Endoglycoceramidase (EC 3.2.1.123) is an enzyme capable of cleaving the glycosidic linkage between oligosaccharides and ceramides in various glycosphingolipids. We report here the purification, characterization, and cDNA cloning of a novel endoglycoceramidase from the jellyfish, *Cyanea nozakii*. The purified enzyme showed a single protein band estimated to be 51 kDa on SDS-polyacrylamide gel electrophoresis. The enzyme showed a pH optimum of 3.0 and was activated by Triton X-100 and Lubrol PX but not by sodium taurodeoxycholate. This enzyme preferentially hydrolyzed gangliosides, especially GT1b and GQ1b, whereas neutral glycosphingolipids were somewhat resistant to hydrolysis by the enzyme. A full-length cDNA encoding the enzyme was cloned by 5′- and 3′-rapid amplification of cDNA ends using a partial amino acid sequence of the purified enzyme. The open reading frame of 1509 nucleotides ends using a partial amino acid sequence of the purified enzyme. The open reading frame of 1509 nucleotides encoded a polypeptide of 503 amino acids including a signal sequence of 25 residues and six potential N-glycosylation sites. Interestingly, the Asn-Glu-Pro sequence, which is the putative active site of *Rhodococcus* endoglycoceramidase, was conserved in the deduced amino acid sequences. This is the first report of the cloning of an endoglycoceramidase from a eukaryote.

Glycosphingolipids (GSLs), amphilic compounds consisting of oligosaccharides and ceramides, are characteristic components of plasma membrane. They are considered to be receptors for microorganisms and their toxins, as well as modulators of cell growth and differentiation (1). Recently, GSLs were found to be enriched with other sphingolipids and cholesterol and to form microdomains on the ectoplasmic membrane (2). These lipid domains assemble receptors and signaling molecules coupled to Src family kinases and G-proteins on their inner surface and mediate membrane trafficking and signaling activities (3).

Endoglycoceramidase (EGCase) is a GSL-specific enzyme that catalyzes the hydrolysis of the glycosidic linkage between oligosaccharides and ceramides of various GSLs (4). This enzyme was first discovered in a culture supernatant of the actinomycete, *Rhodococcus* sp. strain G-74-2 (5), and later found in bacteria (6). A similar enzyme, called ceramide glucanase (CGase), has been found in leeches (7), earthworms (8), and clams (9). Three isoforms of EGCase (EGCase I, II, and III), each differing in molecular weight, pI, and substrate specificity, were isolated from the culture supernatant of *Rhodococcus* sp. M-750, a mutant of the wild strain G-74-2 (4).

Recently, we cloned and sequenced the gene encoding EGCase II of *Rhodococcus* sp. M-777 and showed that the deduced amino acid sequence contained the Asn-Glu-Pro (NEP) sequence, which is commonly conserved as part of the active site region of family A cellulases (endo-1,4-β-glucanase) (10). The NEP sequence was also found in the deduced amino acid sequence of the newly cloned EGCase gene of *Rhodococcus* sp. C9 (11). Replacement of the Glu residue in the NEP sequence with Gln or Asp by site-directed mutagenesis caused drastic loss of enzymatic activity in both M-777 and C9 EGCases, indicating that the Glu residue in the NEP sequence of EGCase plays an important role in its enzymatic activity (11). Molecular cloning of EGCase/CGase from eukaryotes has not yet been conducted. In this paper, we report a novel EGCase from the jellyfish, *Cyanea nozakii*, which is a glycoprotein with N-glycans and acts on GSLs most efficiently at pH 3.0. The substrate specificity of jellyfish EGCase is completely different from those of other EGCases and CGases reported so far; i.e. the jellyfish enzyme hydrolyzed gangliosides, especially b-series polysialogangliosides, much faster than neutral GSLs. This paper also reports the first molecular cloning of a eukaryotic EGCase and reveals that the NEP sequence is commonly conserved in the sequences of EGCases of not only prokaryotes but also eukaryotes.

**EXPERIMENTAL PROCEDURES**

**Materials**

Jellyfish (*C. nozakii*) were collected at Nishinoura in Wakayama, Japan, and stored at −80 °C until use. GM1 was prepared from crude bovine brain ganglioside using sialidase-producing bacteria, *Pseudomonas* sp. YF-2, by the method described in Ref. 12. GQ1b, GT1b, and globoside were obtained from Iatron Laboratories Inc. (Tokyo, Japan), and stored at 2 °C until use. GD1a, GD1b, GD3, GM3, asialo-GM1, lactosylceramide (LacCer), galactosylceramide, and glucosylceramide were from Wako Pure Chemical Industries (Osaka, Japan). Synthetic lactoside, 2-N-dodecanoyl-4-nitrophenoxy-β-d-glucoside, was kindly donated from Dr. T. Yamagata (Japan Institute of Leather Research). Lyso-GM1 was prepared by digestion of GM1 with sphingolipid ceramide N-deacylase (13) by the...
method described in Ref. 14. Triton X-100 and Lubrol PX were obtained from Sigma and Nacalai Tesque Inc. (Kyoto, Japan), respectively. A precoated Silica Gel 60 TLC plate was obtained from Merck. All other reagents were of the highest purity available.

**EGCase Assay**

The reaction mixture contained 10 nmol of GM1 and an appropriate amount of the enzyme in 20 μl of 25 mM sodium acetate buffer, pH 5.0, containing 0.2% (v/v) Triton X-100. Following incubation at 37 °C for the times indicated, the reaction was stopped by heating in a boiling water bath for 5 min. The reaction mixture was evaporated to dryness, redissolved in 10 μl of 50% methanol, and applied to TLC plates, which were then developed with chloroform, methanol, 0.2% CaCl₂ (2/3/1, v/v/v). For determination of hydrolysis of polysialogangliosides, the TLC plates were developed with chloroform, methanol, 0.2% CaCl₂ (2/3/1, v/v/v). GSLs and oligosaccharides were visualized by spraying the TLC plates with orcinol-H₂SO₄ reagent and scanning them with a Shimadzu CS-9300 chromatoscanner with the reflectance mode set at 540 nm. The extent of hydrolysis was calculated as follows: hydrolysis (%) = (peak area for oligosaccharide released) × 100/(peak area for remaining substrate) × (peak area for oligosaccharide released). One unit of the EGCase was defined as the amount of enzyme that catalyzed the release of 1 μmol of GM1/min under the conditions described above. The hydrolysis of 2-N-dodecanoylamino-4-nitrophenyl-β-D-lactoside was examined according to the method described by Miura et al. (15).

**Purification of EGCase from the jellyfish, C. nozakii**

**Step 1: 80% Ammonium Sulfate Precipitation—**Jellyfishes (2.5 kg) were homogenized in a Waring blender and centrifuged at 8,000 rpm for 30 min. The supernatant was then saturated with ammonium sulfate. After standing overnight, the precipitate was dissolved in 500 ml of 20 mM sodium acetate buffer, pH 5.0, containing 0.2% Lubrol PX. After gentle stirring overnight, the supernatant solution (500 ml) was subjected to further purification procedures.

**Step 2: Phenyl-Sepharose Fast Flow Chromatography—**The enzyme solution obtained above was applied to a phenyl-Sepharose 6 Fast Flow column (75 ml; Amersham Pharmacia Biotech) equilibrated with 20 mM sodium acetate buffer, pH 5.0, containing 2 μM NaCl and 0.2% Lubrol PX. After washing the column, the enzyme was eluted with the same buffer saturated with ammonium sulfate. The enzyme was passed through the column, while contaminating proteins (molecular mass in parentheses) were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (20.1 kDa).

**Step 3: SP-Sepharose Fast Flow Chromatography—**The active fractions from the phenyl-Sepharose chromatography (300 ml) were subjected to chromatography using a SP-Sepharose Fast Flow column (55 ml; Amersham Pharmacia Biotech) previously equilibrated with 20 mM sodium acetate buffer, pH 5.0, containing 0.1% Lubrol PX, at a flow rate of 1 ml/min using a BPLC-600FC HPLC system (Yamazen Co., Osaka, Japan), and the effluent was fractionated into each 10-ml portion. After the column was washed with 20 mM sodium acetate buffer, pH 5.0, the enzyme was eluted from the column with 20 mM sodium acetate buffer, pH 5.0, containing 1% Lubrol PX.

**Step 4: Chelating Sepharose Chromatography—**The eluted enzyme solution (250 ml) was applied to a HiTrap Heparin column (chelated with Cu²⁺, 5 ml; Amersham Pharmacia Biotech), equilibrated with 20 mM sodium acetate buffer, pH 5.0, containing 0.1% Lubrol PX at a flow rate of 5 ml/min using a P.S.L.C. system (Amersham Pharmacia Biotech), and 10-ml fractions were collected. After washing the column with the same buffer, the enzyme was eluted from the column with a linear gradient of 0–0.4 M NaCl in the same buffer at a flow rate of 5 ml/min. Fractions each of 10 ml were collected, and those containing enzyme activity were pooled and dialyzed against 20 mM sodium acetate buffer, pH 6.0.

**Step 5: CM-5PW Chromatography—**The dialyzed enzyme solution (195 ml) was applied to a TSKgel CM-5PW column (5 × 50 mm; TOSOH Co., Tokyo, Japan) that had been equilibrated with 20 mM sodium acetate buffer, pH 5.0, containing 0.1% Lubrol PX, at a flow rate of 1 ml/min using the BioCAD SPRINT system (PerSeptive Biosystems, Inc.). The column was washed with the same buffer and developed with a linear NaCl gradient from 0 to 0.1 M in the same buffer. The fractions containing the enzyme were concentrated, and the buffer was replaced with 20 mM sodium acetate buffer, pH 6.0, using a centrifugal concentrator (Centriprep-10, Amicon, Inc.).

**Step 6: Heparin-Sepharose Chromatography—**The concentrated enzyme (21 ml) was applied to a HiTrap Heparin column (1 ml; Amersham Pharmacia Biotech) previously equilibrated with 20 mM sodium acetate buffer, pH 5.0, containing 0.1% Lubrol PX at a flow rate of 1 ml/min. The enzyme was washed through the column, while contaminating proteins were adsorbed and eluted with 1 M NaCl in the same buffer.

**Superdex 200HR Gel Filtration of the Purified EGCase**

100 μl of the purified enzyme was applied to a Superdex 200HR column (10 × 300 mm; Amersham Pharmacia Biotech), which had been equilibrated with 20 mM sodium acetate buffer, pH 6.0, containing 0.3% Lubrol PX and 0.1 M NaCl at a flow rate of 0.5 ml/min using the BioCAD system, and fractions of 1.0 ml each were collected and subjected to the assay of EGCase.

**Protein Assay and SDS-PAGE**

Measurement of protein was determined by the bicinchoninic acid protein assay (Pierce) with bovine serum albumin as the standard. SDS-PAGE was carried out according to the method of Laemmli (16). The proteins on SDS-PAGE were visualized by a silver-staining solution (17) and determined with a Shimadzu CS-9300 chromatoscanner with the refraction mode set at 540 nm.

**Native PAGE**

The purified EGCase was applied onto 4–20% Tris-glycine gel (NOVEX). A duplicate gel was cut into 3-mm slices. Each slice was crushed in 150 μl of 20 mM sodium acetate buffer, pH 5.0, containing 0.1% Triton X-100. After centrifugation at 15,000 rpm for 5 min, an aliquot (10 μl) of the supernatant of the extract was assayed for enzyme activity using GM1 as the substrate. The reaction mixture was incu-
bated at 37 °C for 18 h. The extent of hydrolysis was determined as described above.

**Lectin Blotting**

The purified enzyme was subjected to SDS-PAGE under reducing conditions and then transferred onto polyvinylidene difluoride membranes (Hybond-P; Amersham Pharmacia Biotech). The membranes were blocked with 1% bovine serum albumin in TBS (20 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl) for 1 h with gentle shaking. For specific detection of glycan moieties, the membranes were treated with 1 mg/ml of horseradish peroxidase-labeled lectins (Seikagaku Co., Tokyo, Japan) in the same buffer for 1 h at room temperature. After washing three times with TBS containing 0.1% Tween 20 for 10 min, the membranes were incubated in ECL Plus solution (Amersham Pharmacia Biotech) and determined with a STORM (Molecular Dynamics, Inc., Sunnyvale, CA).

**Glycopeptidase F Treatment**

The purified enzyme was denatured in boiling water bath for 3 min in 5 μl of 50 mM Tris-HCl buffer, pH 8.6, containing 0.5% SDS and 0.75% β-mercaptoethanol. The denatured enzyme was then incubated at 37 °C for 18 h with 0.5 milliunits of glycopeptidase F (Takara Shuzo Co., Shiga, Japan) in 20 μl of the same buffer containing 1% Nonidet P-40.

**Measurement of Other Enzymes**

Exoglycosidases and proteases were assayed using p-nitrophenyl glycoside (18) and Azocoll (19), respectively, as the substrates. Neuraminidase was assayed using 4-methylumbelliferyl-N-acetylneuraminic acid as substrate. The activities of sphingomyelinase and ceramidase were measured using C12-4-nitrobenzo-2-oxa-1,3-diazole-sphingomyelin and C12-4-nitrobenzo-2-oxa-1,3-diazole-ceramide, respectively, by the method described in Refs. 14 and 20.

**Amino Acid Microsequencing**

The purified enzyme was treated with glycopeptidase F and then concentrated using a Y-shaped gel (160 × 160 × 2 mm) modified form of a funnel-shaped one (21). Two protein bands (45 and 46 kDa) were visualized by staining with Coomassie Brilliant Blue, and thus each band was cut out separately, reduced with dithiothreitol, and loaded again on a well of a normal SDS-PAGE. After electrophoresis, the gel was blotted on a polyvinylidene difluoride membrane (ProBlott, PE-Biosystems) and stained with Coomassie Brilliant Blue. The 45- and 46-kDa protein bands were cut out and treated in situ with lysylendopeptidase AP-1 (Wako Pure Chemical Industries). Peptides released from the membrane were fractionated with a reversed-phase HPLC column of C8 (RP-300, 1.0 × 100 mm; PE-Biosystems) and sequenced with a pulse-liquid phase protein sequencer (Procise 492 cLc, PE-Biosystems).
As described under “Experimental Procedures.”

Globo series

pH 3.0, containing 0.4% Triton X-100. The curve.

NAAYCCNGARACNCA-3

strands were designed. Sense oligonucleotide primer, NSout (5'-ATNCGYAG-

FIG. 4. General properties of the jellyfish EGCase. The pH dependence of EGCase activity was assayed using GM1 (A) and asialo-GM1 (B) as the substrates as described under “Experimental Procedures.” [ ], KCl-HCl buffer, pH 1.5–2.0; ▲, glycine-HCl buffer, pH 2.0–3.0; ●, sodium acetate buffer, pH 3.0–6.0; ■, sodium phosphate buffer, pH 6.0–8.0. C, effects of detergents on EGCase. The activity was determined using GM1 as the substrate as described under “Experimental Procedures.” O, Triton X-100; ▲, Lubrol FX; ▲, taurodeoxycholate. D, Lineweaver-Burk plots for the action of the jellyfish EGCase on GM1. GM1 at the indicated concentrations was incubated with 20 n mole of the enzyme at 37 °C for 60 min in 20 μl of 25 mM sodium acetate buffer, pH 3.0, containing 0.4% Triton X-100. The inset shows the substrate saturation curve.

TABLE II

Specificity of EGCase from the jellyfish, C. nozakii

Various GSLs were incubated at 37 °C for 30 min (condition I) or 16 h (condition II) with 0.1 milliunits of the enzyme in 20 μl of 25 mM sodium acetate buffer, pH 3.0, containing 0.2% Triton X-100. GSLs and oligosaccharides were separated by TLC and visualized by spraying with orcinol-H2SO4 reagent and determined as described under “Experimental Procedures.”

| Class and name | Structure | Condition I | Condition II |
|----------------|-----------|-------------|--------------|
| Ganglio series | NeuAcα2–8 NeuAcα2–3Galβ1–13GalNAcβ1–4 NeuAcα2–8 NeuAcα2–3Galβ1–13Galβ1–1′ Cer | 31.2 | 100 |
| GT1b | NeuAcα2–3Galβ1–13GalNAcβ1–4 NeuAcα2–8 NeuAcα2–3Galβ1–1′ Cer | 56.9 | 95.2 |
| GD1a | NeuAcα2–3Galβ1–13GalNAcβ1–4 NeuAcα2–8 NeuAcα2–3Galβ1–1′ Cer | 15.8 | 90.7 |
| GD1b | Galβ1–3GalNAcβ1–4 NeuAcα2–8 NeuAcα2–3Galβ1–1′ Cer | 17.7 | 90.2 |
| GD3 | NeuAcα2–8 NeuAcα2–3Galβ1–1′ Cer | 11.3 | 98.4 |
| GM1 | Galβ1–3GalNAcβ1–4 NeuAcα2–3Galβ1–1′ Cer | 29.2 | 77.7 |
| GM3 | NeuAcα2–3Galβ1–1′ Cer | 12.7 | 97.4 |
| Asialo-GM1 | Galβ1–3GalNAcβ1–4 Galβ1–1′ Cer | <5 | 49.1 |
| Globo series | GalNAcβ1–3Galα1–4 Galβ1–1′ Cer | 0 | <5 |
| Lacto series | GalCer | Galβ1–1′ Cer | <5 | 25.7 |
| Cerebroside | GalCer | Galβ1–1′ Cer | 0 | 0 |
| GlcCer | GalCer | Galβ1–1′ Cer | 0 | 0 |
| Lyso-GSL | GalNAcβ1–3GalNAcβ1–4 NeuAcα2–3 Galβ1–1′ Cer | 0 | 0 |
| Lyso-GM1 | Galβ1–3GalNAcβ1–4 NeuAcα2–3 Galβ1–1′ Cer | 0 | 0 |

First Strand cDNA Synthesis

To obtain the partial cDNA sequence encoded the EGCase, we synthesized the first strand cDNA from the jellyfish. Total RNA and mRNA were obtained from 4 g of the tentacle portion of the jellyfish using Sepasol-RNA I (Nacalai Tesque, Japan) and FastTrack 2.0 kit (Invitrogen), respectively. First strand cDNA was synthesized from 2 μg of mRNA using an AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Life Sciences, Inc.).

Isolation of a Partial cDNA Encoding the EGCase

Based on the amino acid sequences of peptides derived from the purified EGCase, degenerate primers of both sense and antisense strands were designed. Sense oligonucleotide primer, NSout (5'-CTGCGGATTCAGGGGGTATTT-3’), was synthesized from the N-terminal peptide sequences, and for the second round nested PCR, NSin (5'-CCNGA-RACNCARYT-3') was designed. Antisense primers, 559Aout (5'-ATHTAYAGNNNGCGYTT-3') and 547Aout (5'-CCRAYATYATYATYAT-3'), were synthesized based on the internal amino acid sequences of C-559 and C-547/562, respectively. For the second round nested PCR, 559Ain (5'-CGYTNCCRAGIAHTA-3') and 547Ain (5'-ATNCGYAG-NGGNGNCC-3') were used. The first round PCR was performed using two sets of the primers (NSout and 559Aout; NSout and 547Aout) with first strand cDNA as a template in a GeneAmp PCR System 9700 (PE-Biosystems) using AmpliTaq Gold (PE-Biosystems). The second round nested PCR was primed with each first round PCR product as a template using nested primers (NSin and 559Ain, NSin and 547Ain). The PCR products were cloned into pGEM T-easy vector (Promega), and their DNA sequences were determined.

To obtain the 5'- and 3'-end, the SMART RACE cDNA Amplification kit (CLONTECH) was used. 5'-RACE first strand cDNA was primed from 0.75 μg of mRNA with Superscript II reverse transcriptase (Life Technologies, Inc.) using a SMART II oligonucleotide and a 5'-RACE cDNA synthesis primer. The 5'-end of the cDNA was amplified by PCR using gene-specific primer 1 (5'-CTGCGGATTCAGGGGGTATTT-GTTTCA-3') and a universal primer mix and 5'-RACE first strand cDNA as a template, using the Advantage 2 PCR kit (CLONTECH). The 3'-RACE first strand cDNA was primed using 3'-RACE cDNA synthesis primer. The 3'-end of the cDNA was obtained by PCR using a universal
primer mix and gene-specific primer 2 (5'-CTTGGTATGATGCTGC-CAGGCTATGTGC-3') and 3'-RACE first strand cDNA as a template. The 5'- and 3'-RACE PCR products were cloned into the pGEM T-easy vector, and their DNA sequences were determined.

**Construction of a Full-length cDNA**

An upstream (EGC-N) and a downstream primer (EGC-C), each containing an EcoRI site, were designed from the N and C terminus of the open reading frame, respectively. A full-length cDNA was prepared by PCR using these primers and 5'-RACE first strand cDNA as a template with Pyrobest DNA polymerase (Takara Shuzo Co., Japan). After digestion with EcoRI, a PCR product was cloned into a pBlue-script II SK+ (STRATAGENE). 14 clones obtained were sequenced. The full-length PCR product was also directly sequenced to minimize potential PCR artifacts.

**Other Methods**

Nucleotide sequences were determined on both strands by the dideoxynucleotide chain termination method with a Bigdyce Terminator Cycle Sequencing Ready Reaction Kit (PE-Biosystems) and a DNA Sequencer (model 377A, PE-Biosystems). The nucleotide and amino acid sequences were evaluated using the DNASIS computer program developed by Hitachi Software Engineering.

**RESULTS**

**Purification of EGCase from the Jellyfish, C. nozakii**—EGCase was purified from extracts of the jellyfish by ammonium sulfate precipitation, followed by sequential chromatography with phenyl-Sepharose 6 FF, SP-Sepharose FF, HiTrap Chelating, TSKgel CM-5PW, and HiTrap Heparin. As shown in Table I, the enzyme was purified 1,260-fold with 20.5% recovery. The final preparation showed a single, but broad, protein band after staining with silver solution under both reducing and nonreducing conditions (data not shown). Among the metal ions examined, Hg2+, which are potent inhibitors of microbial EGCases, had no significant effects on the activity at 2 mM, whereas Cu2+ and Zn2+, which are potent inhibitors of glycosylated with N-glycans, possibly possessing mannosyl or glucosyl residues.

Properties of the Jellyfish EGCase—Jellyfish EGCase exhibited maximal activity around pH 3.0 with GM1 (Fig. 4A) and asialo GM1 (Fig. 4B) as the substrates. The EGCase required detergents for hydrolysis of GSLs. The optimal concentrations of Triton X-100 and Lubrol PX were 0.2 and 0.4%, respectively. Taurodeoxycholate strongly inhibited the enzyme activity (Fig. 4C), and SDS completely inhibited the EGCase activity at a concentration of 0.1% (data not shown). Among the metal ions examined, Hg2+ completely inhibited the enzyme activity at 2 mM, whereas Cu2+ and Zn2+, which are potent inhibitors of microbial EGCases, had no significant effects on the enzyme activity at the final concentration of 5 mM. Li+, K+, Mn2+, Ni2+, Ca2+, Mg2+, and EDTA had no significant effects on the activity at 5 mM. Fig. 4D shows the Lineweaver-Burk plot and substrate saturation curve (inset). Apparent Km and Vmax values of this enzyme for GM1 were estimated to be 0.35 mM and 4.4 units/mg, respectively.

**Substrate Specificity**—Table II summarizes the extent of the hydrolysis of various GSLs by the jellyfish EGCase under conditions that may reflect the relative initial reaction velocity (condition I) and for determining the degree of hydrolysis after exhaustive digestion (condition II). Of all GSLs tested, gangliosides appeared to be good substrates under both conditions, whereas other co-eluted bands were observed (Fig. 2A). The purified enzyme also showed a single protein band on the native PAGE that corresponded to the active fractions (Fig. 2B). These results indicated that the 51-kDa protein identified on SDS-PAGE was definitely the EGCase. The protein was stained with horseradish peroxidase-labeled concanavalin A, but hardly with *Ricinus communis* agglutinin I (RCA I) and wheat germ agglutinin (WGA) (Fig. 3A), and converted to 45- and 46-kDa bands on SDS-PAGE after exhaustive digestion with glycopeptidase F (Fig. 3B). These results showed that the jellyfish EGCase was glycosylated with N-glycans, possibly possessing mannosyl or glucosyl residues.

![Figure 5](image-url)

**Peptide sequences of EGCase from the jellyfish, C. nozakii**—Peptide sequences were determined by Edman sequencing.

| Peptide Sequence | N terminal |
|------------------|------------|
| C-549/C-552      | DOASLISVPN | PETQQLX |
| Lysylendopeptidase digest | C-547/C-552 | VPYYSDGEYDDTSFXVE |
| C-558            | VPGGDAYRDSVLHVHYYP |
| C-559            | SIVGEYLIPEFGDIYRDFXL |

![Figure 6](image-url)
A Novel Acidic Endoglycosidase from C. nozakii

**Fig. 6.** Nucleotide and deduced amino acid sequences (A) and hydropathy plot (B) of the jellyfish EGCase. A, the deduced amino acid sequence is shown in one-letter amino acid code below the nucleotide sequence. Amino acid residues are numbered beginning with the first methionine, and the translation termination codon is denoted by an asterisk. The signal sequence is in boldface type. Amino acids determined by peptide sequencing are underlined. Potential N-linked glycosylation sites are boxed, and the putative active site (NEP sequence) is double-boxed. The double underline in the 3’-untranslated region indicates the polyadenylation site. B, the deduced amino acid sequence of the EGCase was analyzed by the method of Kyte and Doolittle for hydropathy plotting (23). Amino acid residues are numbered beginning with the first methionine.
GT1b being most favored by the enzyme, followed by QG1b and GM1. Neutral GSLs, such as asialo-GM1 and LacCer, were hydrolyzed considerably more slowly than gangliosides. Gb4Cer was only slightly hydrolyzed. No hydrolysis was observed for cerebrosides, lyso-GM1, 2-N-dodecanoylamino-4-nitrophenyl-3.5-lactoside, sphingomyelin, and ceramide even under condition II. Fig. 5 shows the time course for hydrolysis of GM1, GT1b, and LacCer by the jellyfish enzyme and Rhodococcus EGCase II. It was found that the jellyfish enzyme hydrolyzed GT1b much more rapidly than did EGCase II (Fig. 5B), although the reaction velocity for GM1 by both enzymes was almost the same (Fig. 5A). LacCer, by contrast, was hydrolyzed by the jellyfish enzyme considerably more slowly compared with the microbial enzyme (Fig. 5C).

Amino Acid Sequence of the Purified EGCase—The 45- and 46-kDa polypeptides generated after glycopeptidase F treatment were separately digested with lysylendopeptidase and subjected to amino acid microsequencing. The N-terminal amino acid sequences of both native polypeptides were also determined. Table III shows the partial amino acid sequences of the EGCase, in which C-549, C-547, and C-558 were obtained from the 45-kDa polypeptide, and C-552, C-562, and C-559 were obtained from the 46-kDa polypeptide. The N terminus as well as one of the internal amino acid sequences of both polypeptides were completely identical (Table III). Furthermore, the peptide map of the 45-kDa polypeptide after digestion with lysylendopeptidase was almost identical to that of the 46-kDa one (data not shown). These results indicated that both polypeptides were derived from the same 51-kDa protein.

Cloning of the EGCase cDNA—PCR was performed using two sets of primers (NSout and 559Aout, NSout and 547Aout) and the first strand cDNA as the template. The first round PCR product was then used as a template for nested PCR amplification using nested primers (NSin and 559 Ain, NSin and 547 Ain). As a result, the 620- and 820-bp PCR products were specifically amplified. The former contained C-549/552, C-558, and the later contained C-549/552, C-558, and C-547/562 (Table III). To obtain the 5′- and 3′-terminal segments of the cDNA, 5′- and 3′-RACE were performed. The 650-bp PCR product was amplified from the 5′-RACE. The sequences of the overlapping cDNA fragments contained an initiation codon in agreement with the Kozak rule (22) and a termination codon. The full-length cDNA amplified by PCR was cloned into pBlue-script II SK+ vector, and 14 independent clones were sequenced. As a result, the open reading frame of eight clones showed the identical nucleotide sequences as shown in Fig. 6A (J-EGCase; AB047321). However, other six clones showed minor variation, in which T at position 164, C at 311, T at 526, and C at 794 were replaced with C, T, C, and T, respectively, with the result that Leu172 could be replaced with Pro (J-EGCase P; AB047322). This variation was also confirmed by direct cycle sequencing of the full-length PCR product, suggesting that two variants of EGCase gene are present in the jellyfish.

DNA and Deduced Amino Acid Sequence of the EGCase—Fig. 6A shows the nucleotide and the deduced amino acid sequences of the jellyfish EGCase. The 1,509-bp open reading frame encodes 503 amino acid residues containing six potential N-glycosylation sites. A polyadenylation site was found in the 3′-untranslated region, indicating that the EGCase is not derived from symbiotical bacteria or actinomycetes. From the deduced amino acid sequence, the molecular mass and pI of the EGCase were calculated to be 54,641 and 5.61, respectively. The deduced amino acid sequence contained all the sequences obtained from the purified enzyme after digestion with N-glycosyltransferase of the jellyfish enzyme could be located in lysosomes. The jellyfish enzyme was not affected by Zn2+ and Cu2+ at a concentration of 5 mM, while other EGCases/CGases were strongly inhibited by these metal ions (4, 6–9). Further, the hydrolysis of GM1 by the jellyfish enzyme was enhanced by Triton X-100 but strongly inhibited by taurodeoxycholate.

DISCUSSION

Purified EGCase from the jellyfish, C. nozakii, showed several unique characteristics compared with other EGCases and CGases reported so far. Lectin blotting using concanavalin A as well as glycopeptidase F treatment of the purified jellyfish EGCase showed that the enzyme was glycosylated with Nglycans. The deduced amino acid sequence of the jellyfish EGCase indicated six potential N-glycosylation sites. By contrast, there have been no reports of the presence of glycans in other EGCases/CGases (4, 6–9). Second, the jellyfish EGCase showed a pH optimum at 3.0, while other EGCases/CGases were strongly inhibited by these metal ions (4, 6–9). Fourth, the reaction velocity for GM1 by both enzymes was almost the same (Fig. 5A). LacCer, by contrast, was hydrolyzed by the jellyfish enzyme considerably more slowly compared with the microbial enzyme (Fig. 5C). A polyadenylation site was found in the 3′-untranslated region, indicating that the jellyfish enzyme is the most acidic EGCases/CGases reported so far.
taurodeoxycholate activated both the microbial and leech enzymes (4, 7). Finally, the jellyfish enzyme hydrolyzed gangliosides, especially b-series polysialogangliosides, such as GT1b and GQ1b, much more rapidly than neutral GSLs such as LacCer and asialo-GM1, while both the microbial and leech enzymes hydrolyzed neutral GSLs much more rapidly than these polysialogangliosides (4, 7). Jellyfish EGCase did not hydrolyze lyso-GM1 under the experimental conditions we employed. On the other hand, the leech and Rhodococcus enzymes cleaved sphingosine glycans (4, 7), including lyso-GM1, suggesting that the whole ceramide structure is required for hydrolysis of substrates by the jellyfish EGCase but not by other enzymes.

We previously reported that the NEP sequence, the active site of family A cellulases, was also the putative active site of Rhodococcus EGCase II (10). The present study clearly shows that the NEP sequence was also completely conserved in the jellyfish EGCase. In addition, two regions at positions Leu131–Val137 and Tyr414–Gly420 of the deduced amino acid sequence of the jellyfish enzyme were also highly conserved in Rhodococcus EGCase, suggesting that these portions are also important for the catalytic and/or substrate-binding functions of EGCase.

It should be noted that EGCase activity was found in all species of jellyfish tested (Spirocodon saltator, Aequorea coerulescens, Aurelia aurita, Chrysaora melanaster, Rhopilema esculenta, and C. nozakii). In addition, the enzyme activity was also detected in a freshwater hydra, Hydra magnipapillata. Both jellyfish and hydra are members of the Cnidaria, a group of animals that arose very early in metazoan evolution. Some jellyfish including C. nozakii eat small fish that are digested in the gastro-vascular cavity by digestive enzymes such as proteases, lipases, and chitinases. Then digested particles are solubilized in vesicles/lysosomes. Further study is necessary to confirm the precise location of EGCase in the jellyfish and hydra.

This study is the first to isolate a full-length cDNA encoding a eukaryotic EGCase, which shows an extremely acidic pH optimum. The availability of the cDNA sequence of the EGCase should make it possible to isolate new EGCases/CGases from other invertebrates, and possibly vertebrates, and should help to define the physiological roles of EGCases/CGases in eukaryotes. The jellyfish EGCase efficiently hydrolyzed polysialogangliosides, which were somewhat resistant to hydrolysis by the EGCases and CGases reported previously, and will thus facilitate further research into polysialogangliosides.

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