enzyme. With G_{M1}-ganglioside the adsorbed enzyme isolated in this study had a pH optimum of 4.3 and a K_m of 78 μM. Hydrolysis of Gal-Glc-Cer showed a pH optimum near 4.5 and the K_m was 17 μM. These results are in close agreement to those obtained by other workers with crude partially purified preparations from human liver [2,13] and rat brain [7]. Miyatake and Suzuki [9] found K_m values for the hydrolysis of G_{M1}-ganglioside and ceramide lactose of 820 μM and 190 μM respectively with crude rat brain β-galactosidase. These values are 10-fold higher than the values determined in the present work. However, these workers used citric acid-sodium citrate buffers whereas our values and those of others [2,7,13,22] were determined in acetate buffer. The apparent discrepancies can likely be ascribed to differences in type of buffer used.

The substrate specificities of these enzymes hold importance for our understanding of the metabolism of Gal-Glc-Cer and G_{M1}-ganglioside in normal and diseased brain. The same rabbit enzyme hydrolyzes both compounds and thus may be the pivotal enzyme in the catabolism of gangliosides in this animal. In the human, a G_{M1}-ganglioside storage disease results from impaired breakdown of this lipid due to the lack of β-galactosidase. Suzuki and co-workers [24] noted in this disease increased tissue content of Gal-Glc-Cer in liver and have demonstrated concomitant decrease in the enzymatic hydrolysis of this glycolipid [12]. This contrasts with the data of Brady and co-workers who found that the hydrolysis of specifically-labelled Gal-Glc-Cer was normal or elevated in the brain of patients with this disease [25]. Recent results from our laboratory show that hydrolysis of both synthetic substrates and G_{M1}-ganglioside and Gal-Glc-Cer is reduced in leukocytes and cultured skin fibroblasts from a patient with a third clinical variant of G_{M1}-gangliosidosis [26]. Hydrolysis of galactocerebroside was not affected. Recent data of Wenger et al. [27] and Suzuki [12,28] have defined the substrate specificities of the major β-galactosidases acting on glycolipids in crude extracts of human tissues. The substrate specificity of the brain β-galactosidase isolated in this work is in excellent agreement with these workers. However, purification of the human brain enzyme is necessary to confirm these observations.

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