Vol. 149, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1987 Pages 167-172

OCCURRENCE OF CERAMIDE-GLYCANASE IN THE EARTHWORM, LUMBRICUS TERRESTRIS

Yu-Teh Li, Yukitoshi Ishikawa and Su-Chen Li

Department of Biochemistry, Tulane University School of Medicine, New Orleans, La. 70112

Received October 19, 1987

Summary: We have detected the presence of ceramide-glycanase in the earthworm, Lumbricus terrestris. We have also devised a simple method for the preparation of this enzyme from the earthworm. This enzyme cleaved the linkage between the ceramide and the glycan chain in LacCer, $GbOse_3Cer$, $GbOse_4Cer$, $GbOse_5Cer$, GM3, GM2, GM1 and GD1a. By using tritium-labeled GM2 as substrate, the optimum pH of this enzyme was found to be between pH 4 and 4.5. In the earthworm, the ceramide-glycanase was mainly found in the muscle. The intestine was found to contain a very low level of this enzyme. Because of their easy availability, earthworms should become a convenient source for the preparation of ceramide-glycanase.

We have recently reported the presence of ceramide-glycanase (Cer-glycanase) in an animal origin, the leech (1). This novel enzyme hydrolyzes the linkage between the ceramide and the glycan chain in various glycosphingolipids. A similar enzyme called endoglycoceramidase has been induced in a microorganism, Rhodococcus sp, by using bovine brain gangliosides as the inducer (2). As it is unlikely that the leech would be the only animal which contains this new enzyme, we undertook the search for the presence of this enzyme in other animal tissues. In this communication we report the occurence of Cer-glycanase in the earthworm, Lumbricus terrestris.

EXPERIMENTAL PROCEDURE

Materials - LacCer, $GbOse_3Cer$ and $GbOse_4Cer$ were isolated from human erythrocytes (3). GM2 was isolated from Tay-Sachs brain (4) and $GbOse_5Cer$ from dog intestine (5). GM2, tritium labeled at the terminal GalNAc was prepared according to the method described by Radin (6). Oligosaccharides from various glycosphingolipids were prepared by the hydrolysis of the parent glycosphingolipids with leech Cer-glycanase (1). The following were purchased from commercial sources: the earthworms (Lumbricus terrestris), Carolina Biological Supply Company; GM3, GM1 and GD1a, Supelco; P-nitrophenyl-glycosides, ceramide Type III (primarily nonhydroxy fatty acids), ceramide Type IV (mainly α-hydroxy fatty acids), lactose and sialyllactose, Sigma; sodium taurodeoxycholate, Calbiochem; Sepharose 6B and Octyl-Sepharose CL-4B, Pharmacia; silica gel 60 pre-coated plates, Merck, Darmstadt, Germany.

Enzyme Assays - The assay for Cer-glycanase was carried out essentially according to the method described previously (1). The incubation mixture contained the following components in 0.2 ml: glycolipid substrate, 20 nmol; sodium acetate buffer (50 mM), pH 4.5; sodium taurodeoxycholate, 400 μg, and an appropriate amount of the enzyme. After incubating at 37°C for a preset

0006-291X/87 $1.50

time, 5 volumes of chloroform:methanol (2:1) were added to the reaction mixture to terminate the reaction. The mixture was vortexed and centrifuged to separate the organic phase (lower) from the aqueous phase (upper). These two phases were separately evaporated to dryness. For the 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 (7). Sialic acid-containing glycoconjugates were revealed by resorcinol spray (8), while the glycoconjugates which contained neutral sugars were visualized by diphenylamine spray (9). For the detection of the ceramides released by Cer-glycanase, 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 (10). For quantitative analysis of the oligosaccharide released, the reaction mixture was similar to that described above, except 10 nmol of [^{3}H]-labeled GM2 (1.5×10^4 cpm) was used as the substrate. After incubation, the reaction was terminated by heating the tube in a bath of boiling water for 3 min. The liberated radioactive oligosaccharide was determined by the dialysis method (11). Briefly, the reaction mixture was dialyzed against 2 ml of distilled water overnight at room temperature in a shaker. Then, an aliquot of the dialysate containing the liberated radioactive oligosaccharide was mixed with scintillation fluid, and the radioactivity was measured by liquid scintillation counting (11). One unit of Cer-glycanase is defined as the amount which hydrolyzes 1 nmol of GM2 per h under the above assay conditions. Exo-glycosidases were assayed by using p-nitrophenyl-glycosides as substrates (12). Protein was determined by the method of Lowry et al (13), using bovine serum albumin as standard.

Preparation of Cer-glycanase from Earthworms - Earthworms were dissected by making a longitudinal dorsal incision, separating the muscle tissue to reveal the intact digestive tract. The digestive tract and other organs were carefully removed from the muscle. The muscle was rinsed with distilled water to wash off the digestive fluid. Unless otherwise indicated, the preparation of the enzyme was carried out at a temperature between 0° and 5°C. Centrifugation was routinely carried out at 13,000xg for 20 min using a Sorvall RC5C refrigerated centrifuge. In a typical isolation, the muscle (14g) from 6 earthworms was homogenized with 5 volumes of distilled water with a Polytron homogenizer to obtain 70 ml of clear extract. The extract was brought to 80% saturation with solid ammonium sulfate. The precipitated protein was collected the next day by centrifugation, dissolved in 12 ml of 50 mM sodium acetate buffer, pH 6.0, to obtain a crude enzyme preparation which contained 179 mg protein. This crude enzyme preparation was then applied to a Sepharose 6B column (2.5 x 80cm) which had been equilibrated with 50 mM sodium acetate buffer, pH 6.0. The column was eluted with the same buffer at 40 ml per h, and 5-ml fractions were collected. Fig. 1 shows the elution profile monitored for the absorbance at 280 nm (protein), and for the activities of Cer-glycanase, α-N-acetylgalactosaminidase and β-hexosaminidase. The effluent from the column was divided into five fractions as shown in Fig. 1. Proteins in these five fractions were separately precipitated by the addition of solid ammonium sulfate to 80% saturation, dissolved in a minimal amount of 50 mM sodium phosphate buffer, pH 6.0 and dialyzed against the same buffer to remove ammonium sulfate. Table I summarizes the amount of protein and Cer-glycanase in these fractions. As shown in this table, among the five fractions, the specific activity of Cer-glycanase was found to be highest in Fraction III. However, this fraction also contained a considerable amount of α-N-acetylgalactosaminidase, β-galactosidase and β-hexosaminidase activities. In order to remove exoglycosidases, Fraction III was passed through an Octyl-Sepharose column (0.7 x 7cm) previously equilibrated with 50 mM sodium phosphate buffer, pH 6.0. The column was then washed with the same buffer. Under this condition, all exoglycosidases were not retained by the column. The Cer-glycanase retained by the column was eluted with 10 ml of 50 mM sodium phosphate buffer, pH 6.0 containing 0.1% Triton X-100 and 40% ethylene glycol. The eluate was subsequently concentrated by ultrafiltration using an Amicon

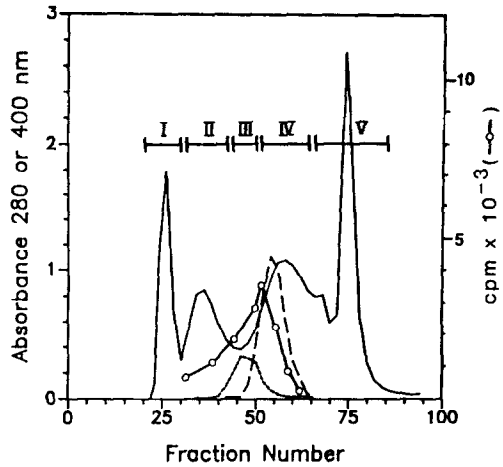

Fig. 1. Sepharose-6B filtration of the earthworm crude enzyme preparation according to the conditions described in the text. ß-Galactosidase (---) was assayed by incubating 10 µl-aliquot of each fraction with P-nitrophenyl-ß-Gal for 60 min, while for ß-hexosaminidase (– – –), 10 µl-aliquot was incubated with P-nitrophenyl-ß-GlcNAc for 30 min. Cer-glycanase (-o-) was assayed by incubating 50 µl-aliquot of each fraction with tritium-labeled GM2 for 16 h. Detailed conditions for assaying exoglycosidases and Cer-glycanase are described in the text.

PM-10 membrane and dialyzed exhaustively against 50 mM sodium phosphate buffer, pH 6.0 to yield 2.2 ml, which contained 52.3 units of Cer-glycanase and 2.1 mg of protein.

RESULTS

General Properties of Earthworm Cer-glycanase - The earthworm Cer-glycanase prepared above was found to be completely free from exoglycosidases. By using tritium labeled GM2 as the substrate, the optimal pH of earthworm Cer-glycanase was found to be around pH 4.5. As in the case of the leech

TABLE I. CERAMIDE-GLYCANASE ACTIVITY IN THE FIVE FRACTIONS OBTAINED FROM SEPHAROSE 6B CHROMATOGRAPHY AS SHOWN IN FIG. 1

Fraction	Volume	Protein	Cer-glycanase	Specific activity
	ml	mg	Units[a]	Units/mg Protein
I	3.7	9.4	7.5	0.80
II	5.6	24.4	36.8	1.51
III	5.6	17.0	72.8	4.28
IV	7.0	63.0	91.3	1.45
V	6.5	25.4	2.9	0.11

[a]1 unit = The amount of enzyme that hydrolyzes 1 nmol of GM2 per h.

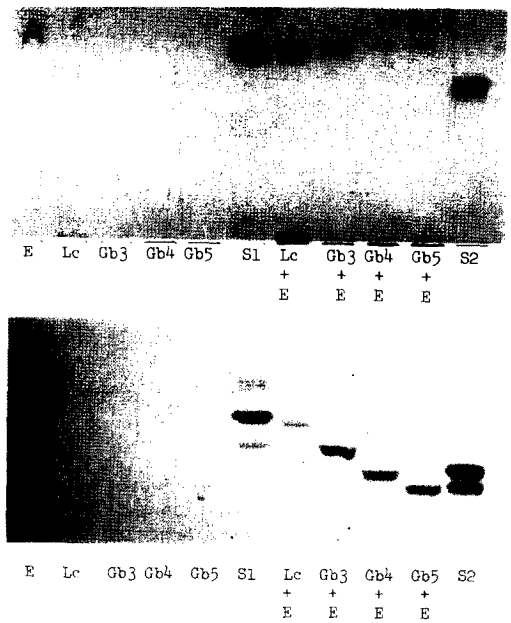

Fig. 2. Thin-layer chromatograms showing the release of the ceramides (A) and the oligosaccharides (B) from various neutral glycosphingolipids. One unit of the partially purified Cer-glycanase containing 40 μg protein were separately incubated with various neutral glycosphingolipids at 37°C for 17 h under the conditions described in the text. (A): E, enzyme; S1, standard ceramide containing nonhydroxy fatty acids; S2, standard ceramide containing α-hydroxy fatty acids; Lc, LacCer; Gb3, $GbOse_3Cer$; Gb4, $GbOse_4Cer$; Gb_5, $GbOse_5Cer$. (B): abbreviations used are identical to (A) except S. S1, standard containing GalNAc, lactose, trisaccharide from $GbOse_3Cer$ (top to bottom); S2, oligosaccharides from $GbOse_4Cer$ (top) and $GbOse_5Cer$.

Cer-glycanase (1), the earthworm Cer-glycanase was also retained by Octyl-Sepharose and the enzyme requires the presence of sodium taurodeoxycholate to carry out the hydrolysis. LacCer was the shortest glycosphingolipid hydrolyzed by the enzyme. DiGalCer (Galα1→4GalCer), however, was not hydrolyzed. The two most common monohexosylceramides, GalCer and GlcCer, were also found to be refractory to the earthworm Cer-glycanase.

Hydrolysis of Neutral Glycosphingolipids by Earthworm Cer-glycanase - As shown in Fig. 2A, earthworm Cer-glycanase was able to release ceramides from $GbOse_5Cer$, $GbOse_4Cer$, $GbOse_3Cer$ and LacCer. Fig. 2B shows the release of the intact oligosaccharide from these glycolipids. The enzyme liberated a pentasaccharide, a tetrasaccharide, a trisaccharide and a disaccharide, respectively, from $GbOse_5Cer$, $GbOse_4Cer$, $GbOse_3Cer$ and LacCer. The TLC-mobility of these oligosaccharides were found to be identical to that derived from these glycolipids by the leech Cer-glycanase. In all cases no release of monosaccharide was detected in the reaction mixture.

Hydrolysis of Gangliosides by Earthworm Cer-glycanase - Earthworm Cer-glycanase was also able to release ceramides from GM3, GM2, GM1 and GD1a as

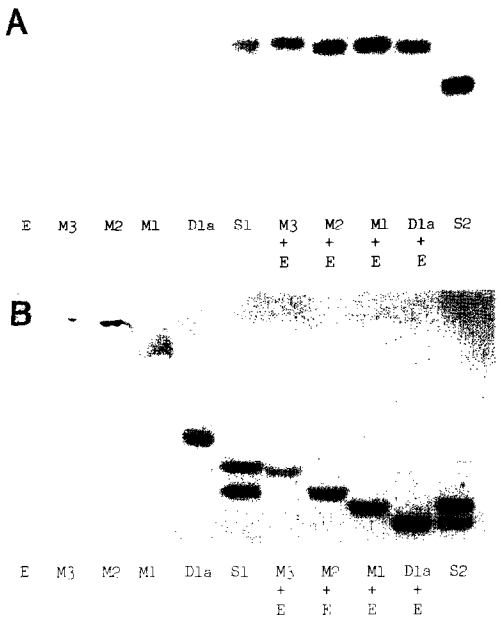

Fig. 3. Thin-layer chromatograms showing the release of the ceramides (A) and the oligosaccharides (B) from various gangliosides. The incubation conditions are identical to those described in Fig. 2. (A): E, enzyme; S1, standard ceramide containing nonhydroxy fatty acids; S2, standard ceramide containing α-hydroxy fatty acids; M3, GM3; M2, GM2; M1, GM1; D1a, GD1a. (B): abbreviations used are identical to (A) except S. S1, standard containing sialyllactose (top), oligosaccharides from GM2; S2, oligosaccharides from GM1 (top) and GD1a.

shown in Fig. 3A. Fig. 3B shows the liberation of the intact sialic acid-containing oligosaccharide from each of the above mentioned gangliosides by the earthworm Cer-glycanase. From GM2, GM1 and GD1a, the enzyme released an intact sialic acid-containing oligosaccharide with TLC-mobility identical to the standard oligosaccharide prepared from the respective ganglioside.

DISCUSSION

The above results clearly show that the earthworm, Lumbricus terrestris, contains Cer-glycanase. The earthworms collected locally were also found to contain this enzyme. It is well known that earthworms are also rich in other glycosidases (14). Among them α-N-acetylgalactosaminidase (15,16) and β-hexosaminidase (16) have been used for the structural analysis of sugar chains in glycoproteins. We found that the earthworm β-galactosidase, α-galactosidase and α-N-acetylgalactosaminidase hydrolyzed GM1, $GbOse_3Cer$ and $GbOse_5Cer$, respectively. However, for some unknown reason the earthworm β-hexosaminidase was not able to cleave GM2. We, therefore, chose to use tritium labeled GM2 as the substrate for the quantitative assay of earthworm Cer-glycanase. From the results presented in this communication, one can not overemphasize the importance of being alerted against the possible presence of

an unanticipated enzyme contamination, such as Cer-glycanase, when glycosidases are used for the structural determination of glycosphingolipids.

After the revelation of the presence of Cer-glycanase in leeches, we have examined the possible presence of this enzyme in other organisms. Since leeches are blood sucking, we initially thought that Cer-glycanase might occur in other blood sucking organisms. However, this enzyme was not found in such blood sucking organisms as mosquitoes (Aedes aegypti (Linnaeus) and Culex quinquefasciatus Say, either male or female), ticks (Ornithodoros tartakovskyi) or triatomine bug (Rhodnius prolixus). Since both leeches and earthworms are annelids and are phylogenetically closely related, it seems reasonable that earthworms also contain Cer-glycanase. It is interesting to note that the digestive tract of the earthworm contains very little Cer-glycanase activity. As earthworms are neither blood sucking nor carnivorous, the presence of Cer-glycanase in the tissues of earthworm must have other biological functions which remain to be revealed.

Since Cer-glycanase can release the intact oligosaccharide and ceramide from glycosphingolipids, it should become a very useful tool for the structural analysis of glycosphingolipids. Because of their easy availability, earthworms should become a convenient source for the preparation of Cer-glycanase.

Acknowledgements - We thank Dr. Robert C. Lowrie for providing mosquitos and ticks and Dr. R.G. Yaeger, for providing triatomine bug. Thanks are also due to Drs. J.E. Muldrey and Paul Siegel for their valuable discussion and careful reading of the manuscript. This investigation was supported by Grants DMB-8617033 from the National Science Foundation and NS 09626 from the National Institutes of Health.

REFERENCES

1. Li, S.-C., DeGasperi, R., Muldrey, J.E., and Li, Y.-T. (1986) Biochem. Biophys. Res. Comm. 141, 346-352.
2. Ito, M., and Yamagata, T. (1986) J. Biol. Chem. 261, 14278-14282.
3. Esselman, W., Laine, R.A., and Sweeley, C.C. (1972) Methods Enzymol. 18, 140-156.
4. Svennerholm, L. (1972) Methods Carbohydr. Chem. 6, 464-474.
5. McKibbin, J.M. (1969) Biochemistry 8, 679-684.
6. Radin, N.S. (1972) Methods Enzymol. 28, 300-306.
7. Kitamikado, M., Ito, M., and Li, Y.-T. (1981) J. Biol. Chem. 256, 3906-3909.
8. Svennerholm, L. (1957) Biochim. Biophys. Acta 24, 604-611.
9. Harris, G., and MacWilliams, I.C. (1954) Chem. Ind. (Lond.) P. 249.
10. Nakamura, K., and Handa, S. (1984) Anal. Biochem. 142, 406-410.
11. Li, S.-C., and Li, Y.-T. (1976) J. Biol. Chem. 251, 1159-1163.
12. Li, Y.-T., and Li, S.-C. (1972) Methods Enzymol. 28, 702-713.
13. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-274.
14. Li, Y.-T., and Shetlar (1965) Comp. Biochem. Physiol. 14, 275-279.
15. Schauer, H., and Gattschalk, A. (1968) Biochim. Biophys. Acta 156, 304-310.
16. Donald, A.S.R. (1980) Biochem. J. 185, 327-337.