A Novel Endoglycoceramidase Hydrolyzes Oligogalactosylceramides to Produce Galactooligosaccharides and Ceramides*

Enzymes capable of hydrolyzing the β-glycosidic linkage between oligosaccharides and ceramides in various glycosphingolipids has been found in microorganisms and invertebrates and designated endoglycoceramidase (EC 3.2.1.123) or ceramide glycanase. Here we report the molecular cloning, characterization, and homology modeling of a novel endoglycoceramidase that hydrolyzes oligogalactosylceramides to produce galactooligosaccharides and ceramides. The novel enzyme was purified from a culture supernatant of Rhodococcus equi, and the gene encoding 488 deduced amino acids was cloned using peptide sequences of the purified enzyme. Eight residues essential for the catalytic reaction in microbial and animal endoglycoceramidases were all conserved in the deduced amino acid sequence of the novel enzyme. Homology modeling of the enzyme using endocellulase E1 as a template revealed that the enzyme displays a (β/α)8 barrel structure in which Glu234 at the end of β-strand 4 and Glu341 at the end of β-strand 7 could function as an acid/base catalyst and a nucleophile, respectively. Site-directed mutagenesis of these glutamates resulted in a complete loss of the activity without a change in their CD spectra. The recombinant enzyme hydrolyzed the β-galactosidic linkage between oligosaccharides and ceramides of 6-gala series GSLs that were completely resistant to hydrolysis by the enzymes reported so far. In contrast, the novel enzyme did not hydrolyze ganglio-, globo-, or lacto-series glycosphingolipids. The enzyme is therefore systematically named “oligogalactosyl-N-acylphosphosine 1,1’-β-galactohydrolase” or tentatively designated “endogalactosylceramidase.”

Glycosphingolipids (GSLs),2 amphipathic compounds consisting of oligosaccharides and ceramides, are ubiquitous components of the plasma membrane (1). Recently, it was revealed that GSLs are enriched with other sphingolipids and cholesterol to form microdomains on ectoplasmic membranes. These lipid microdomains, known as detergent-insoluble sphingolipid-enriched domains, so-called DIs or rafts (2), assemble receptors and signaling molecules such as glycosylphosphatidylinositol-anchored proteins, Src family kinases, and G-proteins (3).

6-Gala series GSLs possessing the structure R-Galβ1-6Galβ1-1’-Cer have been found in the mollusk (Turbo cornutus) (4), the leech (Hirudo nipponia) (5), the earthworm (Phereetima sp.) (6), and some pathogenic cestode parasites (Echinococcus multilocularis) (7), Taenia crassiceps (8), Spirometra erinacei (9), and Metrloasthes cotarunix (10). Recently, it was revealed that aureobasidin A-resistant Zyomyces species synthesized 6-gala series GSLs (tentatively designated CDS, CTS, CTeS, and CPS) instead of inositol phosphorylceramide (11). Because aureobasidin A, a well known and widely used antifungal agent, was found to inhibit the synthesis of inositol phosphorylceramide, the resistance to the agent may stem from the lack of inositol phosphorylceramide in Zyomyces. Thus, an inhibitor for the synthesis of 6-gala series GSLs seems to be a promising antibiotic for aureobasidin-resistant fungus as well as pathogenic cestode parasites synthesizing the GSLs. On the other hand, 6-gala series GSLs from E. multilocularis inhibited the proliferation of human peripheral blood mononuclear cells simultaneously with a decrease in the synthesis of interleukin 2 (12). Thus, the Echinococcus GSLs are considered to play immunologically relevant roles in alveolar hydatid disease.

Endoglycoceramidase (EGCase) or ceramide glycanase (EC 3.2.1.123) is a GSL-specific enzyme that hydrolyzes acidic and

* This work was supported by Grants-in-aid for Basic Research B 17380068 from the Ministry of Education, Culture, Sports, Science and Technology, and New Energy and Industrial Technology Development Organization, Japanese Government. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank/EMBL Data Bank with accession number(s) AB270591.

2 The abbreviations used are: GSL, glycosphingolipid (the structures of GSLs are summarized in Table 3); CBB, Coomassie brilliant blue; EGALC, endogalactosylceramidase; EGCase, endoglycoceramidase; GC, gas chromatography; 6-gala series GSLs, GSLs containing a R-Galβ1-6Galβ1-1’-Cer structure; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; TBS, Tris-buffered saline; TGC, trigalactosylceramide (neogalatriaosylceramide), Galβ1-6Galβ1-6Galβ1-1’-Cer; CDS, Galβ1-6Galβ1-1’-Cer; CTS, Galβ1-6Galβ1-6Galβ1-1’-Cer; CTeS, Galβ1-6Galα1-6Galβ1-6Galβ1-1’-Cer; GalCer, monogalactosylceramide.
neutral GSLs to produce intact oligosaccharides and ceramides of various GSLs. This enzyme was found in a culture supernatant of the actinomycete *Rhodococcus* sp. G-74-2 (13), and then purified from *Rhodococcus* sp. M-777 (14), a mutant strain of G-74-2, and cloned (15). The recombinant EGCase was found to hydrolyze the β-glucosidic linkage between oligosaccharides and ceramides of various GSLs except glucocerebrosides. However, the recombinant enzyme did not hydrolyze gala series GSLs whose oligosaccharides are attached to ceramide by β-galactosyl linkages. EGCase (ceramide glycanase) was also found in bacteria (16), actinomycetes (17), leeches (18), earthworms (19), short-neck clams (20), jellyfish (21), and hydra (22). Among them, the jellyfish and hydra enzymes were cloned and characterized as an animal EGCase. Interestingly, critical amino acid residues in the catalytic module of glycoside hydrolase family 5 endo-β,1,4-glucanase (cellulase) were completely conserved in microbe as well as animal EGCases (21–23), suggesting that both cellulase and EGCase could have evolved from the same ancestral gene. It is noteworthy that EGCase is distributed in animals belonging to *Cnidaria, Mollusca,* and *Annelida,* and in a hydra the enzyme is involved in a unique pathway catabolizing GSLs (22).

Rhodococcal EGCase (EGCase II) serves as a useful tool for studying the structure and functions of various neutral and acidic GSLs owing to its unique ability to remove oligosaccharides from ceramides without damaging either (24–26). An acidic GSLs owing to its unique ability to remove oligosaccharides and in a hydra the enzyme is involved in a unique pathway catabolizing GSLs without damaging either (24–26). An acidic GSLs owing to its unique ability to remove oligosaccharides and in a hydra the enzyme is involved in a unique pathway catabolizing GSLs (22). An acidic GSLs owing to its unique ability to remove oligosaccharides and in a hydra the enzyme is involved in a unique pathway catabolizing GSLs (22).

**Molecular Cloning of Endogalactosylceramidase**

**EXPERIMENTAL PROCEDURES**

*Materials*—The LA Taq and TaKaRa LA PCR *in vitro* cloning kit was purchased from Takara Bio Inc. (Shiga, Japan). The plasmid pET22b was obtained from Novagen (Madison, WI). The restriction enzymes and Ligation-Convention Kit were obtained from Nippon Gene (Tokyo, Japan). Pre-coated Silica gel 60 TLC plates were purchased from Merck (Darmstadt, Germany). Neogalatriciaosylceramide (trigalactosylceramide, TGC) was prepared from a turban shell, *Turbo cornutus,* by a method described previously (4). GT1b, GD1a, GM1a, Gb4Cer, Gb3Cer, LacCer, GalCer, and GlcCer were purchased from Wako Pure Chemical Industries (Osaka, Japan). CDS, CTS, and CTEs from a fungus, *Mucor hiemalis* (11), were kindly donated by Dr. K. Yamamoto, Kyoto University, Japan. GM4 was prepared from red sea bream, *Pagrus major,* by a method described previously (27). *Escherichia coli* strains DH5α and BL21(DE3) were purchased from Takara Bio Inc. Triton X-100 and Lubrol PX were obtained from Sigma-Aldrich Co. and Nacalai Tesque Inc. (Kyoto, Japan), respectively.

**Synthesis of GSLs**—For the synthesis of Galβ1-6Glc derivative (GSC-590), phenyl-2,3,4,6-tetra-O-benzyl-β-D-galactopyranoside and 2-(trimethylsilyl)ethyl-2,3,4-tri-O-benzyl-β-D-glucopyranoside were employed as the glycosol donor and acceptor, respectively. After the coupling, the disaccharide was converted into the trichloroacetimidate derivative as the corresponding glycosyl donor and was then coupled with 3,4-di-O-benzoylphytoceramide prepared from commercially available phytosphingosine. The complete deprotection of the protected glycolipid gave the desired compound. For the synthesis of the Galβ1-4Gal derivative (GSC-591), p-methoxy-2,4,6-tri-O-benzyl-β-D-galactopyranoside was used. A similar procedure described for the synthesis of Galβ1-6Glc derivative gave the desired compound. The structures were supported with the spectral data. The detailed procedure of the compounds will be described elsewhere.

**EGCase Assay**—The activities of EGALC (this study) and EGCase were measured using TGC and GM1a as a substrate, respectively, as described (14). Briefly, 10 nmol of substrate was incubated at 37 °C for a specific period with an appropriate amount of the enzyme in 20 μl of 50 mM sodium acetate buffer, pH 5.5, containing 0.1% (w/v) Triton X-100. The reaction was stopped by heating in a boiled water bath for 5 min. After drying, the sample was dissolved in 10 μl of methanol and then applied to TLC plates, which were developed with chloroform/methanol/0.02% CaCl2 (2/3/1, v/v). The GSLs remaining and oligosaccharide generated were visualized by spraying the TLC plate with orcinol-H2SO4 reagent and scanned in a Shimadzu CS-9300 chromatoscaner with the reflection mode set at 540 nm. The extent of hydrolysis was calculated as follows: hydrolysis (%) = (peak area for oligosaccharide released) × 100/(peak area for oligosaccharide released + peak area for remaining substrate). One unit of EGALC was defined as the amount of enzyme that catalyzed the hydrolysis of 1 μmol of GSL/min under the conditions described above.

**Purification of EGALC**—The culture supernatant (1.8 liters) of *Rhodococcus equi* was saturated with 80% ammonium sulfate and left overnight. The precipitate was collected with centrifugation at 8000 rpm for 30 min at 4 °C, and the precipitate obtained was dissolved in 170 ml of 20 mM sodium acetate buffer, pH 6.0, containing 0.1% Lubrol PX (buffer A). The sample solution was then applied to a column of Octyl-Sepharose CL-4B (200 ml, GE Healthcare Bio-Sciences Corp.) equilibrated with buffer A. The enzyme was eluted from the column with the same buffer containing 1% Lubrol PX. The active fractions showing the hydrolytic activity for TGC were pooled and subjected to chromatography using DEAE-Sepharose FF (70 ml, GE Healthcare Bio-Sciences Corp.) previously equilibrated with buffer A. The enzyme was eluted from the column with the same buffer containing 1.0 M NaCl. The active fractions were pooled and then applied to a column of Chelating-Sepharose FF (30 ml, Cu2+ form, GE Healthcare Bio-Sciences Corp.), which was previously equilibrated with buffer A containing 2.0 M Cu2+.
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NaCl. The major enzyme activity was eluted with the same buffer containing 2.0 M NH₄Cl, and then active fractions were pooled and desalted by chromatography with Octyl-Sepharose FF (70 ml, GE Healthcare Bio-Sciences Corp.). These rounds of chromatography were performed using a BPLC-600FC HPLC system (Yamazen Co., Osaka, Japan). The fractions containing enzyme activity were then applied to a column of DEAE-5PW (7.5 mm diameter/75 mm length, Tosoh Co., Tokyo, Japan) equilibrated with buffer A. The column was washed with buffer A and eluted with a linear gradient of NaCl (0 to 0.2 M) in the same buffer at a flow rate of 1 ml/min. Finally, the active fractions were applied to a POROS HQ (4.6 mmD/100 mM, 1.7 ml, PE-Biosystems) equilibrated with buffer A and eluted with a linear gradient of NaCl (0 to 0.2 M/20 min, and 0.2 to 0.6 M/250 min) in the same buffer at a flow rate of 1 ml/min using a BioCAD SPRINT system (PE-Biosystems).

Protein Assay and SDS-PAGE—Protein content was determined by the bicinchoninic acid protein assay (Pierce) with bovine serum albumin as a standard. SDS-PAGE was carried out according to the method of Laemmli (28). The proteins were stained with silver-staining solution (Atto Co., Tokyo, Japan) or Coomassie Brilliant Blue (CBB) according to the amount of protein loaded.

Determination of Amino Acid Sequence—Peptide mapping was performed according to the method of Cleveland et al. (29). After digesting the purified enzyme with V₈ endoprotei nase (Wako), peptide fragments were separated on a SDS-PAGE gel and electrically transferred to a polyvinylidene difluoride membrane. The transferred peptide fragments of the enzyme were sequenced using a protein sequencer (Pro- cise 492 cLc, PE-Biosystems).

Cloning of the Gene Encoding EGALC.—The internal peptide sequences were used to design degenerate PCR primers for amplification of a partial DNA fragment of the enzyme. The sense primer, C-1479-S1 (5'-GGNGCNAAYTNAAYGGG-3'), (5'-TARAANGCYTGRAANGC-3'), was designed from the peptide sequence of C-1485. Genomic DNA of *R. equi* was prepared as described before (15) and used as a template. PCR was conducted in a T gradient thermocycler (Biometra, Goettingen, Germany) for 35 cycles (each consisting of denaturation at 98 °C for 10 s, annealing at 50 °C for 1 min, and extension at 72 °C for 30 s) using TaKaRa LA TaqDNA polymerase. The amplified PCR product was purified from agarose gel, cloned into a pGEM-T Easy vector (Promega, Madison, WI), and sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE-Biosystems) and a DNA sequencer (model 377A, PE-Biosystems). The full-length egalc gene was cloned by the cassette ligation-mediated PCR method (30) using the LA PCR in vitro cloning kit according to the instructions of the manufacturer. Briefly, *R. equi* genomic DNA (2.0 μg) was digested with BamHI and ligated to a double-stranded DNA Cassette with the restriction site of BamHI. The cassette-ligated restriction fragment was then subjected to nested PCR using cassette- and gene-specific primers in a T gradient thermocycler. The gene-specific primers were as follows: BamA1 (5'-TAGAATGCTTGAATGCCCT) and BamA2 (5'-GTGAGCCTTAGCTGTG-3') for upstream, BamS1 (5'-TCAGATCGGAGCCTTCTACC-3') and BamS2 (5'-GTCGATCAA-GTCAGCAGT) for downstream amplification. The amplification was carried out for 25 cycles (each consisting of denaturation at 94 °C for 10 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min). A second PCR was done with 1 μl of the first PCR product as a template. The second PCR products were cloned into pGEM-T Easy vector, and their DNA sequences were determined.

Sequence Analysis—The nucleotide and deduced amino acid sequences were evaluated using DNASIS software (Hitachi Software Engineering, Tokyo, Japan). The homology search of deduced amino acid sequences was performed with BLAST (31). The alignment of amino acid sequences was performed with ClustalW (32) and JALVIEW (33). The alignment was shaded in BoxShade 3.21 (www.ch.embnet.org/software/BOX_form.html).

Homology Modeling of EGALC—A three-dimensional model structure of EGALC was built using MODELLER 8v2 (34) based on the known crystal structure of the catalytic domain of family 5 endo-β1,4-glucanase (endocellulase E1) of *Acidothermus cel lulolyticus* (35) (PDB ID 1ECE, chain A). Sequence alignment between EGALC and endocellulase E1 was performed by ClustalW. Based on the secondary structure of EGALC predicted by PSIPRED (36), the sequence alignment was adjusted manually by JALVIEW. The final model structure was validated by the PROCHECK program (37).

Construction of the Expression Plasmid Vector for the Recombinant EGALC—To introduce the restriction site for HindIII at the 5'-end and NotI at the 3'-end of the egalc gene, PCR was carried out using genomic DNA of *R. equi* as a template and the following two primers: rEGC-S2 (5'-GGAAAGCTTTCGCGTCGGAGC-3') and rEGC-HisA (5'-GGCCGGCCCGAGTGTGTTAGCAGTAC). These primers contained a HindIII site (underlined) and a NotI site (double underlined), respectively. The conditions for amplification were 25 cycles (each consisting of denaturation at 98 °C for 1 min, annealing at 56 °C for 30 s, and extension at 72 °C for 2 min). The amplified product encoding amino acid residues 23–488 without the signal peptide was digested with HindIII and NotI and was inserted into the corresponding sites of pET22b(+) to generate a C-terminal His₆-tagged protein. The recombinant plasmid was designated as pET22-EGC.

Expression and Purification of Recombinant EGALC—E. coli strain BL21(DE3) cells transformed with pET22-EGC were grown at 37 °C for 12 h in 5 ml of medium A (Luria-Bertani medium supplemented with 100 μg/ml ampicillin) with shaking. The culture was then transferred into 200 ml of medium A and incubated until the A₆₀₀ reached ~0.5. Then isopropyl β-d-thiogalactopyranoside was added to the culture at a final concentration of 1 mM to cause transcription. After 5 h at 25 °C, the cells were harvested by centrifugation (8000 × g for 10 min) and suspended in 100 ml of 30 mM Tris-HCl, pH 8.0, containing 20% sucrose and 1 mM EDTA, then incubated at room temperature for 10 min with gentle shaking. The cells were harvested and re-suspended in 100 ml of cold 5 mM MgSO₄ and incubated on ice for 10 min, and the solution was centrifuged. The supernatant containing the periplasmic fraction was applied to a column of HiTrap Chelating HP (GE Healthcare Bio-Sciences Corp.), which was chelated with Ni²⁺, then the column was
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Site-directed Mutagenesis—The following sense primers were designed to introduce the mutations into the egalc gene (the mutated regions are underlined): for the substitution of Glu\(^{234}\) with Asp or Gln, E234D (5'-CTGCTCAACGAGCCGAC-3') and E234Q (5'-CTGCTCAACGAGCCGAC-3'); for the substitution of Glu\(^{341}\) with Asp or Gln, E341D (5'-TGGA-CCGTTGATTTGGGC-3') and E341Q (5'-TGGAACCGTGCTAGTGGGC-3'). The mutations were introduced by the megaprimer method (38). The first PCR was performed with rEGC-HisA and one of the sense primers containing the mutations and pET22-EGC as a template. The amplified product was used as a megaprimer in the second PCR with rEGC-S2 and pET22-EGC as a template. The final PCR products were cloned into pETE234D, pETE234Q, pETE234E, and pETE341Q, respectively. After confirmation of the desired mutations by DNA sequencing, the mutant recombinant enzyme in 200\(\mu\)l of buffer, pH 7.4, containing 0.5 M NaCl and 25 mm imidazole. The enzyme was expressed and purified by the method described above.

Western Blot Analysis—To perform Western blotting, proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane using a semi-dry blotter (Bio-Rad). The membrane then was blocked with 3% (w/v) skim milk in TBS for 1 h and washed three times with T-TBS (TBS containing 0.02% Tween 20). The membrane was incubated with anti-His (C-terminal) antibodies (Invitrogen) for 2 h at room temperature and washed three times with T-TBS. Then it was incubated with horseradish peroxidase-conjugated anti-mouse IgG antibodies for 1.5 h at room temperature. After three washes with T-TBS, the membrane was stained with a peroxidase staining kit (Nacalai Tesque) and washed three times with T-TBS (TBS containing 0.02% Tween 20). The membrane was cut into two pieces (duplicate samples were prepared at 5 mm sodium acetate buffer, pH 5.5). Two pieces were then transferred to a polyvinylidene difluoride membrane. After the sample was mixed with the matrix on an occluded system using 2,5-dihydrobenzoic acid (Sigma-Aldrich) as a matrix, MALDI-TOF MS analysis was conducted by a Voyager-DE mass spectrometer (PE-Bio-Scientific) at 170°C as trimethylsilylated derivatives. The structure of TGC-derived oligosaccharide was also examined using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). To prepare the sample for MALDI-TOF MS, TGC-derived oligosaccharide were separated on a TLC plate as described above, and then the plate was immersed in a solvent composed of isopropanol/methanol/0.2% CaCl\(_2\) (40/7/20, v/v) for 20 s (39). Sugar-containing compounds were then transferred to a polyvinylidene difluoride membrane at 180°C for 30 s in a TLC blower (Atto Inc.). The membrane was cut into two pieces (duplicate samples were loaded for TLC). One piece (membrane I) was used for the detection of samples, and the other (membrane II) was used for the extraction of samples. The samples were detected with immersing membrane I in orcinol-H\(_2\)SO\(_4\) reagent diluted 50% (v/v) with methanol, following heating at 80°C for 10 min. Corresponding to the area on membrane I, samples were extracted from membrane II with 20 μl of 50% (v/v) methanol with sonication for 1 min.

Analysis of Ceramide Released from TGC—A mixture of 100 nmol of TGC and 5 milliliters of the recombinant enzyme in 200 μl of 50 mm sodium acetate buffer, pH 5.5, containing 0.1% Triton X-100 was incubated at 37°C for 16 h. After incubation, 800 μl of chloroform/methanol (2/1, v/v) was added, then the upper phase was applied to a Sep-Pak Plus C18 cartridge (Waters) previously equilibrated with distilled water. The passed fraction was dried with a rotary evaporator. To reduce the released oligosaccharide, 500 μl of 1% sodium borohydride in 0.01 M NaOH and 50 μg of ninyaminoisol (internal standard) were added, and then the mixture was incubated at room temperature for 1 h. After reduction, the reduced oligosaccharide was passed through a Dowex 50 WX8 (H\(^+\) form, 2 ml, Dow Chemical) column to destroy the excess borohydride. The partial alditol oligosaccharide was evaporated dry, and then evaporated with methanol 5 times. The residue was dissolved in 200 μl of 5%-hydrogen chloride methanol solution (Nacalai Tesque), and the mixture was incubated at 80°C for 16 h. After methanolation, the solution was dried under a stream of nitrogen and dissolved in siblender-HTP (Nacalai Tesque), and the mixture was incubated at room temperature for 30 min. The solution was then dried under a stream of nitrogen and dissolved in 50 μl of hexane. The alditois and other sugar components were analyzed by gas chromatography on a Shimadzu GC-2014 gas chromatograph equipped with a fused silica capillary column (DB-5, 0.25 mm × 25 m, J. & W. Scientific) at 170°C as trimethylsilylated derivatives. The structure of TGC-derived oligosaccharide was also examined using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). To prepare the sample for MALDI-TOF MS, TGC-derived oligosaccharide were separated on a TLC plate as described above, and then the plate was immersed in a solvent composed of isopropanol/methanol/0.2% CaCl\(_2\) (40/7/20, v/v) for 20 s (39). Sugar-containing compounds were then transferred to a polyvinylidene difluoride membrane at 180°C for 30 s in a TLC blower (Atto Inc.). The membrane was cut into two pieces (duplicate samples were loaded for TLC). One piece (membrane I) was used for the detection of samples, and the other (membrane II) was used for the extraction of samples. The samples were detected with immersing membrane I in orcinol-H\(_2\)SO\(_4\) reagent diluted 50% (v/v) with methanol, following heating at 80°C for 10 min. Corresponding to the area on membrane I, samples were extracted from membrane II with 20 μl of 50% (v/v) methanol with sonication for 1 min.

Analysis of Ceramide Released from TGC—A mixture of 100 nmol of TGC and 5 milliliters of the recombinant enzyme in 200 μl of 50 mm sodium acetate buffer, pH 5.5, containing 0.1% (w/v) Triton X-100 was incubated at 37°C for 16 h. After partition of the reaction mixture using chloroform/methanol (2/1, v/v) three times, the lower phase was dried, dissolved in chloroform/methanol (2/1, v/v), and then applied to a TLC plate, which was developed with chloroform/methanol/NH\(_4\)OH (90/10/1, v/v). The visualization of ceramide was performed with CBB-R250. The sample after the partition was also analyzed by MALDI-TOF MS.

MALDI-TOF MS Analysis—The MALDI-TOF MS analysis was conducted by a Voyager-DE mass spectrometer (PE-Biosystems) using 2,5-dihydroxybenzoic acid (Sigma-Aldrich) as a matrix. After the sample was mixed with the matrix on an occupied sample plate, it was dried completely. The operation was
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conducted in the positive polarity mode and the accelerating voltage was 20 kV.

RESULTS

Purification and Partial Amino Acid Sequence of EGALC—An enzyme capable of hydrolyzing TGC (Galβ1-6Galβ1-6Galβ1-1′Cer) was purified from the culture supernatant of R. equi by ammonium sulfate precipitation, followed by various chromatography as described under ”Experimental Procedures.” Fig. 1A shows the final step in the purification process using POROS HQ. In a typical experiment, the enzyme was purified 2100-fold with 8% recovery (Table 1), and the final preparation showed a single protein band after visualization using a silver staining solution, the molecular mass estimated to be 54 kDa based on SDS-PAGE (Fig. 1B). The 54-kDa protein band was subjected to amino acid microsequencing after in-gel digestion with V8 endoproteinase and eight different peptide sequences, including the possible N-terminal sequence were determined as shown in Table 2.

Cloning of the Gene Encoding EGALC—When a set of primers designed from C-1479 and C-1485 peptide sequences (Table 2) were used for the genomic PCR using the R. equi genomic DNA as a template, a 431-bp nucleotide fragment was specifically amplified. To obtain the full-length sequence of the gene, the cassette ligation-mediated PCR was performed as described under ”Experimental Procedures.” As a result, a putative 1,464-bp open reading frame encoding 488 deduced amino acid residues was obtained. The molecular mass and pl of the enzyme were estimated to be 52,299 and 4.28, respectively. A putative ribosome-binding site (Shine-Dalgarno sequence, AGGAAG) and a hydrophobic motif composed of 22 amino acid residues was obtained. The molecular mass of the enzyme was estimated to be 54 kDa on SDS-PAGE when stained with CBB (Fig. 4). The purified recombinant enzyme (specific activity, 9.5 units/mg) gave a single protein band corresponding to a molecular mass of 54 kDa on SDS-PAGE when stained with CBB (Fig. 4).

TABLE 1

Purification of EGALC from R. equi

| Step                          | Total activity (units) | Total protein (mg) | Specific activity (units/mg) | Yield (%) | -fold |
|-------------------------------|------------------------|--------------------|-----------------------------|-----------|-------|
| 80% (NH4)2SO4 precipitation   | 9                      | 370                | 24                          | 100       | 1     |
| Octyl-Sepharose CL-4B         | 8                      | 100                | 80                          | 89        | 3     |
| DEAE-Sepharose FF             | 5                      | 31                 | 160                         | 56        | 7     |
| Chelating-Sepharose FF        | 5                      | 12                 | 420                         | 56        | 17    |
| TSKgel DEAE-5PW               | 4                      | 5.6                | 710                         | 44        | 30    |
| POROS HQ                      | 0.8                    | 0.016              | 50,000                      | 8         | 2,100 |

TABLE 2

Peptide sequences of EGALC

| Peptide no. | N-terminal                | Internal               | Internal               | Internal               |
|-------------|---------------------------|------------------------|------------------------|------------------------|
| C-1440      | APRIL                     | C-1441                 | GFAEPLSLRAYPRAVFGLTEVTANGFR |
| C-1442      | RVQDAK                    | C-1442                 | GQDCFXQLRQOXG           |
| C-1457      | AEPPOVY                  | C-1457                 | ARQPOV                 |
| C-1477      | YSVTL                     | C-1477                 | YSVTL                  |
| C-1479      | LLRGMNVKVLNIDYA           | C-1479                 | LLRGMNVKVLNIDYA         |
| C-1482      | ARAQFAFY                 | C-1485                 | ARAQFAFY                |

FIGURE 1. Purification of EGALC from the culture supernatant of R. equi. A, purification of the enzyme using POROS HQ. An aliquot of protein was applied to a column of POROS HQ equilibrated with buffer A and eluted with a linear gradient of NaCl in the same buffer at a flow rate of 1 ml/min using a BioCAD SPRINT system as described under ”Experimental Procedures.” B, SDS-PAGE showing the purified enzyme. The fractions shown by the underline in panel A were pooled and analyzed by SDS-PAGE. Proteins were visualized with a silver staining solution.
General Properties of the Recombinant EGALC—The recombinant enzyme exhibited maximal activity at around pH 5.5 with TGC as a substrate and required a detergent such as Triton X-100 or Lubrol PX for the hydrolysis of TGC with an optimal concentration of 0.1% (w/v). Taurodeoxycholate, however, strongly inhibited the enzyme activity at a concentration of 0.05%. Among the metal ions tested, Hg$^{2+}$ completely inhibited the enzyme activity, whereas 50–90% inhibition was observed with Fe$^{3+}$, Zn$^{2+}$, and Cu$^{2+}$ at 5 mM. On the other hand, Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, Co$^{2+}$, and EDTA had no significant effect on the activity at the same concentration. The \( K_m \), \( V_{max} \), and \( k_{cat} \) values for TGC were calculated as 0.43 mM, 87.8 pmol/min, and 797.8 min$^{-1}$, respectively.

Mode of the Action of the Recombinant EGALC—The recombinant enzyme hydrolyzed CDS (Gal$_1$-6Gal$_1$-1-Cer), TGC (Gal$_1$-6Gal$_1$-6Gal$_1$-1-Cer), CTS (Gal$_1$-6Gal$_1$-6Gal$_1$-1-Cer), and CTeS (Gal$_1$-6Gal$_1$-6Gal$_1$-1-Cer) to produce oligosaccharides with different R$_f$ values, whereas neither LacCer, Gb3Cer, or GM1a was hydrolyzed by the enzyme (Fig. 5A).

To determine the point of the substrate cleaved by the enzyme, the digestion product of TGC was subjected to TLC after partition with chloroform/methanol (2:1, v/v). The upper layer contained an oligosaccharide released from TGC (data not shown), whereas the lower layer contained a band corresponding to the \( R_f \) of the bovine brain ceramide (Fig. 5B). Then, TGC, the TGC-derived oligosaccharide, and the TGC-derived ceramide were analyzed separately by MALDI-TOF MS. As a result, the major pseudomolecular ions, [M + Na]$^+$, were observed at \( m/z \) 1047.0 for TGC (Fig. 6A), \( m/z \) 527.8 for the oligosaccharide (Fig. 6B), and \( m/z \) 561.1 for the ceramide (Fig. 6C), which correspond to the molecular mass of TGC, galactotriose, and N-palmitoylsphingosine (C16:0 and d18:1), respectively. In agreement with the heterogeneity in ceramide moiety of TGC (4), several
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FIGURE 4. SDS-PAGE showing the purified recombinant EGALC. The purified protein was subjected to SDS-PAGE and visualized by CBB staining. Lane 1, crude extract of E. coli BL21(DE3) transformed with pET22-EGC; lane 2, periplasmic fraction of the transfectants; lane 3, the purified recombinant EGALC. The enzyme was purified using a HiTrap Chelating HP column as described under “Experimental Procedures.”

additional peaks were detected in Fig. 6 (A and B), e.g. m/z 1061.0 (Fig. 6A) and m/z 575.1 (Fig. 6C) could be derived from TGC with N-heptadecanoylsphingosine (C17:0 and d18:1) and the ceramide released, respectively. To confirm that the enzyme hydrolyzes the internal Galβ1-1’Cer linkage, the reducing end of the oligosaccharide was determined. Only galactose was detected when the sugar component of the oligosaccharide was analyzed by GC after reduction (Fig. 6D), whereas galactose and galactiol were detected at a molar ratio of about 2:1 when the sugar component analysis was performed after reduction of the oligosaccharide with NaBH4 (Fig. 6E), indicating that galactose was present at the reducing end of the oligosaccharide. Collectively, the data clearly indicated that the enzyme hydrolyzed the Galβ1-1’Cer linkage of TGC to produce an intact oligosaccharide and ceramide. Thus, the enzyme should be named “oligogalactosyl-N-acylsphingosine 1,1’-β-galactohydrolase” or tentatively designated “endogalactosylceramidase” (EGALC).

**Substrate Specificity of EGALC**—The specificity of the enzyme was further examined using various GSL substrates under two sets of conditions; one reflecting the relative initial velocity (condition I) and the other for determining the degree of hydrolysis after exhaustive digestion (condition II). As shown in Table 3, the recombinant enzyme hydrolyzed the GSLs containing a Galβ1-1’Cer linkage such as GalCer, CDS, TGC, CTS, and CTeS, although the hydrolysis of GalCer was relatively slow. Neither sulfatide (HSO4−-3Galβ1-1’Cer) nor GM4 (NeuAca2-3Galβ1-1’Cer) was hydrolyzed by the enzyme even under condition II, although both GSLs contain a Galβ1-1’Cer structure. In addition, the enzyme did not degrade the chemically synthesized GSLs, GSC-590 (Galβ1-6Glcβ1-1’Cer) and GSC-591 (Galβ1-4Galβ1-1’Cer). These results indicate that the enzyme recognizes the sugar and linkage adjacent to Galβ1-1’Cer, i.e. the R-Galβ1-6Galβ1-1’Cer structure seems to be essential for the enzyme reaction. It should be emphasized that all the GSLs containing Galβ1-1’Cer listed in Table 3 are completely resistant to hydrolysis by rhodococcal EGCase II, and jellyfish and hydra EGCases. In contrast, no GSLs belonging to the ganglio-, globo-, or lacto- series were hydrolyzed by EGALC cloned in this study. The time courses for the hydrolysis of these GSLs by EGALC are shown in Fig. 7. It was found that the enzyme hydrolyzed CTeS most rapidly followed by CDS, TGC, and CTS, whereas GM1a, GM4, and sulfatide were not hydrolyzed at all during the course of incubation.

**Determination of Catalytic Amino Acid Residues of EGALC**—Alignment of the deduced amino acid sequence of the enzyme with sequences of other EGCases (Fig. 2) and endo-β1,4-glucanases (supplemental Fig. S1) and three-dimensional structure predicted by homology modeling (Fig. 3) suggested that Glu234 and Glu341 are an acid/base catalyst and a nucleophile, respectively. To address whether they function as catalytic residues, Glu234 and Glu341 were replaced with Asp (E234D and E341D) or Gln (E234Q and E341Q) by site-directed mutagenesis. Four mutant enzymes and the wild-type enzyme were separately expressed in E. coli strain BL21(DE3), purified, and then analyzed by SDS-
PAGE followed by staining with CBB (Fig. 8A) and anti-His antibody (Fig. 8B). The enzyme activity for TGC was examined using 10 ng of wild-type enzyme or 100 ng of mutant enzyme. Compared with the wild-type enzyme, the activity of mutants E234D and E341D, in which the acidic Glu residue was replaced by a similar acidic Asp residue, was strongly decreased, and no activity was detected in mutants E234Q and E341Q, in which the acidic Glu residue was replaced by a neutral Gln residue (Fig. 8C). It was confirmed that CD spectra of E234Q and E341Q were virtually identical to that of the wild-type enzyme (Fig. 8D), indicating that the point mutation did not affect the secondary structure of mutant enzymes. Taken together, it was revealed in this study that Glu^{234} and Glu^{341} of the EGALC function as an acid/base catalyst and a nucleophile, respectively.

**DISCUSSION**

An enzyme that degrades various acidic and neutral GSLs into oligosaccharides and ceramides by cleaving a glucosyl bond was systematically named “oligoglycosylglucosylceramide glycolyropholase” and given the EC number 3.2.1.123 (43). This type of enzyme (EGCase or ceramide glycanase) has been found in actinomyces (13, 17), bacteria (16), leeches (18), earthworms (19), short-necked clams (20), jellyfish (21), and hydra (22). Rhodococcal EGCase II (15, 17) and

![FIGURE 6. MALDI-TOF MS and GC analyses of the reaction products from TGC generated by the recombinant EGALC. MALDI-TOF MS analysis of TGC (A), the TGC-derived oligosaccharide (B), and the TGC-derived ceramide (C).](image)

### Table 3

**Specificity of the recombinant EGALC**

Various GSLs (10 nmol) were incubated at 37 °C for 30 min with 50 microunits (condition I) or for 16 h with 0.5 milliunit (condition II) of the enzyme in 20 μl of 50 mM sodium acetate buffer, pH 5.5, containing 0.1% Triton X-100. Values are the means ± S.D. (n = 3).

| Substrate | Hydrolysis | Condition I | Condition II | EGCase II† |
|-----------|------------|-------------|--------------|------------|
| 6-Gala series | | | | |
| CDS | Galβ1-1-Galβ1-1’Cer | 17.1 ± 0.9 | 65.9 ± 1.9 | 0
| TGC | Galβ1-1-Galβ1-1’Cer | 17.8 ± 1.7 | 65.9 ± 1.9 | 0
| CTS | Galβ1-1-Galβ1-1’Cer | 21.6 ± 2.7 | 67.2 ± 2.7 | 0
| CTeS | Galβ1-1-Galβ1-1’Cer | 45.0 ± 0.4 | 70.8 ± 2.7 | 0
| Ganglio series | | | | |
| GgCer | NeuAcβ1-3Galβ1-1-Galβ1-1’Cer | 0 | 0 | 100a |
| GD1a | NeuAcβ1-3Galβ1-1-Galβ1-1’Cer | 0 | 0 | 100a |
| GM1a | NeuAcβ1-3Galβ1-1-Galβ1-1’Cer | 0 | 0 | 100a |
| Globoside | | | | |
| GbCer | GalNAcβ1-3Galβ1-1-Galβ1-1’Cer | 0 | 0 | 5.3a |
| Ganglio series | | | | |
| GgCer | GalNAcβ1-3Galβ1-1-Galβ1-1’Cer | 0 | 0 | 12.4a |
| Lactoserie | | | | |
| LacCer | Galβ1-1-Galβ1-1’Cer | 0 | 0 | 100a |

* For EGCase II, 1.5 milliunits of enzyme was incubated with substrates at 37 °C for 16 h.
† Values were from Ref.15.
‡ 6 milliunits of the enzyme was used.
§ Chemical synthesized GSLs.
the jellyfish (21) and hydra (22) enzymes were cloned. These enzymes did not hydrolyze gαla series GSLs, which contain a Galβ1-1’Cer core structure. We report here a novel EGCase having a completely different substrate specificity, i.e. the enzyme cloned in this study specifically cleaves the Galβ1-1’Cer linkage in 6-gala series GSLs, producing oligosaccharides and ceramides. However, the enzyme did not cleave the Glcβ1-1’Cer linkage in ganglio-, globo-, or lacto-series GSLs in contrast to EGCases previously reported (Fig. 9).

Thus, the systematic name of the novel enzyme should be “oligogalactosylceramide galactohydrolase,” and we have tentatively designated it “endogalactosylceramidase” (EGALC). It is worth noting that the enzyme also hydrolyzed GalCer, but not GkCer, although the extent of the hydrolysis extent of GalCer by the enzyme was very low compared with that of Galβ1-6Galβ1-1’Cer and Galβ1-6Galβ1-6Galβ1-1’Cer.

EGALC specifically targets 6-gala series GSLs and is not able to degrade GM4 (NeuAcα2-3Galβ1-1’Cer) or sulfatide (HSO₄-3Galβ1-1’Cer), although they both contain a β-galactosylceramide linkage. Because 6-gala series GSLs commonly have at least one β,1-galactosyl linkage adjacent to the core Galβ1-1’Cer structure, it is speculated that the minimum structure required for hydrolysis by the enzyme is Galβ1-6Galβ1-1’Cer. Thus, two GSLs were newly synthesized to examine the specificity of the Trichodema endo-β,1,6-galactanase, which degrades β-1,6-galactooligosaccharides but not β-1,4-galactooligosaccharides (41, 42).

The similarity of the primary structure as well as the identical catalytic amino acid residues suggest that EGALC proceeds with the hydrolytic reaction in a very similar, if not identical, manner to EGCases of the glycoside hydrolase family 5 family in CAZY (afmb.cnrs-mrs.fr/CAZY/fam/GH5.html). Actually, a three-dimensional model of EGALC revealed that the enzyme possesses a (β/α)₉ barrel structure and proceeds with the hydrolysis reaction via a retaining double-replacement mechanism (Fig. 3). Furthermore, site-directed mutagenesis confirmed that Glu190 and Glu246 possibly function as an acid/base catalyst and a nucleophile, respectively, very similar to Rhodococcus EGCase II, despite a clear difference in substrate specificity. However, it is unclear at present why EGALC specifically hydrolyzes GSLs containing Galβ1-6Galβ1-1’Cer, but not Galβ1-4Glcβ1-1’Cer, as a core structure. To address this question, the x-ray structure of the enzyme-substrate complex should be solved.

The specificity of EGALC toward the ceramide moiety of GSLs is likely to be broad, because the extent of hydrolysis of
the GSL from mollusk (TGC) and that of GSLs from fungus (CDs, CTS, and CTeS) by the enzyme were similar, although the structures of the cerebrosides of these GSLs are clearly different; i.e. the cerebroside of TGC contains mainly C16:0 fatty acid and d18:1 sphingosine (4), whereas that of fungal GSLs consist of C24:0, C25:0, and C26:0 2-hydroxy fatty acids and t18:0 phytosphingosine (11). Furthermore, the enzyme degraded GalCer as well as galactosylsphingosine (Galβ1-1’Sph), which lacks a fatty acid moiety, although the extent of hydrolysis of both GSLs was very low after exhaustive digestion with the enzyme (Table 3).

*操作点 A* is not likely to participate in the metabolism of its own cellular components of the microorganism, because *R. equi* does not synthesize 6-gala GSLs. Alternatively, we found that *R. equi* can grow in synthetic medium containing TGC as the sole source of carbon (data not shown), indicating that the microorganism can utilize 6-gala GSLs as a carbon source after degradation of the GSLs with EGALC. Because *R. equi* secretes EGALC outside of the cells, it is assumed that the enzyme could function to catabolize the 6-gala GSLs in the cellular membrane of various fungi, mollusks, and others after death or possibly in a living form.

Recently, much attention has been paid to 6-gala series GSLs possessing the structure Galβ1-6Galβ1-1’Cer, because many pathogenic fungi and parasites synthesize these GSLs (7–11). The pathway of biosynthesis for 6-gala series GSLs could therefore be a target for antibiotics. It should be noted that *Mucor hiemalis* is resistant to aureobasidin A because the fungus synthesizes 6-gala series GSLs instead of inositol phosphorylceramide, which is a target for the antifungal agent (11). EGALC reported here would be useful for the study of structure and function of 6-gala series GSLs, which seem to be physiologically relevant in pathogenic fungi and parasites.

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