A Novel Enzyme That Cleaves the N-Acyl Linkage of Ceramides in Various Glycosphingolipids as Well as Sphingomyelin to Produce Their Lyso Forms*

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We describe a novel enzyme that hydrolyzes the N-acyl linkage between fatty acids and sphingosine bases in ceramides of various sphingolipids. The enzyme was purified about 300-fold with 5% recovery from the culture filtrate of a newly isolated bacterium (Pseudomonas sp. TK4) by ammonium sulfate precipitation followed by several steps of high performance liquid chromatography. The purified enzyme preparation was completely free of exoglycosidases, sphingomyelinase, and proteases, and showed a single protein band corresponding to a molecular mass of 52 kDa on SDS-polyacrylamide slab gel electrophoresis after staining with Coomassie Brilliant Blue. The enzyme shows quite wide specificity, i.e., it hydrolyzes both neutral and acidic glycosphingolipids, and simple glycosphingolipid cerebrosides to polysialogangliosides such as GQ1b. Furthermore, the enzyme also hydrolyzes sphingomyelin to produce the respective lyso form. However, the enzyme shows hardly any activity on ceramides, indicating that it is completely different from the ceramidase (EC 3.5.1.23) reported previously. This enzyme, which is tentatively named sphingolipid ceramide N-deacylase, should greatly facilitate the further study of sphingolipids as well as lysosphingolipids.

In vertebrates, glycosphingolipids (GSLs) are located on the outer leaflet of the plasma membranes and may function as mediators of cell-cell interaction, attachment, proliferation, and differentiation (1). Lyso-GSLs, which are GSLs N-deacylated in the ceramide moiety, have been detected in normal tissues at very low levels but are accumulated in inherited sphingolipidoses (2). Furthermore, sphingosine, a deglycosylated form of lyso-GSLs, has been found to modulate protein kinase C-dependent cell functions (5) as well as a number of other systems (6).

Although one possible mechanism by which intracellular lyso-GSLs may be removed by direct N-deacylation has been proposed (7), the molecular mechanism of lyso-GSL generation in situ remains unclear. Recently, Hirabayashi et al. reported the presence of lyso-GSL-generating hydrolase activity in actinomycetes (8). The enzyme was, however, difficult to solubilize from the cells, and thus the enzyme protein has not yet been characterized. In this paper we report that the novel enzyme, purified as an apparently homogeneous protein from a newly isolated bacterium, cleaves the N-acyl linkage of ceramides in various GSLs as well as sphingomyelin to produce their lyso forms. This is the first report describing the generation of lyso-sphingomyelin from sphingomyelin by a specific hydrolase. Lysosphingomyelin has been shown to exert potent mitogenic activity (9) and to modulate cytosolic protein phosphorylation (10). The enzyme, tentatively designated sphingolipid ceramide N-deacylase, should facilitate the further study of sphingolipid as well as lysosphingolipid functions.

EXPERIMENTAL PROCEDURES

Materials—A mixture of gangliosides was prepared from bovine brain using a method described previously (11). GM1 and asialo GM1 were prepared from a mixture of gangliosides by digestion with neuraminidases isolated from Clostridium perfringens (Sigma) and Arthrobacter ureafaciens (Nakarai Chemical Co., Japan), respectively, followed by purification with DEAE-Sephrose and latex bead column chromatography. Other GSLs were purchased from Iatron Laboratories, Inc. (Japan). Sphingomyelin and ceramide from bovine brain were purchased from Matreya, and Triton X-100 was from Sigma. Precocated Silica Gel 60 TLC plates were obtained from Merck (Germany). Endoglycoceramidase was prepared as described previously (12, 13) or purchased from Takara Shuzo Co. (Japan).

Enzyme Assay—The activity of sphingolipid ceramide N-deacylase was measured using asialo GM1 as the substrate as described below. The reaction mixture contained 10 nmol of asialo GM1 and an appropriate amount of the enzyme in 20 mM sodium acetate buffer, pH 5.0, containing 0.8% Triton X-100. Following incubation at 37 °C for 30 min, the reaction was stopped by heating in a boiling water bath for 3 min. The reaction products were freeze-dried by a Speed Vac concentrator (Savant Instruments, Inc.), redissolved in 5 μl of chloroform/methanol (1:2, v/v), and analyzed by TLC using chloroform, methanol, 10% acetic acid (5:4:1, v/v/v) as the developing solvent. GSLs and lyso-GSLs were visualized by spraying the TLC plates with orcinol-H2SO4 reagent and scanning them with a Shimadzu CS-9300 chromatoscaner with the reflectance mode set at 540 nm. The extent of hydrolysis was calculated as follows: hydrolysis (%) = (peak area for lysoasialo GM1 generated) × 100/(peak area for remaining asialo GM1 + peak area for lysoasialo GM1 generated). One enzyme unit was defined as the amount capable of catalyzing the release of 1 μmol of lysoasialo GM1/min from the asialo GM1 under the conditions indicated.
Sphingolipid Ceramide N-Deacylase

above. Exoglycosidases and proteases were assayed using p-nitrophenylglycosides (14) and azocoll (15), respectively, as substrates.

TLC Analysis—Lysosphingolipids and sphingolipids were analyzed by TLC using chloroform, methanol, 10% acetic acid (5/4/1, v/v/v) as the developing solvent. Lysy-GSLs were visualized with either orcinol-H$_2$SO$_4$ reagent or ninhydrin. Lysosphingomyelin was visualized with either Coomassie Brilliant Blue (16) or ninhydrin. Oligosaccharides released from the product by endoglycocerebrosidase were analyzed by TLC using n-butyl alcohol/acetic acid/H$_2$O (2/1/1, v/v/v) (17) and visualized with orcinol-H$_2$SO$_4$ reagent.

FAB-MS Analysis—Lysy-GSLs were analyzed by negative FAB-MS using a JEOL JMS-HX-100 mass spectrometer (JEOL, Ltd., Japan) with triethanolamine as the matrix. For lysosphingomyelin, analysis was conducted in the positive mode using diethanolamine as the matrix (18).

SDS-Polyacrylamide Slab Gel Electrophoresis and Protein Assay—SDS-Polyacrylamide gel electrophoresis was conducted on a slab gel with 10% acrylamide according to Laemmli (19). The sample was heated at 100°C for 3 min before electrophoresis except for detection of the activity. For this purpose, the sample was left at room temperature for 10 min. The duplicate gel was cut into 4-mm slices without staining. Each gel slice was crashed with a glass bar in an Eppendorf tube containing 1 ml of 20 mM sodium acetate buffer, pH 5.0, containing 0.3% Triton X-100 and shaken at 4°C for 2 h. After centrifugation at 10,000 rpm for 10 min, the supernatant was dialyzed against 2 mM sodium acetate buffer, pH 6.0, in order to remove SDS, and this was found to be effective for restoration of enzyme activity. The enzyme activity was determined by the method described under “Experimental Procedures” using asialo GM1 as the substrate. The incubation time for this experiment was 16 h. The protein was stained with Coomassie Brilliant Blue, and the protein content at each step of purification was determined by the bicinchoninic acid protein assay (Pierce) with bovine serum albumin as the standard. Sugar Composition Analysis—Lysosialo GM1 (100 nmol) was hydrolyzed with 2.5 nM trifluoroacetic acid at 100°C for 6 h and analyzed using a Dionex HPLC system with a CarboPac PA column (Dionex) and pulsed amperometric detection (20).

Isolation and Cultivation of Pseudomonas sp. TK-4—A strain (TK-4) capable of producing sphingolipid ceramide N-deacylase was isolated from pond water using a synthetic medium containing gangliosides as the sole source of carbon. The bacterium was assigned to the genus Pseudomonas on the basis of morphological and biochemical characteristics, which will be reported in detail elsewhere. In order for this strain to retain its ability to produce the enzyme, it must be maintained in a medium containing gangliosides (0.5% polypeptone, 0.1% yeast extract, 0.2% NaCl, 0.1% bovine brain gangliosides, and 1.6% agar, pH 7.0), as is presently being done at our laboratory. For preparation of sphingolipid ceramide N-deacylase, inocula from an agar slant of the strain TK-4 were introduced into a cotton-plugged 50-ml flask containing 20 ml of 20 mM sodium acetate buffer, pH 6.0, and the same buffer containing 2% Lubrol PX. The culture was then transferred to a molecular mass of 52 kDa on SDS-polyacrylamide slab gel electrophoresis after staining with Coomassie Brilliant Blue (Fig. 1). The duplicate gel was cut into 4-mm slices, the protein was eluted and dialyzed, and the enzyme activity was measured as described under “Experimental Procedures.” The activity was detected only at the position corresponding to the 52-kDa band.

RESULTS

Purification of Sphingolipid Ceramide N-Deacylase—In a typical experiment, the enzyme was purified about 300-fold from a culture filtrate of the newly isolated Pseudomonas sp. TK4 strain with 5% recovery. The purified enzyme preparation was completely free from the following enzyme activities: α- and β-galactosidases, β-N-acetylgalactosaminidase, β-N-acetylgalactosaminidase, β-N-acetylgalactosaminidase, β-fucosidase, α- and β-mannosidases, α- and β-glucosidases, sialidase, endoglycocerebrosidase, sphingomyelinase, and proteases. The enzyme preparation showed a single protein band corresponding to a molecular mass of 52 kDa on SDS-polyacrylamide slab gel electrophoresis after staining with Coomassie Brilliant Blue (Fig. 1). The duplicate gel was cut into 4-mm slices, the protein was eluted and dialyzed, and the enzyme activity was measured as described under “Experimental Procedures.” The activity was detected only at the position corresponding to the 52-kDa band.

General Properties—The general properties of the enzyme are as follows: optimal activity at pH 5.0–6.0 and stable between pH 4.0 and 9.0; potently inhibited by Hg$^2+$, Cu$^2+$, and Zn$^2+$ (2 mM) but not by Ca$^{2+}$, Mn$^{2+}$, Mg$^{2+}$, and EDTA, all at the same concentration. The enzyme retained 80% of its activity when kept at 60°C for 30 min and can be kept at −85°C for 2 months without any loss of activity. Addition of Triton X-100 at a concentration of 0.4–0.8% (w/v) increased the enzyme activity about 10-fold in comparison with that in the absence of the detergent.

Characterization of Enzymatic Digestion Products—To elucidate the action mode of the enzyme, asialo GM1, GalCer, and sphingomyelin were digested with the enzyme, and the digestion products were separately purified by HPLC followed by TLC analysis. The digestion products migrated on the TLC plate more slowly than native sphingolipids and were stained with either orcinol-H$_2$SO$_4$ (those from asialo GM1 and GalCer;
parental asialo GM1 released by endoglycoceramidase (Fig. 2). lyso-GSLs (12), had the same mobility on TLC as that from the ceramidase, which releases sugar chains from both GSLs and that the sugar chain released from the product by endoglyco-

Gal:Glc sugar chain was intact even after enzyme treatment (GalN: factor). In sphingolipids by the enzyme treatment. Sugar composition

lane 3, product released from sphingomyelin by the enzyme; lanes 1 and 2, Coomassie Brilliant Blue staining; lanes 3 and 4, ninyhdrin staining. C, oligosaccharide released from the product by endoglycoceramidase; lanes 1 and 5, product from asialo GM1; lanes 4 and 6, oligosaccharide released from the product by endoglycoceramidase; lanes 3 and 7, asialo GM1; lanes 4 and 8, oligosaccharide released from asialo GM1 by endoglycoceramidase; lane 9, sphingosine standard. Lanes 1–4, orcinol-H$_2$SO$_4$ staining; lane 5, ninyhdrin staining. Chloroform, methanol, 10% acetic acid (5:4:1, v/v/v) was used as the developing solvent for TLC for all substances after enzyme treatment (Fig. 2). All sphingolipids tested were changed to ninhydrin-positive substances after enzyme treatment (Fig. 2). This demonstrated the generation of free aminogroups in sphingolipids by the enzyme treatment. Sugar composition analysis of the product from asialo GM1 revealed that the sugar chain was intact even after enzyme treatment (GalN: Gal:Glc = 1.01:2.0:0.87). This result was confirmed by the fact that the sugar chain released from the product by endoglycoceramidase, which releases sugar chains from both GSLs and lyso-GSLs (12), had the same mobility on TLC as that from the parental asialo GM1 released by endoglycoceramidase (Fig. 2C, lanes 2 and 4). The product from asialo GM1 released by the enzyme was stained with ninyhdrin (Fig. 2C, lane 5), but the oligosaccharides released from the product by endoglycoceramidase were not stained with ninyhdrin (Fig. 2A, lanes 1 and 2). Thus, the products from asialo GM1 and GalCer released by the enzyme were differentiated by ninyhdrin staining (Fig. 2C, lanes 2 and 4). The product from asialo GM1 released by the enzyme was stained with Coomassie Brilliant Blue (that from sphingomyelin; Fig. 2B, lane 2). The product from GalCer released by the enzyme was identical to the galactosylsphingosine (psychosine) standard on TLC (Fig. 2A, lanes 4 and 5).

All sphingolipids tested were changed to ninhydrin-positive substances after enzyme treatment (Fig. 2A, lanes 7 and 9, and Fig. 2B, lane 4), whereas the parental sphingolipids were not stained with ninyhdrin (Fig. 2A, lanes 6 and 8, and Fig. 2B, lane 3). The product from GalCer released by the enzyme was stained with Coomassie Brilliant Blue (that from sphingomyelin; Fig. 2B, lane 2). The product from asialo GM1 released by the enzyme was stained with ninyhdrin (Fig. 2C, lane 5), but the oligosaccharides released from the product by endoglycoceramidase were not stained with ninyhdrin (Fig. 2C, lane 6), suggesting that the acetyl group at the C-2 position in GalNAc could not be removed by the enzyme, i.e. the enzyme is specific to the N-amide linkage in ceramide but not to that in the carbohydrate moiety. Sphingo-
sine, which was stained by ninyhdrin, was generated from the product by endoglycoceramidase treatment (Fig. 2C, lane 6). Finally, the products released from asialo GM1, GalCer, and sphingomyelin were identified using a FAB-MS. As shown in Fig. 3, A and B, the characteristic pseudomolecular ions (M$_{-}$H)$^+$ were found at m/z 989 for the product released from asialo GM1 (M, 1256; C18:0, d 18:1), and m/z 461 for the product released from GalCer (M, 728; C18:0, d 18:1) using the negative ion mode. On the spectra of the product released from asialo GM1 by the enzyme, the fragment ions m/z 827 (corresponding to lysoasialo GM2) and m/z 624 (corresponding to lysoasialo GM3) were also observed (Fig. 3A). For the product released from sphingomyelin (M, 732; C18:0, d 18:1), (M + H)$^+$ was found at m/z 467 using...
Sphingolipids having a d18:1 sphingosine base as the major component of all sphingolipid substrates tested even after prolonged incubation. The reason for this is unknown at present but may be due to feedback inhibition of the enzyme by the fatty acids generated, since addition of stearic acid to the reaction mixture inhibited the enzyme activity (data not shown).

**Discussion**

Lysosphingolipids are present at low levels in normal tissues but are abnormally accumulated in cells in various lysosomal storage diseases (2). For example, in Gaucher’s disease, which is caused by a deficiency of glucosylceramidase, abnormal accumulation of glucosylceramide as well as its lyso form, glucosylsphingosine, is observed (24). Intracellular generation of psychosine is seen in cases of Krabbe’s disease (25), which is a progressive and fatal neurogenic disorder. Both psychosine and glucosylsphingosine are considered to be synthesized by the glycosylation of sphingosines in situ, although Yamaguchi et al. (26) reported very recently that glucosylsphingosine is formed not only through the glycosylation of sphingosine but also through the deacylation of glucosylceramide in cultured fibroblasts. They suggested the participation of acidic ceramidase in glucosylsphingosine formation. The enzyme presented here, however, seems to be completely different from the ceramidase reported so far, since the enzyme hydrolyzes various sphingolipids efficiently but hardly acts on ceramide (Fig. 4), which is hydrolyzed by endoglycoceramidase (12). The mode of action of sphingolipid ceramide N-deacylase is presented in Fig. 5. This enzyme cleaves the linkage between ceramides and oligosaccharides in GSLs (12).

**Table I**

| Name                  | Substrate structure                  | Hydrolysis (%) |
|-----------------------|--------------------------------------|----------------|
| Ceramide              | Glcβ1-1’Cer                         | 48             |
| Glucosylceramide      | Galβ1-1’Cer                          | 42             |
| Sulfatide             | Galβ1-4Glcβ1-1’Cer                   | 64             |
| Lactosylceramide      | Galβ1-3Galaβ1-1’Cer                  | 64             |
| Asialo GM1            | GalNAcβ1-4Galβ1-1’Cer                | 53             |
| Globotetraosylceramide| GalNAcβ1-3Galαβ1-4Galβ1-1’Cer        | 45             |
| GM2                   | GalNAcβ1-4(NeuAca2-3)Galβ1-1’Cer     | 69             |
| GM1                   | Galβ1-3GalNAcβ1-4(NeuAca2-3)Galβ1-1’Cer | 61             |
| GD1a                  | NeuAca2-3Galβ1-3GalNAcβ1-4(NeuAca2-3)Galβ1-1’Cer | 49             |
| GQ1b                  | NeuAca2-8NeuAca2-3Galβ1-3GalNAcβ1-4(NeuAca2-8NeuAca2-3)Galβ1-1’Cer | 49             |
| Sphingomyelin         | Choline phosphate-Cer                | 28             |

* Cer, ceramide.

**Fig. 5. Mode of action of sphingolipid ceramide N-deacylase on asialo GM1.** Sphingolipid ceramide N-deacylase hydrolyzes the N-acyl linkage between fatty acids and sphingosine bases in ceramides of sphingolipids, while endoglycoceramidase hydrolyzes the glycosidic linkage between ceramides and oligosaccharides in GSLs (12).

**Endoglycoceramidase (EGCase)**

**Sphingolipid ceramide N-deacylase (SCDase)**
inhibit protein kinase C and have suggested that the accumulation of lyso-GSLs would eventually lead to cell death due to dysfunction of the signal transduction system (4). Besides inherited lysosomal disease, lyso-GM3 has also been found in a human epidermoid carcinoma cell line, A431, and was shown to inhibit EGF-dependent EGF receptor phosphorylation (3). However, the mechanism of lysoganglioside formation in situ has not yet been elucidated. Whether sphingolipid ceramide \(N\)-deacylase (or a similar enzyme) responsible for lysoganglioside generation is present in mammalian tissues should be clarified carefully.

The discovery of sphingolipid ceramide \(N\)-deacylase should provide advantages for the study of sphingolipids as well as lysosphingolipids. The preparation of lysosphingolipids will become much easier by using this enzyme. To date, the preparation of lyso GSLs has been done using purely chemical procedures (28), which are somewhat troublesome, time-consuming, and give a low yield, especially in the case of polysialogangliosides. Using the sphingolipid ceramide \(N\)-deacylase we were able to obtain easily the lyso forms of all species of GSLs tested without any alternation of their carbohydrate and sphingoid moieties, allowing preparation of new GSL derivatives containing appropriately labeled fatty acids. Furthermore, utilizing the amino groups newly generated in lyso-GSLs, they can be coupled with either appropriate proteins or gel matrix for the affinity column. In addition to the GSLs, the fact that the enzyme can be applied to sphingomyelin should be noted.

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