Purified Human Liver Acid β -D-Galactosidases Possessing Activity towards G_{M1} -Ganglioside and Lactosylceramide

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Our studies with purified human liver acid β -D-galactosidases (EC 3.2.1.23) indicate that 4-methylumbelliferyl β -D-galactosidase and G_{M1} -ganglioside β -D-galactosidase activities are identical with lactosylceramidase II activity. Evidence for this includes co-purification of all enzyme activities by affinity chromatography to yield a single band on polyacrylamide-gel electrophoresis and coincident elution from Sepharose 6B of all three enzyme activities.

Acid β -D-galactosidases which hydrolyse synthetic substrates have been demonstrated in various human tissues and extracellular fluids (Furth & Robinson, 1965; Hultberg & Ockerman, 1969; Ho & O'Brien, 1970; Meisler, 1972; Ho *et al.*, 1973; Norden & O'Brien, 1973; Norden *et al.*, 1974). These enzymes also hydrolyse terminal β -D-galactoside linkages of naturally occurring glycolipids (Meisler, 1972; Ho *et al.*, 1973; Norden & O'Brien, 1973; Norden *et al.*, 1974; Wenger, 1974; Tanaka *et al.*, 1975) and glycoproteins (MacBrinn *et al.*, 1969; Mahadevan *et al.*, 1969; Norden *et al.*, 1974). Previous studies have shown that human liver acid β -D-galactosidases occur as the 'A' protein (mol.wt. 60×10^3 – 70×10^3) and the 'B' protein (mol.wt. 600×10^3 – 700×10^3) (Furth & Robinson, 1965; Hultberg & Ockerman, 1969; Ho & O'Brien, 1970; Ho *et al.*, 1973; Norden & O'Brien, 1973; Norden *et al.*, 1974), which share antigenic determinants (Norden *et al.*, 1974) and many biochemical properties (Ho *et al.*, 1973; Norden & O'Brien, 1973).

Previous reports suggest that human G_{M1} -ganglioside* β -D-galactosidase cleaves lactosylceramide under certain conditions; however, this fact has not been established with certainty, since crude enzyme preparations were studied (Norden *et al.*, 1974; Tanaka *et al.*, 1975; Tanaka & Suzuki, 1976). We have reported a specific affinity-chromatographic procedure for the purification of human liver acid β -D-galactosidases 'A' and 'B' (Miller *et al.*, 1976). The studies presented in the present report with purified acid β -D-galactosidase demonstrate that G_{M1} -ganglioside β -D-galactosidase and lactosylceramidase II activities are properties of the same enzyme.

* Abbreviation: G_{M1} -ganglioside, galactosyl-N-acetyl-galactosaminyl-(N-acetylneuraminy)galactosylglucosylceramide.

Experimental

Materials

Sepharose 6B and concanavalin A-Sepharose 4B were purchased from Pharmacia (Uppsala, Sweden). D-Galactono- γ -lactone, 4-methylumbelliferyl β -D-galactopyranoside and purified sodium taurocholate were obtained from Koch-Light Laboratories (Colnbrook, Bucks., U.K.), and D-galactose and crude taurocholate were supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.). Lactosylceramide was a product of Miles-Yeda (Kankakee, IL, U.S.A.), and NaB^3H_4 (10 Ci/mmol) was obtained from Amersham-Searle (Arlington Heights, IL, U.S.A.). Galactose oxidase was supplied by Kabi (Stockholm, Sweden). Chemicals for polyacrylamide-gel electrophoresis were purchased from Miles Laboratories (Elkhart, IN, U.S.A.). Purified sodium taurodeoxycholate was supplied by Calbiochem (La Jolla, CA, U.S.A.). Diaflo ultra-filtration device and membranes were purchased from Amicon Corp. (Lexington, MA, U.S.A.).

Methods

All procedures were carried out at 0–4°C unless otherwise specified. Human liver acid β -D-galactosidases were isolated and purified by affinity chromatography as previously described (Miller *et al.*, 1976). The procedure involved a sequence of concanavalin A-Sepharose 4B column chromatography, dialysis at pH 5.0 and affinity column chromatography (2 cm $\times$ 22 cm, 69 ml bed volume) on Sepharose 4B-6-aminohexyl 1-thio- β -D-galactopyranoside (thiogalactosyl-Sepharose 4B). After application to the affinity column of 70.5 ml of acid β -D-galactosidase (375 units of 4-methylumbelliferyl β -D-galactosidase; 883 mg of protein) eluted from the concanavalin A-Sepharose 4B column, it

was washed with 0.01 M-sodium acetate/acetic acid (pH 5.0), containing 0.1 M-NaCl, until the A_{280} was zero (see Fig. 1). The acid β -D-galactosidase activity was then eluted with the same buffer containing 0.1 M-D-galactono- γ -lactone. After the fractions containing β -D-galactosidase activity (nos. 76–83; Fig. 1) had been combined, solid D-galactose was added to a concentration of 0.1 M to stabilize the enzyme. The pool of acid β -D-galactosidase containing 2.92 mg of protein was concentrated, by using a Diaflo UM-10 membrane, to 1.2 ml (260 units of acid 4-methylumbelliferyl β -D-galactosidase) and stored at 0–4°C in the presence of 0.02% NaN_3 . Samples of the preparation eluted from the affinity column were used for Sepharose 6B column chromatography, kinetic studies with various substrates and polyacrylamide-gel electrophoresis.

A 0.1 ml sample of the purified enzyme (22 units of acid 4-methylumbelliferyl β -D-galactosidase; 0.24 mg of protein) was applied to a Sepharose 6B column (0.9 cm $\times$ 100 cm, 60 ml bed volume), and eluted with 0.01 M- NaH_2PO_4 , pH 7.0, containing 0.1 M-NaCl, 0.1 M-D-galactose, 0.1 mM-EDTA (disodium salt) and 0.02% NaN_3 .

Acid β -D-galactosidase was assayed as previously described (Ho *et al.*, 1972) with 4-methylumbelliferyl β -D-galactopyranoside. G_{M1} -ganglioside β -D-galactosidase was assayed by using G_{M1} -ganglioside purified and specifically tritiated in the terminal galactose moiety as described by Norden & O'Brien (1973). Lactosylceramidases I and II were assayed as described by Wenger *et al.* (1974) and Tanaka *et al.* (1975) respectively, by using lactosylceramide tritiated in the terminal galactose moiety by the galactose oxidase/ NaB^3H_4 procedure for the G_{M1} -

ganglioside (Norden & O'Brien, 1973). The fractions from the affinity and Sepharose 6B columns were assayed by the G_{M1} -ganglioside-assay procedure of Norden & O'Brien (1973) and the lactosylceramidase II-assay procedure of Tanaka *et al.* (1975). For these assays the substrate concentrations were 0.1 mM for G_{M1} -ganglioside and 0.05 mM for lactosylceramide. In certain studies, modifications of the aforementioned assay systems for lactosylceramide were used: (1) crude taurocholate (1.25 mg/assay) was substituted for the purified taurocholate in the Wenger *et al.* (1974) procedure; (2) purified sodium deoxytaurocholate (0.5 mg/assay) was substituted for the crude taurocholate in the Tanaka *et al.* (1975) procedure. One unit of enzyme activity is defined as the hydrolysis of 1 μ mol of substrate/min per ml at 37°C.

The acid β -D-galactosidases eluted from the affinity column were subjected to electrophoresis in tube 5% (w/v) polyacrylamide gels (8.8 cm $\times$ 0.5 cm) at pH 8.3 (Tris/glycine) by the method of Davis (1964). The gels were stained for protein with Coomassie Blue after fixation with 12.5% (w/v) trichloroacetic acid.

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

Results

The 4-methylumbelliferyl β -D-galactosidase, G_{M1} -ganglioside β -D-galactosidase and lactosylceramide β -D-galactosidase activities co-chromatographed on the thiogalactosyl-Sepharose 4B column and were specifically eluted with 0.1 M-D-galactono- γ -lactone as coincident symmetrical peaks of activity (Fig. 1). Recovery from the affinity column

Fig. 1. Affinity-column chromatography on a Sepharose 4B-6-aminoethyl 1-thio- β -D-galactopyranoside column of concentrated α -methyl D-mannoside eluted from concanavalin A-Sepharose 4B

The column (2 cm $\times$ 22 cm) was eluted as described under 'Methods'. Fractions (21 ml) were collected and 10 μ l portions were assayed. At the arrow the column was eluted with 0.1 M-D-galactono- γ -lactone. $\blacktriangle$, 4-Methylumbelliferyl β -D-galactopyranoside (5 min); $\triangle$, G_{M1} - β -D-galactopyranoside (20 min); $\circ$, lactosylceramide (20 min); $\bullet$, A_{280} . Inset photo reveals the Coomassie Blue staining of a polyacrylamide gel containing 23 μ g of the concentrated enzyme preparation eluted from the Sepharose 4B-6-aminoethyl 1-thio- β -D-galactopyranoside column.

Fig. 2. Sepharose 6B column chromatography of the concentrated preparation eluted from Sepharose 4B-6-amino-hexyl 1-thio- β -D-galactopyranoside with D-galactono- γ -lactone

The column was eluted as described under 'Methods' at a flow rate of 3 ml/h at pH 7.0. Fractions (0.3 ml) were collected and 10 μ l portions were assayed for the various enzyme activities as described under 'Methods'. ●, 4-Methylumbelliferyl β -D-galactopyranoside (30 min); Δ , G_{M1} -ganglioside (60 min); ○, lactosylceramide (60 min).

was 69% with respect to the 4-methylumbelliferyl β -D-galactosidase. The purity of the enzyme is demonstrated by the inset photograph, which reveals the presence of a single band of protein in the sample eluted from the thiogalactosyl-Sepharose 4B column on polyacrylamide-gel electrophoresis at pH 8.3.

Sepharose 6B column chromatography of the purified acid β -D-galactosidase preparation revealed that 4-methylumbelliferyl β -D-galactosidase, G_{M1} -ganglioside β -D-galactosidase and lactosylceramidase II activities were eluted coincidentally with the 'A' and 'B' β -D-galactosidase isoenzymes (Fig. 2).

The purified acid β -D-galactosidase preparation was active against lactosylceramide and G_{M1} -ganglioside (Table 1). However, large differences in activity towards lactosylceramide were found when the assay system was varied. Under the assay conditions of Tanaka *et al.* (1975), the specific activity of lactosylceramidase II was almost equivalent to that of G_{M1} -ganglioside β -D-galactosidase. With the assay procedure of Wenger *et al.* (1974), lactosylceramidase I was 1% of that found with the assay procedure of Tanaka *et al.* (1975). Substitution of crude taurocholate for purified taurocholate in the former system resulted in a 37-fold enhancement of lactosylceramidase I activity in the purified β -D-galactosidase preparation.

Discussion

Results obtained in other laboratories have suggested that human lactosylceramidase I and

Table 1. Activity of purified acid β -D-galactosidase with natural and synthetic substrates

Appropriately diluted portions of the preparation eluted from the Sepharose 4B-6-amino-hexyl 1-thio- β -D-galactopyranoside column were assayed with 4-methylumbelliferyl β -D-galactopyranoside, G_{M1} -ganglioside and lactosylceramide at 37°C for 10 min, under the optimal assay conditions indicated under 'Methods'. The activity with each substrate is expressed as μ mol of galactose hydrolysed/min per mg of protein.

Substrate	Activity
4-Methylumbelliferyl β -D-galactopyranoside	83.40
G_{M1} -ganglioside	18.50
Lactosylceramide:	
Tanaka <i>et al.</i> (1975) system	15.30
Modified Tanaka <i>et al.</i> (1975) system*	16.40
Wenger <i>et al.</i> (1974) system	0.17
Modified Wenger <i>et al.</i> (1974) system†	6.36

* Substitution of purified taurodeoxycholate for crude taurocholate as described under 'Methods'.

† Substitution of crude taurocholate for purified taurocholate as described under 'Methods'.

lactosylceramidase II are different enzymes, the former being the enzyme that cleaves galactosylceramide and the latter being the enzyme that cleaves G_{M1} -ganglioside (Wenger, 1974; Wenger *et al.*, 1974, 1975a,b; Tanaka *et al.*, 1975; Tanaka & Suzuki, 1975, 1976, 1977). However, the use of crude homogenates and partially purified enzyme preparations has not provided sufficient evidence for the identity of either lactosylceramidase I or II with the respective β -D-galactosidases. The present report provides convincing evidence that, in the presence of either crude bile salts or purified taurodeoxycholate, the purified G_{M1} -ganglioside β -D-galactosidase can actively cleave lactosylceramide at significant rates. These results are consistent with the conclusion that lactosylceramidase II activity is due to G_{M1} -ganglioside β -D-galactosidase.

The low activity of lactosylceramidase I in our purified acid β -D-galactosidase preparation (Table 1) is consistent with the proposal by Tanaka & Suzuki (1976) that lactosylceramidase activity in human liver is due primarily to lactosylceramidase II, with lactosylceramidase I present as a minor component. Differences were observed in the degree of stimulation of lactosylceramidase I by crude taurocholate when the purified β -D-galactosidase was used compared with previous studies with crude liver homogenates (Tanaka & Suzuki, 1976). The enhanced lactosylceramidase I activity (37-fold) obtained on substituting crude taurocholate for purified taurocholate in the standard Wenger

et al. (1974) assay system was 7-fold greater than Tanaka & Suzuki (1975) had observed with crude liver enzyme preparations.

The remarkable effect of detergent stimulation of hydrolysis of galactosphingolipids by the purified enzyme is worth further study, especially since little effect is noted for the cleavage of synthetic substrates when various detergents are used. A better understanding of the molecular interaction between glycosidases and glycosphingolipids in aqueous systems *in vivo* may be obtained with the use of purified glycosidases and the appropriate bile salt. Enhancement of hydrolysis of galactosphingolipids by detergents may be analogous to the 'co-hydrolase' proposal described by Radin (1975).

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