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A UNIQUE GLYCOSPHINGOLIPID-SPLITTING ENZYME (CERAMIDE-GLYCANASE FROM LEECH) CLEAVES THE LINKAGE BETWEEN THE OLIGOSACCHARIDE AND THE CERAMIDE

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Summary: A novel type of enzyme which hydrolyzes the linkage between the ceramide and the sugar chain in various glycosphingolipids has been found in the leech, Hirudo medicinalis. This enzyme releases the intact oligosaccharide from LacCer, $GbOse_3Cer$, $GbOse_4Cer$, $GbOse_5Cer$, $nLcOse_4Cer$, GM3, GM2, GM1, GD1a and GT1 with the concurrent release of ceramides. By using tritium-labeled GM1 as substrate we found the optimum pH of this enzyme to be between pH 4 and 5. Since the enzyme cleaves the linkage between the ceramide and the sugar chain in various glycosphingolipids with no apparent preference toward the sugar chain, we propose to call this enzyme ceramide-glycanase.

Erythrocytes of higher animals are rich in glycosphingolipids (1), and leeches consume animal blood. It occurred to us that as corollary to these two facts, leech might be expected to catabolize glycosphingolipids. When this hypothesis was tested, the results far exceeded our expectations. This communication reports the presence in the leech, Hirudo medicinalis, of a unique enzyme which hydrolyses the linkage between the ceramide and the sugar chain in various glycosphingolipids.

In contrast with previously known glycosidases, the highly specific exo- and endo-glycosidases, which produce either monosaccharides or partially degraded oligosaccharides, this enzyme releases the entire oligosaccharides intact, together with ceramide. We propose to call this enzyme a ceramide-glycanase, and we abbreviate this to "Cer-glycanase".

EXPERIMENTAL PROCEDURE

Materials - LacCer and $GbOse_3Cer$ were isolated from human erythrocytes (2). $nLcOse_4Cer$ was isolated from bovine erythrocytes (3) and GM2 was isolated from Tay-Sachs brain (4). GM1, tritium labeled at the terminal Gal was prepared according to the method described by Radin (5). Oligosaccharides from $GbOse_4Cer$, $GbOse_3Cer$, GM1 and GM2 were prepared by ozonolysis of the parent glycosphingolipids (2). β-Galactosidase and β-hexosaminidase were isolated from jack bean (6). α-Galactosidase was isolated from fig (7) and α-N-acetyl-

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galactosaminidase from limpet (8). The following were generous gifts: lacto-N-neotetraose, Dr. C. A. Bush, Illinois Institute of Technology; oligosaccharides prepared from GM1 and GD1a (9), Dr. C.-L. Schengrund, Hershey Medical Center, the Pennsylvania State University. The following were purchased from commercial sources: leeches (Hirudo medicinalis), Carolina Biological Supply Company; GM3, GM1, GD1a, GT1 and $GbOse_4Cer$, Supelco; ConA-Sepharose, Octyl-Sepharose, p-nitrophenyl-glycosides, ceramide Type III (primarily nonhydroxy fatty acids), ceramide Type IV (mainly α-hydroxy fatty acids), lactose, sialyllactose, and neuraminidase Type X from Clostridium perfringens, Sigma; Nucleosil C18 beads (silica beads bonded with octadecyl groups, particle size 30μ), Rainin; sodium taurodeoxycholate, Calbiochem; Sepharose-6B, Pharmacia; silica gel 60 pre-coated plates, Merck, Darmstadt, Germany.

Enzyme Assays - The assays were performed in 10 x 75 mm glass tubes. The incubation mixture contained the following components in 0.2 ml: glycolipid substrate, 30 nmol; sodium acetate buffer (50 mM), pH 4.5; sodium taurodeoxycholate, 200 μg, and an appropriate amount of enzyme. After the mixture was incubated at 37°C for a preset time, the reaction was terminated by adding 5 volumes of chloroform:methanol (2:1). The mixture was vortexed and briefly centrifuged to separate the organic phase (lower) from the aqueous phase (upper). Under this condition the enzyme protein became insoluble and stayed at the interface. The organic and the aqueous phases were separately withdrawn from the tube and evaporated to dryness. For detection of the released oligosaccharide, the aqueous phase was analyzed by TLC using n-butanol:acetic acid:H_2O (2:1:1) as the developing solvent (10). Sialic acid containing glycoconjugates were revealed by resorcinol spray (11), while glycoconjugates which contained neutral sugars were visualized by diphenylamine spray (12). For the detection of the ceramides released by the enzyme, the organic phase was analyzed by TLC using chloroform:methanol (9:1) as the developing solvent. The ceramides were revealed by staining the plate with Coomassie brilliant blue as described by Nakamura and Handa (13). For quantitative analysis of the oligosaccharide released, the assay was performed in a 1.5 ml polypropylene tube; the reaction mixture was similar to that described above except 30 nmol of [^{3}H]-labeled GM1(1.5 x 10^4 cpm) was used. After incubation, the reaction was terminated by heating the tube in a bath of boiling water for 3 min, followed by the addition of 20 μl of 1 M KCl and 200 μl of a slurry of Nucleosil C18 to adsorb the unreacted GM1 and ceramide (14). The mixture was vortexed, allowed to stand for 15 min at room temperature, and then centrifuged in a microcentrifuge at 12,000 r.p.m. An aliquot of the supernatant containing the liberated radioactive oligosaccharide was mixed with scintillation fluid, and the radioactivity was measured by liquid scintillation counting (14). Exo-glycosidases were assayed by using p-nitrophenyl-glycosides as substrates (6). Protein was determined by the method of Lowry et al. (15), using bovine serum albumin as standard.

Isolation of Ceramide-glycanase (Cer-glycanase) from Leech - Unless otherwise indicated, the isolation of the enzyme was carried out at a temperature between 0 and 5°C. Centrifugation was routinely carried out at 13,000 x g for 20 min using a Sorvall RC5C refrigerated centrifuge. In a typical isolation, seven leeches (Hirudo medicinalis) weighing 23 g were rinsed with distilled water, minced and homogenized with 5 volumes of 50mM sodium phosphate buffer, pH 7.0, with a Polytron homogenizer and centrifuged to obtain 120 ml of clear extract. The extract was brought to 30% saturation with solid $(NH_4)_2SO_4$. After standing for 2 h, the mixture was centrifuged to remove the precipitated protein. The supernatant was brought to 80% saturation with solid $(NH_4)_2SO_4$. The precipitated protein was collected the next day by centrifugation, dissolved in 30 ml of 50mM sodium acetate buffer, pH 4.5, to obtain a crude enzyme preparation which contained 588 mg protein. The crude enzyme preparation was divided into 5-ml portions and applied to a Sepharose-6B column (2.5 x 90 cm) previously equilibrated with 50 mM sodium acetate buffer, pH 4.5. The column was eluted with the same buffer at 25 ml per h and 5-ml fractions were collected. The elution profile is shown in Fig. 1. The

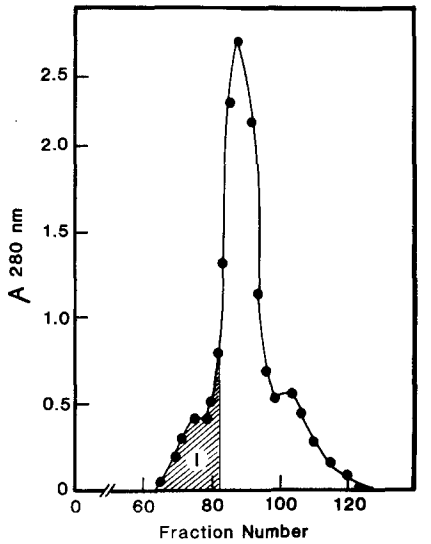

Fig. 1. Sepharose-6B filtration of the crude enzyme precipitated between 30% and 80% $(NH_4)_2SO_4$ saturation according to the conditions described in the text. Cer-glycanase and exo-glycosidases appear in peak I (shaded area).

Cer-glycanase activity together with various exo-glycosidase activities were eluted in the first protein peak (peak I). Peak I was concentrated to a volume of 4 ml which contained 20.3 mg protein, using an Amicon ultrafiltration unit with a PM-10 membrane. This fraction was subsequently applied to an Octyl-Sepharose column (1 x 5 cm) which had been previously equilibrated with 50 mM sodium acetate, pH 4.5. The column was washed with the same buffer to remove the unadsorbed protein. Under this condition, most exo-glycosidases were not retained by the column. The Cer-glycanase retained by the column was eluted with 15 ml 1% octyl-β-glucoside dissolved in the same buffer, concentrated by ultrafiltration using an Amicon PM-10 membrane and dialyzed exhaustively against the same buffer to yield 2 ml of a solution which contained 5.6 mg protein. Twenty microliters of this preparation (56 µg protein) hydrolyzed 30% of GM1 from 30 nmol of [^{3}H]- labeled GM1 in 3 h under our standard assay conditions. This partially purified Cer-glycanase preparation was used for the hydrolysis of various glycosphingolipids and for the optimum pH studies.

RESULTS

General Properties of Cer-glycanase - The partially purified Cer-glycanase was found to contain traces of α- and β-galactosidases and β-hexosaminidase activities. These contaminating exo-glycosidases did not significantly interfere with the analysis of Cer-glycanase activity. The pH optimum of the enzyme was determined to be between pH 4 and 5 for the release of tritiated oligosaccharide from the labeled GM1. The hydrophobic nature of the enzyme is indicated by its adsorption on Octyl-Sepharose. The partially purified enzyme was found to require the presence of sodium taurodeoxycholate

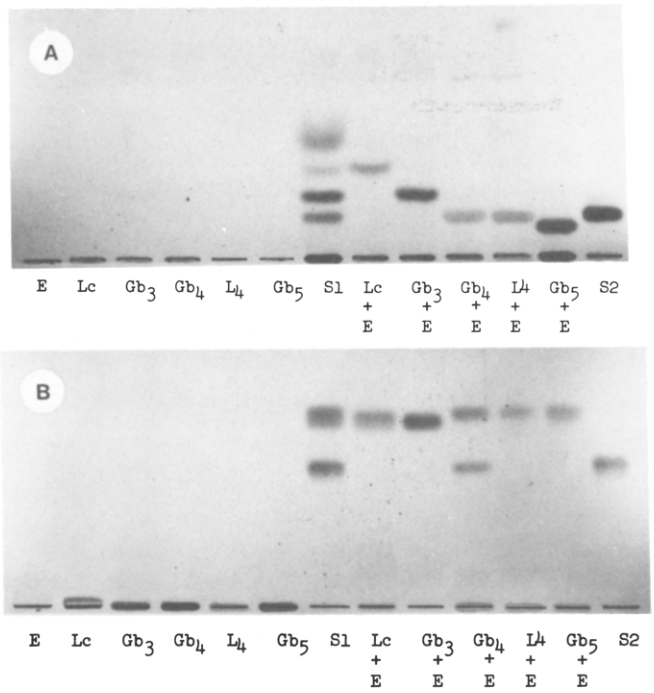

Fig. 2. Thin-layer chromatograms showing the oligosaccharides (A) and the ceramides (B) released by Cer-glycanase from various neutral glycosphingolipids. Twenty microliters of the partially purified Cer-glycanase containing 56 μg protein were separately incubated with various neutral glycosphingolipids for 6 h at 37°C under the conditions described in the text. (A): E, enzyme; Lc, LacCer; Gb_3, $GbOse_3Cer$; Gb_4, $GbOse_4Cer$; L_4, $nLcOse_4Cer$; Gb_5, $GbOse_5Cer$; S1, standard containing GalNAc, lactose, trisaccharide from $GbOse_3Cer$, tetrasaccharide from $GbOse_4Cer$ (top to bottom); S2, lacto-N-neotetraose. (B): abbreviations used are identical to (A) except S1 and S2. S1, standard containing ceramides with nonhydroxy fatty acids (top band) and α-hydroxy fatty acids (bottom band); S2, standard ceramide containing α-hydroxy fatty acids. Detailed conditions for the analysis of the oligosaccharides and the ceramides are described in the text.

to carry out the cleavage of the linkage between the ceramide and the sugar chain in the glycosphingolipids used in this study.

Hydrolysis of Neutral Glycosphingolipids by Cer-glycanase - As shown in Fig. 2A, Cer-glycanase was able to liberate the intact oligosaccharide from LacCer, $GbOse_3Cer$, $GbOse_4Cer$, $GbOse_5Cer$, and $nLcOse_4Cer$. The disaccharide released from LacCer by the the enzyme showed TLC mobility identical to that of lactose and it was converted to Gal and Glc by jack bean β-galactosidase. The enzyme released an oligosaccharide from $nLcOse_4Cer$ with TLC mobility identical to that of lacto-N-neotetraose. The trisaccharide and tetrasaccharide released from $GbOse_3Cer$ and $GbOse_4Cer$, respectively, showed TLC mobilities

identical to the globotriaose and globotetraose prepared from these two glycosphingolipids by ozonolysis. The oligosaccharide released from $GbOse_5Cer$ moved slower on TLC than that from $GbOse_4Cer$; no authentic oligosaccharide from $GbOse_5Cer$ was available in our laboratory. The above oligosaccharides released by the enzyme were subsequently characterized by sequential enzymatic hydrolysis using fig α-galactosidase (7), limpet α-N-acetylgalactosaminidase (8) and the β-galactosidase and β-hexosaminidase both from jack bean (6). Their structures were found to be consistent with that found in the parent glycosphingolipids. The corresponding ceramides liberated from these glycosphingolipids are shown in Fig. 2B. In the case of $GbOse_4Cer$, which contained both types of fatty acids, the enzyme liberated ceramides both with and without α-hydroxy fatty acids. From the other glycosphingolipids only ceramides without α-hydroxy fatty acids were detected.

Hydrolysis of Gangliosides by Cer-glycanase - Fig 3A shows the hydrolysis of gangliosides by Cer-glycanase. The enzyme liberated an intact sialic acid containing oligosaccharide from GM3, GM2, GM1, GD1a and GT1. From GM3, the enzyme released an oligosaccharide with TLC mobility identical to that of sialyllactose. Clostridial neuraminidase converted this sialo-oligosaccharide to a disaccharide which was subsequently hydrolyzed by jack bean β-galactosidase to produce Gal and Glc. From each of the gangliosides, GM2, GM1 and GD1a, the enzyme released an intact sialic acid containing oligosaccharide with TLC mobility identical to the oligosaccharide obtained by ozonolysis from the respective ganglioside. The oligosaccharide released from GT1 was slower in TLC mobility than that from GD1a; no ozonolysis product of GT1 was available in our laboratory. The corresponding liberation of the ceramides from these gangliosides is shown in Fig 3B. The ceramides released from these gangliosides had TLC mobilities corresponding to the standard ceramide with nonhydroxy fatty acids.

DISCUSSION

Our current knowledge of the catabolism of glycosphingolipids in higher animals has grown out of the studies of the partial degradation products accu-

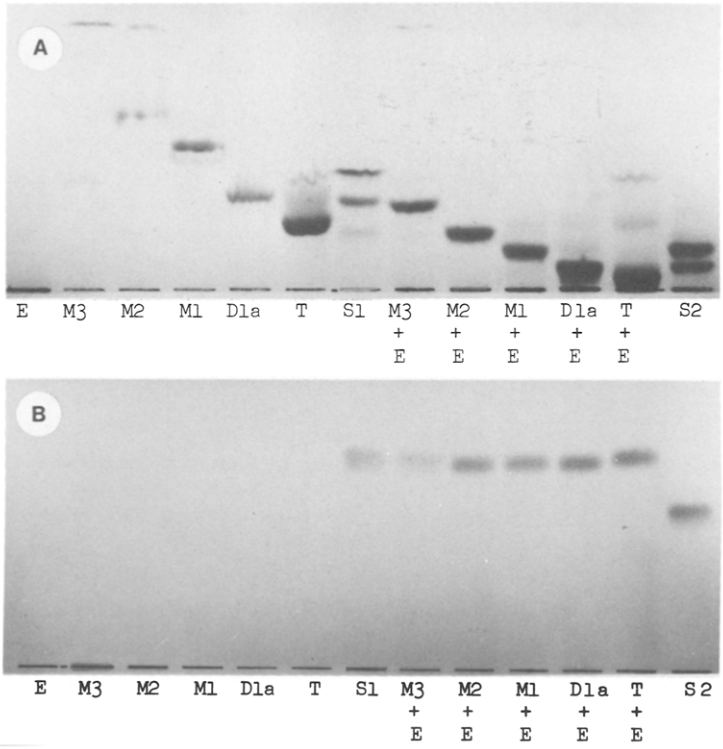

Fig. 3. Thin-layer chromatograms showing the oligosaccharides (A) and the ceramides (B) released by Cer-Glycanase from various gangliosides. The incubation conditions are identical to those described in Fig. 2. (A): E, enzyme; M3, GM3; M2, GM2; M1, GM1; D1a, GD1a; T, GT1; S1, standard containing sialic acid, sialyllactose and the oligosaccharide from GM2 (top to bottom); S2, standard containing oligosaccharides from GM1 (top band) and from GD1a (bottom band). (B): abbreviations used are identical to (A) except S1 and S2. S1, standard ceramide containing non-hydroxy fatty acids; S2, standard ceramide containing α-hydroxy fatty acids. Detailed conditions for the analysis of the oligosaccharides and the ceramides are described in the text. From the substrate controls (lanes without enzyme) it is evident that most of the GM3 and GM2 were partitioned into the organic phase while the more polar gangliosides such as GM1, GD1a and GT1 were predominantly partioned into the aqueous phase.

mulated in various sphingolipidoses due to genetic enzyme deficiencies (16, 17). In higher animals, the catabolism of glycosphingolipids occurs by way of the sequential removal of sugar units from the non-reducing terminal of the sugar chain by specific exo-glycosidases (16, 17). In this catabolic scheme, small water soluble monosaccharides are released from a glycolipid substrate which becomes progressively more hydrophobic. Often, in addition to the enzyme, an activator protein is required to facilitate the reaction (16, 17), probably because of the hydrophobic nature of the substrate.

In contrast to this catabolic pathway, quite a different pathway appears likely in the leech: there the products of the first enzymatic step are the hydrophobic ceramide and the water soluble intact oligosaccharide, which can then be easily hydrolyzed by exo-glycosidases. The fact that the partially purified leech Cer-glycanase requires taurodeoxycholate may well indicate that the leech enzyme has an in vivo requirement for an "activator protein" or a detergent. This question is under pursuit in our laboratory as is the further purification of the enzyme and studies of its specificities. The location of this unique enzyme in leech also remains to be elucidated.

Since this enzyme releases the intact oligosaccharide together with the ceramide moiety in a single step, it should become a very useful tool for structural studies of glycosphingolipids.

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