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# Transglycosylation and Reverse Hydrolysis Reactions of Endoglycoceramidase from the Jellyfish, *Cyanea nozakii*[1]

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**Endoglycoceramidase (EGCase: EC 3.2.1.123) is an enzyme capable of cleaving the glycosidic linkage between oligosaccharides and ceramides in various glycosphingolipids. We report here transglycosylation and reverse hydrolysis reactions of EGCase from the jellyfish *Cynaea nozakii*. Various alkyl-GM1 oligosaccharides (alkyl-II$^3$NeuAcGgOse4) were synthesized when GM1 ganglioside was treated with the EGCase in the presence of 1-alkanols. Among various 1-alkanols tested, methanol was found to be the most preferential acceptor, followed by 1-hexanol and 1-pentanol. GM1 was the best donor, followed by GD1b and GT1b, when methanol was used as an acceptor. However, neither globoside nor glucosylceramide was utilized by the enzyme as a donor substrate. The enzyme transferred oligosaccharides from various glycosphingolipids to NBD-ceramide, a fluorescent ceramide, producing NBD-labeled glycosphingolipids. In addition to the transglycosylation reaction, the enzyme catalyzed the reverse hydrolysis reaction; lactose was condensed to ceramide to generate lactosylceramide in the presence of the enzyme. These results indicate that the jellyfish enzyme will facilitate the synthesis of various neoglycoconjugates and glycosphingolipids.**

**Key words: endoglycoceramidase, fluorescence-labeling, glycosphingolipids, reverse hydrolysis, transglycosylation.**

Glycosphingolipids (GSLs), amphipathic compounds consisting of oligosaccharides and ceramides, are characteristic components of plasma membranes. GSLs 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 to form microdomains on the ectoplasmic membrane (*2*). These lipid microdomains assemble transmembrane-receptors and signaling molecules such as Src-family kinases and G-proteins on their inner surface, and thus possibly modulate the membrane trafficking and inter-/intracellular signaling (*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. (*5*), and later found in the bacterium *Corynebacterium* sp. (*6*). A similar enzyme, called ceramide glycanase (CGase), has been found in leeches (*7*), earthworms (*8*), and clams (*9*).

Previously, 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 from *Rhodococcus* sp. C9 (*11*). Analysis using site-directed mutagenesis revealed that the NEP sequence of both M-777 and C9 EGCases is essential for the expression of activity. Recently, we reported the cDNA cloning of a novel EGCase (J-EGCase) from the jellyfish *Cyanea nozakii* and revealed that the NEP sequence is conserved in not only prokaryotic EGCases but also this eukaryotic enzyme (*12*), suggesting that EGCases and cellulases are derived from the same ancestral gene. J-EGCase shows the most acidic pH optimum (pH 3.0) among EGCases/CGases reported so far and exhibited the highest activity toward sialic acid–containing GSLs (gangliosides) (*12*).

In this paper, we report the enzymatic synthesis of alkylglycosides and fluorescence-labeled GSLs using the transglycosylation reaction of J-EGCase. We also describe the reverse hydrolysis (condensation) reaction of J-EGCase; lactose was condensed to ceramide, yielding lactosylceramide (LacCer).

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Abbreviations: Cer, ceramide; CGase, ceramide glycanase; EGCase, endoglycoceramidase, GM1, gangliotetraosylceramide [Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1'Cer], GSL(s), glycosphingolipid(s), LacCer, lactosylceramide [Galβ1-4Glcβ1-1'Cer], MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NBD, 4-nitrobenzo-2-oxa-1,3-diazole, SCDase, sphingolipid ceramide *N*-deacylase; TLC, thin-layer chromatography.

## MATERIALS AND METHODS

*Materials*—A mixture of crude gangliosides was prepared from bovine brains as described in Ref. *13*. GM1 was prepared from crude gangliosides using a sialidase-producing bacterium, *Psedomonas* sp. YF-2, by the method described in Ref. *14*. GT1b, GD1b, asialo GM1, lactosylceramide (LacCer), glucosylceramide, and 1-alkanols were purchased from Wako Pure Chemical Industries (Osaka). Globoside was obtained from Iatron Lab. (Tokyo). Bovine brain ceramide was from Matreya (USA). Lactose and Triton X-100 were obtained from Sigma (USA). A precoated Silica Gel 60 TLC plate was obtained from Merck (Germany). [$^{14}$C]Ceramide ([$^{14}$C]C16:0/d18:1, 1.85 GBq/mmol) and [$^{14}$C]GM1 (1.85 GBq/mmol) were prepared by the condensation reaction of sphingolipid ceramide *N*-deacylase as described in Ref. *15* C12-NBD-ceramide (NBD-C12:0/d18:1) and C12-NBD-GM1 (NBD-C12:0/d18:1) were also prepared by use of sphingolipid ceramide *N*-deacylase as described in Refs. *16* and *17*, respectively. C6-NBD-Ceramide (NBD-C6:0/d18:1) was purchased from Matreya (USA). All other reagents were of the highest purity available.

*Preparation of J-EGCase and Definition of an Enzyme Unit*—EGCase (J-EGCase) was purified from the jellyfish *Cyanea nozaki* by the method described in Ref. *12*. The enzyme activity was assayed using GM1 as the substrate in the presence of 0.2% of Triton X-100 at pH 3.0 by the method described previously (*12*). One unit of the EGCase was defined as the amount of enzyme which catalyzes the hydrolysis of 1 μmol of GM1 per min under the conditions. A value of $10^{-3}$ units of enzyme was expressed as 1 milliunit in this study.

*Synthesis of Alkyl-Glycosides Using Transglycosylation Reaction of J-EGCase*—Transglycosylation reaction of the enzyme was carried out as follows: the reaction mixture contained 20 nmol of GM1 and 0.5 milliunits of the enzyme in 40 μl of 25 mM sodium acetate buffer, pH 3.0, containing 0.2% Triton X-100 and an appropriate amount of 1-alkanols. After incubation at 37°C for the times indicated, 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% $CaCl_2$ (4:4:1, v/v). GSLs and oligosaccharides were visualized by spraying the TLC plates with orcinol-$H_2SO_4$ reagent and quantified by use of a Shimadzu CS-9300 chromatoscanner with the reflectance mode set at 540 nm.

*Synthesis of NBD-Labeled GSLs by J-EGCase*—The reaction mixture contained 20 nmol of GM1, an appropriate amount of C6- or C12-NBD-ceramide, and 0.1 milliunits of the enzyme in 20 μl of 25 mM sodium acetate buffer, pH 3.0, containing 0.2% Triton X-100. After incubation at 37°C for the times indicated, the mixture was dried, redissolved in 15 μl of chloroform/methanol (2:1, v/v), and applied to TLC plates, which were then developed with chloroform/methanol/0.2% $CaCl_2$ (5:4:1, v/v). Transglycosylation products and remaining NBD-ceramides were quantified with a Shimadzu CS-9300 chromatoscanner (excitation 475, emission 525 nm).

*Synthesis of Lactosylceramide Using Reverse Hydrolysis (Condensation) Reaction of J-EGCase*—The reaction mixture contained 5 mg of ceramide, 10 mg of lactose, and 2.5 milliunits of J-EGCase in 100 μl of 25 mM sodium acetate buffer, pH 3.0, containing 2% Triton X-100. After incubation at 37°C for 40 h, the reaction mixture was evaporated to dryness, redissolved in 1 ml of chloroform/methanol (95:5, v/v), and applied to a Sep-Pak Plus Silica cartridge (Waters, USA) previously equilibrated with chloroform/methanol (95:5, v/v). After washing the column with 10 ml of the same solvent, lactosylceramide (LacCer) was eluted with 10 ml of chloroform/methanol (2:1, v/v). Fractions of 2 ml were collected, and the synthesis of LacCer was analyzed by TLC using chloroform/methanol/0.2% $CaCl_2$ (5 4:1, v/v) as a developing solvent.

*Preparation of Methyl-GM1 Oligosaccharide for MALDI-TOF-MS*—A mixture of 100 nmol of GM1 and 2 5 milliunits of the enzyme in 200 μl of 25 mM sodium acetate buffer, pH 3.0, containing 0.2% Triton X-100 in the presence of 25% methanol was incubated at 37°C for 16 h, then evaporated to dryness, redissolved in 10 ml of $H_2O$, and applied to a Sep-Pak Plus C18 cartridge (Waters, USA) previously equilibrated with $H_2O$. Both methyl-GM1 oligosaccharide and GM1 oligosaccharide passed through the column, while the unhydrolyzed GM1 was adsorbed and subsequently eluted with chloroform/methanol (2:1, v/v). The pass-through fractions were dried and used as a sample for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis as described below.

*MALDI-TOF-MS Analysis*—Equal amounts of 10 μM aqueous solution of sample and matrix solution (10 mg/ml of 2,5-dihydroxybenzoic acid in 1.1 mixture of methanol/water) were mixed, and 1 μl of the mixture was loaded onto the sample plate and allowed to dry. The sample plate was then loaded into the Voyager Vestec apparatus (PerSeptive Biosystem) in a predetermined position. Nitrogen laser (337 nm) was used for the ionization.

## RESULTS

*Transglycosylation Reaction of J-EGCase*—New orcinol-$H_2SO_4$ positive spots having different $R_f$s were observed on TLC when the purified J-EGCase was incubated with GM1 ganglioside in the presence of 1-alkanols, besides those of the GM1-oligosaccharide (hydrolysis product) and GM1 (remaining substrate) (Fig. 1A) These newly generated products seemed to be alkyl-glycosides, which could have been synthesized by transfer of oligosaccharide from GM1 to a 1-alkanol. To confirm whether the J-EGCase catalyzes the transglycosylation reaction using ceramide as an acceptor, the generation of [$^{14}$C]GM1 was examined when the purified enzyme was incubated with GM1 and [$^{14}$C]ceramide ([$^{14}$C]C16:0/d18:1). The newly synthesized [$^{14}$C]GM1 was detected on TLC with an imaging analyzer BAS 1000 (Fig. 1B, lane 1). These results indicate that the J-EGCase transfers oligosaccharide from GM1 to 1-alkanols and ceramide.

The reaction product of J-EGCase with GM1 in the presence of methanol was analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) using 2,5-dihydroxybenzoic acid as the matrix. Intense peaks $[M-H]^-$ were observed at $m/z$ 997 and 1,011, which may be attributed to GM1-oligosaccharide (II$^3$NeuAcGgOse4) and methyl-GM1 oligosaccharide (methyl-II$^3$NeuAcGgOse4), respectively (Fig 2). The former is the hydrolysis product of GM1 by the enzyme, and the latter is

consistent with the total mass of GM1 oligosaccharide with a methyl group.

The acceptor specificity was examined under two different conditions using the purified J-EGCase; condition (I) may reflect the relative initial reaction velocity, and condition (II) the exhaustive reaction after prolonged incubation (Table I). The degree of hydrolysis of GM1 by the enzyme attained 69.4 and 93.0% under conditions (I) and (II), respectively. As shown in Table I, methanol at the concentration of 20% was found to be the most favorable acceptor under both conditions. under condition (I), 18.3% of GM1 was converted to methyl-GM1 oligosaccharide; and under condition (II), 27.5%. 1-Pentanol, 1-hexanol and 1-octanol were also estimated to be good acceptors under condition (I), although under condition (II) the apparent generation of these three alkyl-glycosides rather decreased. This result suggests that the reaction velocity for hydrolysis of alkyl-glycosides could be faster than that of their synthesis if the newly generated alkyl-glycosides had a chain length of five or more carbons. It should be noted that 1-propanol strongly inhibited the both transglycosylation and hydrolysis reactions under the conditions employed (Table I).

The effects of concentration of various 1-alkanols on the transglycosylation activity were examined. The optimal concentration of methanol in the reaction mixture was found to be 25% (v/v), at which 29.6% of GM1 was con-

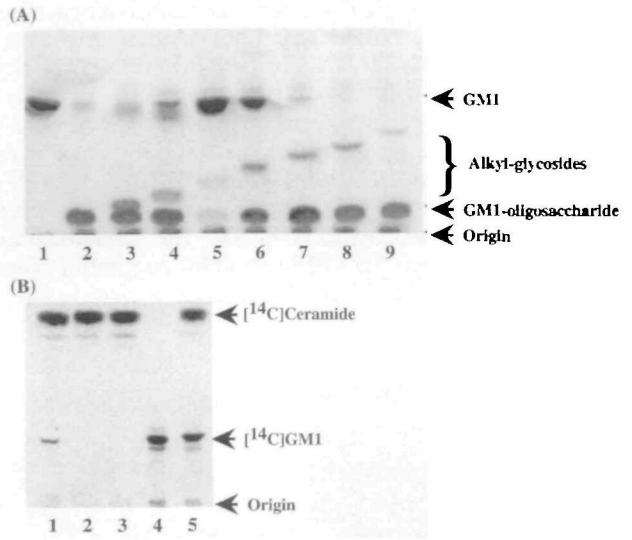


Fig. 1 **Transfer of II³NeuAcGgOse4 from GM1 to various 1-alkanols (A) and [¹⁴C]ceramide (B) by the action of J-EGCase.** (A) The reaction mixtures contained 20 nmol of GM1, 0 5 milliunits of enzyme, and various 1-alkanols at the concentration of 20% in 40 μl of 25 mM sodium acetate buffer, pH 3 0, containing 0 2% Triton X-100. After incubation at 37°C for 16 h, each reaction mixture was applied to a TLC plate, which was developed with chloroform/methanol/0 2% $CaCl_2$ (4 4 1, v/v) Carbohydrate-containing compounds were visualized by spraying the TLC plate with orcinol-$H_2SO_4$ reagent. Lane 1, GM1, lane 2, GM1 + J-EGCase; lane 3, GM1 + J-EGCase + methanol, lane 4, GM1 + J-EGCase + ethanol, lane 5, GM1 + J-EGCase + 1-propanol, lane 6, GM1 + J-EGCase + 1-butanol, lane 7, GM1 + J-EGCase + 1-pentanol, lane 8, GM1 + J-EGCase + 1-hexanol, lane 9, GM1 + J-EGCase + 1-octanol (B) TLC showing the [¹⁴C]GM1 generated after incubation of the enzyme with GM1 and [¹⁴C]ceramide ([¹⁴C]C16 0/d18·1) GM1 (10 nmol) was incubated with [¹⁴C]ceramide (200 pmol) in the presence of 0.1 milliunits of the enzyme in 25 mM sodium acetate buffer, pH 3 0, containing 0 2% Triton X-100 Lane 1, GM1 + [¹⁴C]ceramide + J-EGCase, lane 2, GM1 + [¹⁴C]ceramide, lane 3, [¹⁴C]ceramide, lane 4, [¹⁴C]GM1 (200 pmol), lane 5, [¹⁴C]GM1 (200 pmol) + J-EGCase.

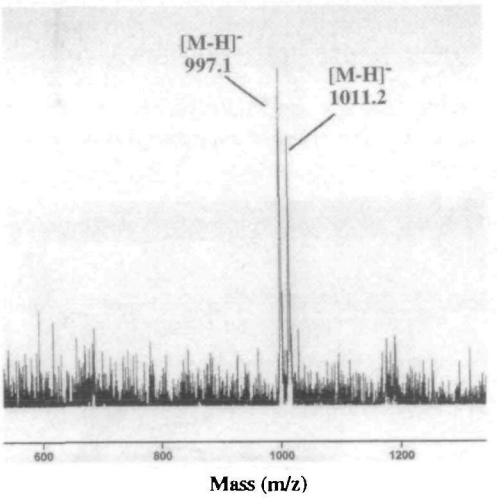


Fig 2 **Negative ion MALDI-TOF-MS spectrum of GM1 oligosaccharide and methyl-GM1 oligosaccharide.** A mixture of 100 nmol of GM1 and 2 5 milliunits of J-EGCase in 200 μl of 25 mM sodium acetate buffer, pH 3 0, containing 0 2% Triton X-100 in the presence of 25% methanol was incubated at 37°C for 16 h, then the reaction products were purified using Sep-Pak Plus C18 cartridge and analyzed by MALDI-TOF-MS as described in "MATERIALS AND METHODS" The mass ions at $m/z$ 997 1 and 1,011.2 are consistent with the molecular mass of GM1-oligosaccharide (II³NeuAcGgOse4) and methyl-GM1 oligosaccharide (methyl-II³NeuAcGgOse4), respectively

TABLE I. **Acceptor specificity for transglycosylation reaction of J-EGCase.**

| Acceptor | Alkyl-GM-1-oligosaccharide (%) | | GM1-oligosaccharide (%) | | Remaining GM1 (%) | |
|---|---|---|---|---|---|---|
| | I | II | I | II | I | II |
| Control | – | – | 69 4 | 93 0 | 30.6 | 7 0 |
| Methanol | 18.3 | 27 5 | 55.2 | 59.9 | 26.5 | 12 6 |
| Ethanol | 8 5 | 13 1 | 41 9 | 63.3 | 49.6 | 23.6 |
| 1-Propanol | 0.6 | 2 1 | 2 3 | 4.0 | 97 1 | 93 9 |
| 1-Butanol | 3.9 | 7 9 | 14 2 | 39.3 | 81 9 | 52 8 |
| 1-Pentanol | 15 3 | 9 3 | 76 1 | 88.9 | 8.6 | 1 8 |
| 1-Hexanol | 16.5 | 9 2 | 77 4 | 90.8 | 6.2 | 0 |
| 1-Octanol | 14 1 | 3.0 | 80 6 | 97.0 | 5.3 | 0 |

GM1 was incubated at 37°C for 1 h (condition I) or 16 h (condition II) with 0 5 milliunits of the enzyme in 40 μl of 25 mM sodium acetate buffer, pH 3.0, containing 0 2% Triton X-100 in the presence of 20% 1-alkanol. Carbohydrate-containing compounds were visualized by spraying with orcinol-$H_2SO_4$ reagent and determined as described under "MATERIALS AND METHODS."

verted to a methyl-GM1 oligosaccharide (Fig. 3A). The optimum concentration of 1-alkanol for the transglycosylation activity differed depending on the 1-alkanol used: for ethanol it was 12% with 11.4% yield of ethyl-GM1 oligosaccharide (Fig. 3B), and for 1-octanol 10–70% with 20% yield of octyl-GM1 oligosaccharide (Fig. 3C). Interestingly, both transglycosylation and hydrolysis reactions were inhibited by excess amounts of methanol and ethanol (Fig. 3, A and B), whereas no inhibitory effects were observed on either reaction when 1-octanol was used (Fig. 3C).

Donor specificities were also investigated using various GSLs in the presence of 25% methanol. As shown in Table II, gangliosides with mono- (GM1a), di- (GD1b), and tri- (GT1b) sialic acid residues appeared to be good donors: the yield of each methyl-oligosaccharide was 30.3, 22.9, and 16.5%, respectively. Neutral GSLs such as asialoGM1 and lactosylceramide (LacCer) were found to be poor donor substrates as compared to gangliosides (Table II). Neither transglycosylation nor hydrolysis reaction was observed for globoside (Gb4Cer) and glucosylceramide (GlcCer).

The optimum pH for the transglycosylation activity of J-EGCase was found to be around 3.0 when GM1 was incubated with the enzyme in the presence of 25% methanol. The optimum pH for the transglycosylation activity was coincided with that for the hydrolysis reaction using GM1 as the substrate (*12*). The transglycosylation reaction did not require the cations or energy donors such as ATP (data not shown).

*Enzymatic Synthesis of Fluorescence-labeled GSLs by the Transglycosylation Reaction of J-EGCase*—Fluorescence-labeled GSLs are useful to analyze the metabolism and intracellular transport of GSLs as well as to determine the activities of various GSL-metabolizing enzymes. Thus, we examined whether fluorescence-labeled ceramide can act as an acceptor in the transglycosylation reaction of J-EGCase. It was found that the enzyme transfers oligosaccharides from various GSLs to NBD-labeled dodecanoylsphingosine, C12-NBD-ceramide (NBD-C12:0/d18:1), yielding fluorescent GSLs (Fig 4). The fluorescent GSL generated from the incubation of GM1 with C12-NBD-ceramide in the presence of the enzyme was subjected to MALDI-TOF-MS analysis. As a result, a pseudomolecular ion $[M-H]^-$ of $m/z$ 1,640 was found, which is consistent with the molecular weight of C12-NBD-GM1. It is noted that the synthesized C12-NBD-GM1 was hydrolyzed by the *Rhodococcus* EGCase to produce C12-NBD-ceramide, suggesting that the C12-NBD-ceramide is coupled to oligosaccharide *via* a

TABLE II **Donor specificity for transglycosylation reaction of J-EGCase.**

| Substrate | Methyl-GSL oligosaccharide (%) | GSL oligosaccharide (%) | Remaining substrate (%) |
|---|---|---|---|
| GT1b | 16 5 | 61 4 | 22 1 |
| GD1b | 22 9 | 60 6 | 16 5 |
| GM1 | 30 3 | 51 6 | 18 1 |
| Asialo GM1 | 7 7 | 12 7 | 79.6 |
| Globoside | 0 | 0 | 100 |
| LacCer | 3 1 | 4 7 | 92.2 |
| GlcCer | 0 | 0 | 100 |

Various GSLs were incubated at 37°C for 16 h with 0 5 milliunits of the enzyme in 20 μl of 25 mM sodium acetate buffer, pH 3 0, containing 0 2% Triton X-100 in the presence of 25% methanol Carbohydrate-containing compounds were visualized by spraying with orcinol-$H_2SO_4$ reagent and determined as described under "MATERIALS AND METHODS"

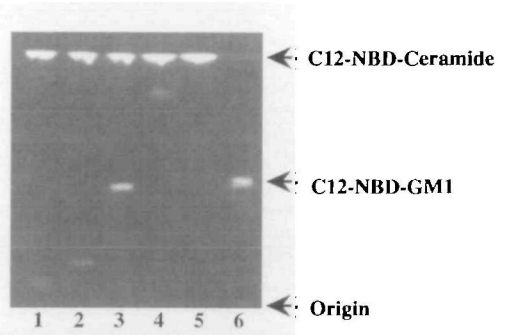


Fig. 4 **Transfer of oligosaccharides from various GSLs to C12-NBD-ceramide by the action of J-EGCase.** The reaction was carried out with 20 nmol of GSL, 0 1 milliunit of enzyme, and 200 pmol of C12-NBD-ceramide (NBD-C12 0/d18:1) in 20 μl of 25 mM sodium acetate buffer, pH 3 0, containing 0 2% Triton X-100 After incubation at 37°C for 1 h, the reaction mixture was evaporated to dryness and analyzed by TLC. Lane 1, GT1b + J-EGCase + C12-NBD-ceramide, lane 2, GD1b + J-EGCase + C12-NBD-ceramide, lane 3, GM1 + J-EGCase + C12-NBD-ceramide, lane 4, lactosylceramide + J-EGCase + C12-NBD-ceramide, lane 5, standard C12-NBD-ceramide, lane 6, standard C12-NBD-GM1

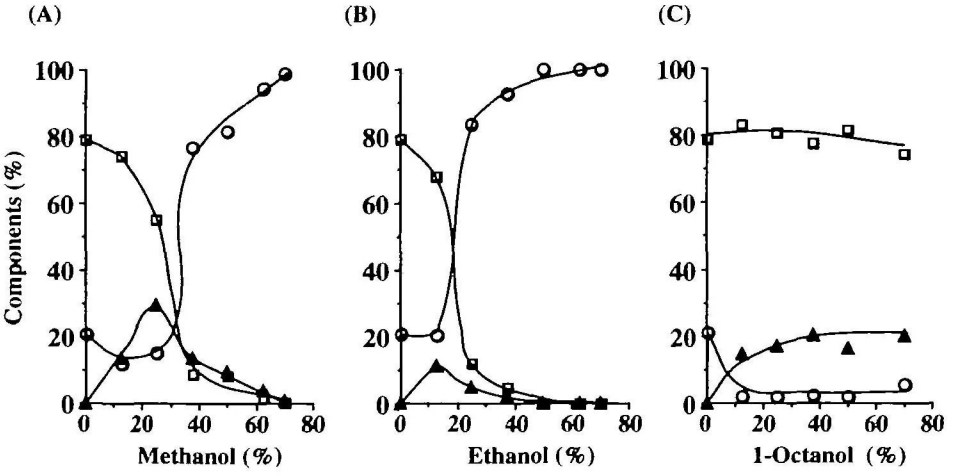


Fig 3 **Effects of concentrations of methanol (A), ethanol (B), and 1-octanol (C) on transglycosylation activity of J-EGCase.** The reaction mixtures contained 20 nmol of GM1, 0.5 milliunit of enzyme and 1-alkanols at the indicated concentrations in 40 μl of 25 mM sodium acetate buffer, pH 3 0, containing 0.2% Triton X-100. After incubation at 37°C for 3 h, the reaction mixtures were evaporated to dryness and analyzed by TLC. ○, remaining substrate (GM1); ▲, transglycosylation product (alkyl- II$^3$NeuAcGgOse4); □, hydrolysis product (II$^3$NeuAcGgOse4). (A), methanol, (B), ethanol, (C), 1-octanol.

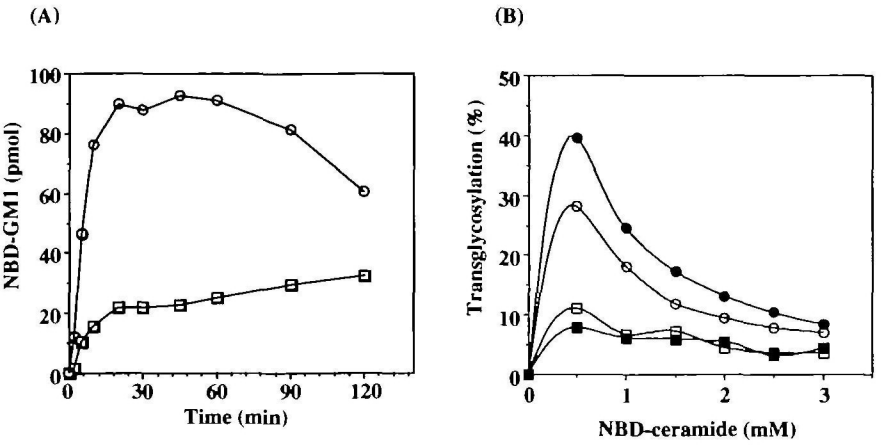


Fig 5 **Time course of generation of NBD-GM1 (A), and effect of NBD-ceramide concentration on transglycosylation activity of J-EGCase (B).** (A) The reaction was performed in a mixture of 20 nmol of GM1, 200 pmol of C6- or C12-NBD-ceramide, and 0 1 milliunit of J-EGCase in 20 μl of sodium acetate buffer, pH 3 0, containing 0 2% Triton X-100. After incubation at 37°C for the times indicated, the reaction mixture was evaporated to dryness and analyzed by TLC □, C6-NBD-GM1; ○, C12-NBD-GM1 (B) The reaction was carried out with 20 nmol of GM1, 0 1 milliunit of J-EGCase, and C6- or C12-NBD-ceramide at the indicated concentrations in 20 μl of sodium acetate buffer, pH 3 0, containing 0.2% Triton X-100 Acetone was added at the final concentration of 15% where indicated After incubation at 37°C for 60 min, the reaction mixture was analyzed by TLC ●, C12-NBD-GM1 in the presence of acetone, ○, C12-NBD-GM1 without acetone, ■, C6-NBD-GM1 in the presence of acetone, □, C6-NBD-GM1 without acetone

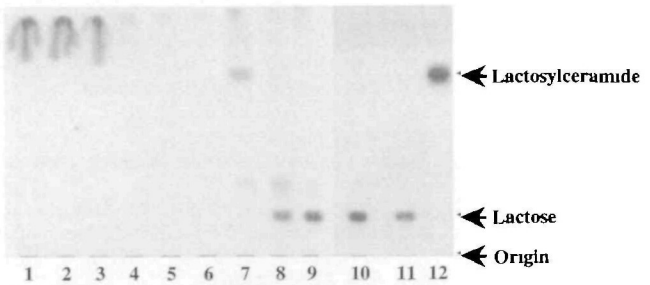


Fig. 6 **Synthesis of lactosylceramide using the reverse hydrolysis reaction of J-EGCase.** The reaction mixture contained 5 mg of bovine brain ceramide, 10 mg of lactose, and 2 5 milliunits of enzyme in 100 μl of sodium acetate buffer, pH 3 0, containing 2% Triton X-100 After incubation at 37°C for 40 h, the LacCer generated was isolated using Sep-Pak Plus Silica column. The eluates from the column were checked by TLC Ceramide was passed thorough the column using chloroform/methanol (95 5) (fractions 1 to 3) LacCer (fraction 7) and unreacted lactose (fractions 8 to 10) were eluted with chloroform/methanol (2 1) Lane 11, standard lactose, lane 12, standard LacCer Details are described in "MATERIALS AND METHODS"

β-glycosidic linkage, because the enzyme was demonstrated to be an endotype 1,1-β-glucanohydrolase [EC 3.2.1.123] (*5, 11*).

Figure 5A shows the time course of generation of fluorescent GM1 using C12-NBD-ceramide or NBD-labeled hexanoylsphingosine (C6-NBD-ceramide) as an acceptor The generation of C12-NBD-GM1 was much faster than that of C6-NBD-GM1, indicating that while both fluorescent ceramides were utilized in the transglycosylation reaction of the enzyme, that with the long-chain fatty acid was preferred. It was confirmed that NBD-sphingosine (NBD-d18:1) was not utilized by the enzyme as an acceptor (data not shown). The maximal generation of C12-NBD-GM1 was observed at 30–60 min incubation, at which time 45% of added C12-NBD-ceramide was converted to the fluorescent GM1. The yield decreased gradually beyond 60 min, possibly due to the hydrolysis of the generated C12-NBD-GM1 by the J-EGCase. On the other hard, the generation of C6-NBD-GM1 increased slowly but continuously with times up to 120 min, possibly because C6-NBD-GM1 is also less favored than C12-NBD-GM1 in the hydrolysis reaction of EGCase. Figure 5B shows the effects of the concentration of NBD-ceramide on the transglycosylation activity of J-EGCase in the presence or absence of acetone. The optimum concentration of NBD-ceramide was found to be 0.5 mM for both C12-NBD-ceramide and C-6-NBD-ceramide as the acceptor, regardless of the presence or absence of acetone. Interestingly, however, with C12-NBD-ceramide as an acceptor, the transglycosylation activity of the enzyme was enhanced when acetone was added to the reaction mixture by 15%. On the other hand, addition of acetone produced no significant increase of transglycosylation activity when C6-NBD-ceramide was used as an acceptor (Fig. 5B).

*Enzymatic Synthesis of Lactosylceramide by the Reverse Hydrolysis Reaction of J-EGCase*—In addition to transglycosylation activities, several glycosidases are known to catalyze the reverse hydrolysis reaction (condensation) (*18*). Condensation will be useful to synthesize GSLs using appropriate sugar chains instead of GSLs. Thus, we examined whether J-EGCase catalyzes the condensation. Bovine brain ceramide and lactose were incubated with the enzyme in the presence of 2% Triton X-100 at 37°C for 40 h, then the reaction mixture was evaporated to dryness, redissolved in 1 ml of chloroform/methanol (95 5, v/v), and applied to a Sep-Pak Plus Silica cartridge. The cartridge was washed with the same solvent solution and then eluted with chloroform/methanol (2:1, v/v). Each fraction was applied to a TLC plate, which was visualized by orcinol-$H_2SO_4$ reagent. Ceramide passed through the column upon washing with chloroform/methanol (95:5) (Fig. 6, fractions 1 to 3), whereas the newly generated product (Fig. 6, fraction 7) and unreacted lactose (Fig. 6, fraction 8 to 10) were eluted from the column with chloroform/methanol (2:1). $R_f$ of the newly generated product on TLC coincided with that of standard LacCer (Fig. 6).

A pseudomolecular ion $[M+H]^+$ of the product was found to have $m/z$ 890 by MALDI-TOF-MS using the positive ion mode with 2,5-dihydroxybenzoic acid as the matrix. This is consistent with the molecular weight of LacCer, in which the ceramide is most likely to be octadecanoylsphingenine (C18:0/d18:1).

## DISCUSSION

Compared with the transglycosylation reactions of other EGCases/CGase, J-EGCase showed several unique characteristics: methanol was found to be the best acceptor for J-EGCase among 1-alkanols tested, whereas the leech CGase was reported to be strongly inhibited by short chain 1-alkanols and no transfer product was observed when methanol was used as an acceptor (*19*). Ethanol was also utilized by J-EGCase as an acceptor, whereas EGCase from *Corynebacterium* sp. showed no transglycosylation activity with ethanol as an acceptor (*20*). These results indicate that J-EGCase is much more suitable to synthesize short-chain alkyl-glycosides than previously reported EGCases/CGases.

J-EGCase was found to transfer oligosaccharides from various GSLs to not only 1-alkanols but also ceramides. Using fluorescence-labeled ceramide as an acceptor, we prepared various fluorescence-labeled GSLs by the transglycosylation reaction of J-EGCase. C12-NBD-GM1 was found to be generated more rapidly than C6-NBD-GM1 by the enzyme, and no transfer product was detected when NBD-sphingosine was used as an acceptor. Why the enzyme did not utilize NBD-sphingosine as an acceptor remains unclear Fluorescent pigment NBD, which is directly coupled to C2 amino group of sphingoid base, may hinder the access of enzyme to the acceptor substrate.

Recently, we reported the synthesis of radioisotope- and fluorescence-labeled GSLs using a unique condensation reaction of sphingolipid ceramide *N*-deacylase (SCDase), in which labeled fatty acids are condensed to lyso-GSLs (GSLs without fatty acid) (*17*). Compared to the method using SCDase, the present method using transglycosylation activity of J-EGCase is much easier and less time-consuming, because the process for lyso-GSL preparation could be omitted.

In addition to transglycosylation, we also found that J-EGCase catalyzed the reverse hydrolysis reaction: lactose was condensed to ceramide, yielding LacCer. GSL-sugar chains can now be synthesized on a large scale by combination of various glycosyltransferases fixed on polymer (*21*) or by fermentation technology using microbes possessing specific glycosyltransferase genes (*22*). Various sugar chains with glucose at the reducing end are expected to be condensed to ceramide by the reverse hydrolysis reaction of J-EGCase, yielding desired GSLs.

The transglycosylation and condensation reactions of J-EGCase will be useful for preparation of various neoglycoconjugtes and GSLs, and thus facilitate further development in GSL research.

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