# 05 *ABSTRACT*


## Makoto Ito

***Ph.D.***
***Professor***
***Department of Bioscience and Biotechnology, Kyushu University, JAPAN***


# Molecular Evolution of Enzymes Involved in Glycosphingolipid Metabolism
## –Housekeeping and Signaling–

*profile*

**Makoto Ito**

Makoto Ito received his Ph.D. in 1981 from Kyushu University, Fukuoka, Japan, under the supervision of Dr. Manabu Kitamikado. Between 1982 and 1993, he worked at the laboratory of Dr. Tatsuya Yamagata at the Mitsubishi Kasei Institute of Life Sciences first as a postdoctoral fellow, then as senior researcher. He then returned to Kyushu University in 1993 as an associate professor and was appointed full professor in the Department of Bioscience and Biotechnology, Graduate School of Kyushu University in 2002. Dr. Ito received the Young Scientist Award for Biochemists from the Japanese Biochemical Society in 1991 (Title of paper: Discovery of endoglycoceramidase and its application for glycosphingolipid research). His research has focused on the structure and functions of glycoconjugates and their metabolizing enzymes. His recent research interests include the functions of glycosphingolipids and their metabolites, especially signaling functions and microdomains.


*Key words* glycosphingolipids, molecular evolution, endoglycoceramidase, sphingolipid ceramide N-deacylase, ceramidase


Glycosphingolipids (GSLs), amphipathic compounds consisting of oligosaccharide and ceramide (Cer) moieties, have been defined as tumor antigens, receptors for microbes and their toxins, and possible modulators of various cellular activities. Recently, GSLs were found to be enriched with cholesterol and GPI-anchor proteins to form lipid microdomains called rafts on the plasma membrane of vertebrates. In this symposium, the author reports on the molecular evolution of two unique enzymes and one ubiquitous enzyme involved in glycosphingolipid metabolism.

Endoglycoceramidase (EGCase, EC 3.2.1.123) is a glycohydrolase which hydrolyzes the linkage between the oligosaccharide and Cer of various GSLs (Fig. 1A). The enzyme was first found in actinomycetes *Rhodococcus* sp., then in the leech, short-necked clam and jellyfish. The genes encoding EGCase were cloned from *Rhodococcus* sp. M-750 and jellyfish in our laboratory. Interestingly, 8 amino acid residues essential for the activity of family A/5 cellulases (endo-β1,4-glucanase), including proton donor and catalytic nucleophile, are completely conserved in the deduced amino acid sequences of both prokaryotic and eukaryotic EGCases. By site-directed mutagenesis, these conserved amino acids were found to be integral for the catalytic reaction of EGCases. Furthermore, homology modeling of *Rhodococcus* EGCase using *Clostridium* A/5 cellulase as a template demonstrated that both enzymes share a common catalytic domain in the form of a $(\beta/\alpha)_8$-barrel, *i.e.*, a barrel of eight parallel β-strands surrounded by eight α-helices. These results indicate that EGCase catalyzes the general acid/base reactions in a similar manner for family A/5 cellulases and suggest that both enzymes are generated from the same ancestral gene. The distinct difference in substrate specificity of both enzymes, one hydrolyzes GSLs and one cellulose, may stem from the substrate-binding cleft in the enzyme molecules.

EGCases were found in eukaryotes and prokaryotes including Cnidaria. To clarify the biological functions of EGCase in Cnidaria, we cloned the enzyme from hydra, *Hydra magnipapillata*. *In situ* hybridization revealed that the EGCase gene was expressed in the endodermal layer of tentacle and peduncle and budding polyp in hydra. The EGCase, which seems to be located primarily in phagosomes/lysosomes, showed extremely acidic pH optima (3-3.5) and was secreted into the gastric cavity when brine shrimp was fed to hydra. It was confirmed that the GSLs in brine shrimp were digested by hydra EGCase, suggesting that the enzyme participates as a housekeeping enzyme in the digestive process.

Rhodococcal EGCase is now being successfully used by many researchers including those in our laboratory to elucidate the structure and functions of GSLs. In this symposium, the author will briefly summarize the recent contributions of EGCase in GSL research.

The second topic is an enzyme capable of generating lyso-forms of GSLs, *i.e.*, *N*-deacylated derivatives of GSLs. Lyso-GSLs are detected in normal tissues at very low levels, but accumulated in several types of inherited sphingolipid storage diseases. Recently, the biological significance of lyso-GSLs has drawn the attention of cell biologists because the G protein-coupled receptors for lyso-GSLs have been cloned and characterized as OGR and TDAG8. Lyso-GSL generating enzymes have been found in several bacteria and cloned from a bacterium, *Shewanella alga*. The recombinant enzyme expressed in *E. coli* hydrolyzed the *N*-acyl linkage of Cer of various GSLs and sphingomyelin to produce their lyso-forms and fatty acids (Fig. 1A). Thus the enzyme was tentatively designated Sphingolipid Cer *N*-Deacylase (SCDase). Unexpectedly, there were no homologous sequences for the enzyme found in human or other mammalian DNA databases when searched by BLAST and FASTA. Thus, the molecular mechanism underlying the generation of lyso-GSLs in mammals remains unclear.

The final topic is the ceramidase, which is involved in the final step of GSL catabolism and regulating cellular levels of Cer, sphingosine, and possibly sphigosine-1-phosphate. Ceramidase hydrolyzes the *N*-acyl linkage of free Cer and does not hydrolyze GSLs directly and is thus distinguished from the SCDase described above (Fig. 1B). Ceramidase is classified into three groups; acidic, neutral and alkaline enzymes. The acidic enzyme functions as a housekeeping enzyme to catabolize Cer in lysosomes. A genetic deficiency of the enzyme causes Farber diseases. Neutral ceramidase is found to change the balance of Cer/sphingosine/sphingosine-1-phosphate in response to various stimuli including cytokines and growth factors, and may modulate the sphingolipid-mediated signaling. Recently, we cloned the genes/cDNAs encoding neutral ceramidases from bacteria, drosophila, zebra fish, mouse and rat, demonstrating that the genetic information of the enzyme is well conserved from bacteria to mammals. In this symposium, the author reports that *O*-glycosylation of the mucin-like domain of mammalian neutral ceramidases was required for localization of the enzyme to the surface of the plasma membranes. The deduced amino acid sequences of mammalian enzymes contain a serine-threonine-rich domain (mucin box) which follows the signal sequence, whereas those of bacterial and invertebrate enzymes completely lack the mucin box, suggesting that the specific domain has been acquired during evolution. In HEK293 cells overexpressing ceramidase, the enzyme was not only secreted into the medium after cleavage of the $NH_2$-terminal region, but also localized at the plasma membrane as a type II integral membrane protein. Lectin blot analysis using peanut agglutinin demonstrated that the mucin box of the enzyme is highly glycosylated with *O*-glycans. Interestingly, a deletion mutant lacking the mucin box was secreted into the medium without being retained at the cell surface. The addition of porcine stomach mucin blocked the recruitment of wild-type enzyme to the cell surface. Furthermore, a mucin box-fused GFP with a signal sequence was distributed on the surface of the cells, suggesting that the mucin box functions as a signal for the localization of proteins to plasma membranes. We also found that the 112-kDa membrane-bound enzyme from mouse kidney is *O*-glycosylated whereas the 94-kDa soluble enzyme from the liver is not. These results clearly indicate that posttranslational modification of the enzyme with *O*-glycans is tissue specific and directs the enzyme to the surface of plasma membranes.

In this symposium, the author will discuss the mystery why a certain enzyme (neutral ceramidase) was selected to evolve in mammals, while other enzymes (EGCase and SCDase) were not selected and retained at the early stage of evolution. During the evolution of ceramidase, sugar chains (*O*-glycans) were given by which the enzyme could locate at the cell surface where the enzyme possibly functions as a signal transducer.


**Acknowledgments:** This study was performed in collaboration with graduate students at the Department of Bioscience and Biotechnology, Kyushu University, and the author thanks all of them. The author also thanks Dr. Michiko Go of Nagoya University for assistance in the homology modeling of EGCase.


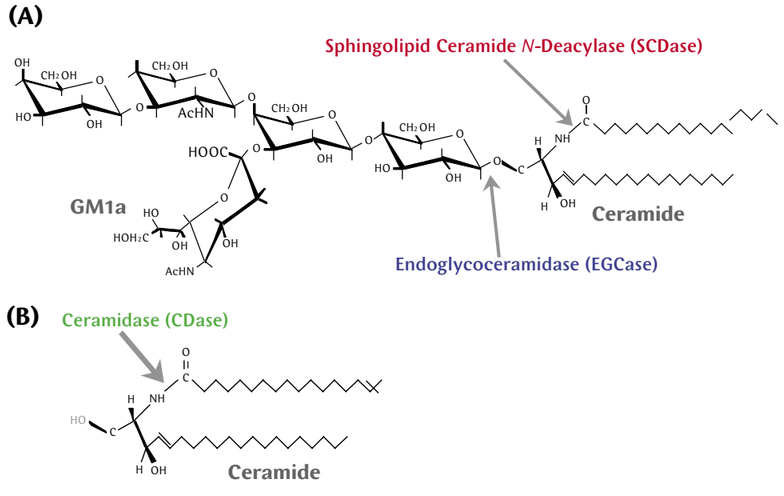


**Figure 1. Action Points of EGCase, SCDase and Ceramidase.**

*References*


(A) Endoglycoceramidase (EGCase)
1) Ito M, Yamagata T.
A novel glycosphingolipid-degrading enzyme cleaves the linkage between the oligosaccharide and ceramide of neutral and acidic glycosphingolipids. *J. Biol. Chem.* (261):14278-14282,1986
2) Li SC, DeGasperi R, Muldrey JE, Li YT.
A unique glycosphingolipid-spilitting enzyme (ceramide-glycanase from leech) cleaves linkage between the oligosacharide and the ceramide. *Biochem. Biophy. Res. Commun.* (141):346-352, 1986
3) Ito M, Komori H.
Homeostasis of cell-surface glycosphingolipid content in B16 melanoma cells. - Evidence revealed by an endoglycoceramidase - *J. Biol. Chem.* (271):12655-12660, 1996
4) Izu Y, Izumi Y, Kurome Y, Sano M, Kondo A, Kato I, Ito M.
Molecular cloning, expression, and sequence analysis of the endoglycoceramidase II gene from *Rhodococcus* sp. strain M-777. *J. Biol. Chem.* (272):19846-19850, 1997
5) Sakaguchi K, Okino N, Izu H, Ito M.
The Glu residue in the conserved Asn-Glu-Pro sequence of endoglycoceramidase is essential for enzymatic activity. *Biochem. Biophys. Res. Commun.* (260):89-93, 1999
6) Horibata Y, Okino N, Ichinose S, Omori A, Ito M.
Purification, characterization and cDNA cloning of a novel acidic endoglycoceramidase from the jellyfish, *Cyanea nozakii*. *J.Biol. Chem.*(275):31297-31304, 2000

(B) Sphingolipid Ceramide *N*-Deacylase (SCDase)
1) Ito M, Kurita T, Kita K.
A novel enzyme that cleaves the *N*-acyl linkage of ceramides in various glycosphingolipids as well as spingomyelin to produce their lyso forms. *J. Biol. Chem.* (270): 24370-24374, 1995
2) Furusato M, Sueyoshi N, Sakaguchi K, Okino N, Ito M. Molecular cloning and characterization of sphingolipid ceramide *N*-deacylase from a marine bacterium, *Shewanella alga* G8. *J. Biol. Chem.* (276): 17300-17307, 2002

(C) Ceramidase
1) Tani M, Okino N, Mori K, Tanigawa T, Izu H, Ito M.
Molecular cloning of the full-length cDNA encoding mouse neutral ceramidase. - Novel but highly conserved gene family of neutral/alkaline ceramidases- *J. Biol. Chem.* (275):11229-11234, 2000
2) Mitsutake S, Tani M, Okino N, Yoshida S, Ito M.
Purification, characterization, molecular cloning and subcellular distribution of neutral ceramidase of rat kidney. *J. Biol. Chem.* (276):26249-26359, 2001