Crystal Structure of GH101 Endo- α -N-acetylgalactosaminidase from *Bifidobacterium longum*

(Received December 2, 2008; Accepted December 24, 2008)

Ryuichiro Suzuki,¹ Takane Katayama,² Shinya Fushinobu,^{1,*} Motomitsu Kitaoka,³ Hidehiko Kumagai,²
Takayoshi Wakagi,¹ Hirofumi Shoun,¹ Hisashi Ashida⁴ and Kenji Yamamoto⁴

¹Department of Biotechnology, The University of Tokyo
(1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan)

²Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University
(1-308, Suematsu, Nonoichimachi, Ishikawa 921-8836, Japan)

³Enzyme Laboratory, National Food Research Institute, National Agriculture and Food Research Organization
(2-1-12, Kannondai, Tsukuba 305-8642, Japan)

⁴Graduate School of Biostudies, Kyoto University
(Kitashirakawa Oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan)

Abstract: Endo- α -N-acetylgalactosaminidase (endo- α), a member of glycoside hydrolase (GH) family 101, catalyzes the hydrolysis of *O*-glycosidic α linkages of mucin-type *O*-glycan. Endo- α can be used in the synthesis of various glycoconjugates because of its transglycosylation activity. Therefore, it is important to elucidate the structure-function relationship of this enzyme. The gene encoding endo- α from *Bifidobacterium longum* JCM1217 has been cloned, and its gene product (EngBF) has been characterized in detail. EngBF releases a galacto-*N*-biose (Gal β 1-3GalNAc, GNB) from glycoconjugates without damaging either the glycan or the core protein. This study presents the crystal structure of EngBF at 2.25 Å resolution. The catalytic domain of EngBF resembles the TIM barrel fold of GH13 α -amylase family. Based on structural comparison with α -amylase family, the catalytic nucleophile and acid/base catalyst residues of EngBF are determined to be Asp 789 and Glu822, respectively. Moreover, the structural basis of substrate recognition by EngBF was predicted by automated docking and mutational studies, and was compared with endo- α from *Clostridium perfringens* strain 13 (EngCP). The difference in substrate specificities between EngBF and EngCP is attributed to variations in amino acid sequences in the regions forming the substrate binding pocket. Results of our present study provide insights into both the reaction and substrate recognition mechanisms of endoglycosidases that liberate mucin-type *O*-glycan from glycoconjugates.

Key words: Bifidobacteria, endo- α -N-acetyl galactosaminidase, glycoside hydrolase family 101, core 1 disaccharide, galacto-*N*-biose, mucin-type *O*-glycan

Glycosylation, one of the major post-translational modifications, consists mainly of *N*-linked and *O*-linked glycosylations. In the “mucin-type” *O*-linked glycans, a GalNAc residue is α -linked to the hydroxyl group of a Ser/Thr residue in the core protein. At least eight different core structures of mucin-type *O*-glycans have hitherto been identified and utilized as basal core structures to build mature glycans such as the ABO blood group antigen.¹⁾ The mature glycans participate in a variety of biological phenomena, cell-cell adhesions, and signal transduction events. It is well known that glycans of carcinoma patients are aberrantly glycosylated.²⁾ These abnormal glycosylations result in expression of the carcinoma markers, Tn-antigen (GalNAc α 1-Ser/Thr) and T-antigen or core 1 (Ga β 1-3GalNAc α 1-Ser/Thr).^{3,4)} These markers are involved in the metastasis and invasion of cancer by acting as cell adhesion molecules.^{4,5)} Thus, T-antigen analogues have the potential to serve as vaccines for cancer.⁶⁾ In this context, many examples of production of the mucin-type glycan analogues containing T-antigen using various

methods, *e.g.*, transglycosylation activity, have been reported.^{7–12)}

Endo- α -N-acetylgalactosaminidase (endo- α ; EC 3.2.1.97) from *Bifidobacterium longum* JCM1217 (EngBF) catalyzes the hydrolysis of *O*-glycosidic α linkage of mucin-type *O*-glycan and releases a galacto-*N*-biose (GNB, Gal β 1-3GalNAc) from core 1. EngBF is an extracellular membrane-bound enzyme that plays a critical role in the degradation of intestinal mucin.¹³⁾ EngBF is possibly linked to the recently found metabolic pathway of bifidobacteria specific for GNB and lacto-*N*-biose (LNB, Gal β 1-3GlcNAc).¹⁴⁾ LNB is abundantly present in human milk oligosaccharides, and the GNB/LNB pathway is suggested to be involved in the intestinal colonization of bifidobacteria. The released core 1 can be transported into the bifidobacterial cells via an ABC-type transporter specific for GNB and LNB,¹⁵⁾ where it is further metabolized by intracellular enzymes.^{14,16)}

EngBF and its homologues are classified into the glycoside hydrolase (GH) family 101 in the Carbohydrate-Active enZyme (CAZy) database (<http://www.cazy.org/>). As illustrated in Fig. 1, EngBF comprises a total 1966 residues; a signal peptide (residues 1–29), a PEGA do-

* Corresponding author (Tel. & Fax. +81-3-5841-5151, E-mail: asfushi@mail.ecc.u-tokyo.ac.jp).

Fig. 1. The overall structure (A) and domain structure (B) of EngBF.

The overall structure of EngBF can be divided into domain N (residues 341–604), domain A (residues 605–721 and 766–919), domain B (residues 722–765), domain D (residues 920–1095), domain E (residues 1096–1257), domain F (residues 1258–1452) and domain G (residues 1453–1524). The catalytically important residues (Asp682, Asp789 and Glu822) and four Mn^{2+} ions are shown as ball-and-stick models and spheres, respectively.

main (residues 295–326), a GH101 conserved region (residues 590–1381), a carbohydrate-binding module (CBM) family 32 domain (residues 1548–1687), a FIVAR domain (residues 1703–1909) and a transmembrane region (residues 1942–1958).^{17–19} EngBF is highly specific for the core 1 structure, and 3 conserved residues, Asp682, Asp789 and Glu822, have been suggested to be important for catalysis. GH101 endo- α s are retaining enzymes, which exhibit a transglycosylation activity. The engineering approaches for the transglycosylation activity of endo- α s are useful for synthesizing a variety of glycoconjugates, because the enzymes display wide acceptor specificities.^{7,8,17} Furthermore, endo- α is a unique enzyme that catalyzes removal of the mucin-type *O*-glycan from the core protein without damaging it; thus, the enzyme can be exploited as a novel tool for glycoproteomics. To date, GH101 endo- α s from *Clostridium perfringens*, *Streptococcus pneumoniae*, *Enterococcus faecalis* and *Propionibacterium acnes* have been cloned and characterized.^{20–22} Endo- α from *C. perfringens* strain 13 (EngCP) displays broader substrate specificity than EngBF.²⁰ After our presentation at the Symposium on Amylases and Related Enzymes, 2008, the crystal structure of selenomethionine-labeled endo- α from *Streptococcus pneumoniae* R6 (SpGH101) at 2.9 Å resolution was reported.²³ However, the detailed mechanism and structural basis for substrate recognition of endo- α remains to be elucidated. Here we report the crystal structure of native EngBF protein at 2.25 Å resolution. Based on the structure and automated docking analysis, we have elucidated the critical residues for substrate recognition by mutational analysis.

Overall structure of EngBF.

A truncated construct of EngBF without the N-terminal PEGA and the C-terminal CBM32 domains (residues 340–1529) that retained equivalent activity against core 1-

*p*NP (Gal β 1-3GalNAc α 1-*p*NP) compared to the wild-type enzyme was used in this study. Native and selenomethionine-labeled EngBF proteins were purified to homogeneity and were then subjected to crystallization screenings. The initial phases were determined by a multiple-wavelength anomalous dispersion method using a selenomethionine derivative. The crystal structure of the native EngBF was determined at 2.25 Å resolution (Table 1 and Fig. 1). The final model comprises residues from 341 to 1524; residues 1476–1481 were not included due to disorder. The EngBF structure was divided into several domains, which were named according to the conventional naming system of α -amylases. The catalytic domain of EngBF (domains A and B) has a $(\beta/\alpha)_8$ barrel-like fold, which resembles the $(\beta/\alpha)_8$ barrel of the GH13 α -amylase family. This domain is surrounded by four β -sandwich domains (domains N, D, E and F). The C-terminal domain G has a three α -helix bundle fold, and the disordered region is within this domain. Before the release of the coordinates of SpGH101 structure in the Protein Data Bank, a structural homology search using the DALI server (<http://www.ebi.ac.uk/dali/>) indicated that the catalytic domain of EngBF (domains A and B) exhibits its highest similarity to the α -amylase TVA II from *Thermoactinomyces vulgaris* R-47 (PDB ID = 1BVZ, RMSD = 2.95 Å for 222 C α atoms).²⁴ The catalytic domain of α -amylase comprises a complete $(\beta/\alpha)_8$ -barrel, whereas that of EngBF lacks β 6– β 8 to form a broken $(\beta/\alpha)_8$ -barrel. The extra domain, the so-called domain B that is conserved amongst the α -amylase family, is inserted between the A β 3 strand and A α 3 helix.²⁵ The long loop region corresponding to domain B of the α -amylase family was also found in EngBF (Fig. 1). Therefore, the catalytic barrel domain of EngBF is divided into domain A and domain B. Four manganese ions (Mn^{2+}) in hexagonal coordination were identified at the interface between domain A

Table 1. Data collection and refinement statistics of the native EngBF crystal structure.

	Native EngBF structure
Data collection	
Space group	$P6_5$
Unit cell dimensions (Å)	$a = b = 192.4$, $c = 122.8$
Resolution ^a (Å)	50.00–2.25 (2.33–2.25)
Measured reflections	3934375
Unique reflections	121950
Completeness ^a (%)	99.9 (99.9)
Mean I/σ ^a	37.4 (4.1)
R_{merge} ^a (%)	6.4 (42.3)
Refinement	
Resolution range (Å)	48.11–2.25
R/R_{free} factor (%)	17.5/20.3
No. of non-hydrogen atoms	9902
Rmsd bond lengths (Å)	0.018
Rmsd bond angles (°)	1.6

^aValues in parentheses are for the highest resolution shell.

and the surrounding domains (Fig. 1), indicating that the Mn^{2+} ions are involved in the stabilization and assembly of domains. Thermal stabilization of EngBF activity was observed in the presence of Mn^{2+} or Mg^{2+} ions (data not shown).

Active site.

Despite extensive efforts to crystallize EngBF in complex with GNB using both soaking and co-crystallization methods, the complex structure could not be obtained. Instead, an automated docking analysis was performed using the program AutoDock 4.0.²⁶⁾ The most plausible docking result, using a GNB model as a ligand and the native EngBF crystal structure as a receptor, is shown in Fig. 2. The docked GNB model was positioned in the central pocket of the broken (β/α)₈ barrel without any steric hindrance. In this docking model, the anomeric C1 atom of GalNAc (subsite -1) is located in proximity of a side chain oxygen atom of Asp789, and the α -C1 hydroxyl group forms a direct hydrogen bond with a side chain oxygen atom of Glu822. Therefore, it can be concluded that Asp789 and Glu822 are the candidates for the nucleophile and acid/base catalyst in EngBF, respectively. This result is also supported by structural comparison with the TAKA-amylase/acarbose complex.²⁷⁾ TAKA-amylase is one of the most extensively characterized enzymes be-

Fig. 2. Stereographic view of the active site of EngBF with a docked GNB model.

The docked GNB molecule and residues around the active site are shown as ball-and-stick models. Possible direct hydrogen bonds are shown as dashed lines. Grouping of the residues for mutational analyses is shown in parentheses in the labels (see text).

longing to the GH13 α -amylase family.^{28–30)} Asp206, Glu230 and Asp297 in TAKA-amylase are known as the nucleophile, the acid/base catalyst and the third conserved residue, respectively.²⁷⁾ Interestingly, the positions of Asp206 (nucleophile) and Glu230 (acid/base) in TAKA-amylase correspond to those of Asp789 and Glu822 in EngBF (Fig. 3). The nucleophile and acid/base catalyst were located at the C-terminal loop of the 4th and 5th β -strands in the barrel scaffold, respectively. Furthermore, the locations of sugars in subsite -1 are well superimposable. Asp682 in EngBF is also shown to be catalytically essential by a mutational analysis.¹⁷⁾ In the docking model, the side chain of Asp682 forms two direct hydrogen bonds with the O4 hydroxyl of GalNAc (subsite -1) and the O6 hydroxyl of Gal (subsite -2). Therefore, it was concluded that Asp682 is the third conserved residue of EngBF. However, in TAKA-amylase, the third conserved residue (Asp297) is located in a different position and instead an essential aromatic residue for stacking interaction at subsite -1, Tyr82, is located at the position corresponding to Asp682 in EngBF.³¹⁾

The hydrophobic β -faces of both the GalNAc and Gal units of the docked GNB molecule face upward and are exposed to solvent (Fig. 2). Since stacking interactions between the sugar β -faces and aromatic residues are often essential for protein-carbohydrate interactions,³²⁾ the interactions in the docked model appears to be incomplete. Interestingly, two aromatic residues, Trp748 and Trp750, form the wall of the pocket, and are likely to form the stacking interaction when they lay down on the sugar β -faces by substrate-induced fit. To provide experimental evidence for the computational docking results and hypothesis of induced fit, kinetic parameters of various mutant enzymes against core 1- p -NP were measured. The residues around the pocket were categorized into four groups (Fig. 2); residues essential for catalysis (group A), those involved in direct substrate recognition (group B), those involved in indirect substrate recognition (group C), and aromatic residues for potential stacking interactions (group D). The group B residues (Tyr787 and Asp1295) form a direct hydrogen bond with the docked GNB molecule. The former interacts with the carbonyl oxygen of the *N*-acetyl group of GalNAc at subsite -1, and the latter interacted with the C3 and C4 hydroxyl groups of Gal at

Fig. 3. Catalytically important residues in the barrel scaffold of EngBF (A) and TAKA-amylase (B).

The docked GNB model in EngBF and acarbose complexed with TAKA-amylase are shown as ball-and-stick models. The two structures are aligned according to the structural superimpositions of the secondary structure elements. The locations of the nucleophile and the acid-base catalyst are identical between EngBF and TAKA-amylase.

subsite -2. The group C residues (Asn720, Gln894 and Lys1199) are positioned around the docked GNB molecule, but they do not form direct interactions. Mutations of the group A residues (D682A, D789A and E822A) were fatal for the core 1-*p*NP hydrolytic activity. In particular, the activities of D682A and D789A were completely abolished. This result indicates that these residues play a critical role in the catalytic activity, which agrees with the previous observation.¹⁷⁾ The $k_{\text{cat}}/K_{\text{M}}$ values of the group B mutants (Y787F and D1295A) also conspicuously diminished. The Y787F mutation resulted in a significant decrease in the k_{cat} value with a minor increase in the K_{M} value. Because the side chain hydroxyl group of Tyr787 is located close to the nucleophile (3.3 Å to a side chain carboxyl oxygen atom of Asp789), elimination of this hydroxyl group may directly influence the catalysis. In contrast, the Asp1295 variant had a slightly elevated k_{cat} value and displayed a remarkable increase in the K_{M} value. This result clearly indicates that Asp1295 plays an important role in substrate binding. The group C mutants (N720A, Q894A and K1199A) showed similar features in their kinetic parameters. The increased k_{cat} and K_{M} values led to almost the same $k_{\text{cat}}/K_{\text{M}}$ values as that of the wild type enzyme. Therefore, the role of these three residues was interpreted as substrate recognition rather than catalytic activity. The group D mutants (W748A and W750A) exhibited significantly decreased $k_{\text{cat}}/K_{\text{M}}$ values, and it was impossible to separately determine K_{M} and k_{cat} values due to significant elevation of the K_{M} value. Therefore, Trp748 and Trp750 were considered as more crucial residues for substrate binding compared to groups B and C residues and the hypothesis for substrate-induced formation of stacking interactions is strongly supported by this result.

Substrate specificity.

Recently, Ashida *et al.* reported that both EngBF and EngCP display high activity against core 1-*pNP* but differ in their substrate specificities.²⁰⁾ For example, EngCP shows higher activities toward core 2-*pNP* (Gal β 1-3(GlcNAc β 1-6)GalNAc α 1-*pNP*), core 3-*pNP* (GlcNAc β 1-3GalNAc α 1-*pNP*), GalNAc β 1-3GalNAc α 1-*pNP* and Glc β 1-3GalNAc α 1-*pNP* than EngBF. Core 2 has a β 1-6 branch of the GlcNAc unit at GalNAc (subsite -1) of core 1. The structures of the latter three substrates are similar to core 1, but the Gal unit at subsite -2 is substituted by GlcNAc, GalNAc and Glc, respectively. The molecular surface of the substrate-binding pocket of EngBF is shown in Fig. 4. Also shown is a pairwise sequence alignment with EngCP at regions around the C6 hydroxyl group of GalNAc at subsite -1 and the C2 and C4 hydroxyl groups of Gal at subsite -2. EngCP has a deletion of five residues in the region around the C6 hydroxyl group of GalNAc, suggesting that EngCP has space to accommodate the β 1-6 GlcNAc branch in this region. Moreover, EngCP has a deletion of seven residues in the region around the C2 hydroxyl group, and deletion of the residue corresponding to Asp1295 of EngBF, which may be involved in recognition of the C4 hydroxyl group of Gal at subsite -2. These deletions might make EngCP more active against core 3-*pNP*, GalNAc β 1-3GalNAc α 1-

Fig. 4. Molecular surface around the catalytic pocket of EngBF, and amino acid sequence alignment of EngBF and EngCP.

Upper panel: Molecular surface of EngBF and stick model of the docked GNB. Predicted binding positions of substrates that have a β 1-6 branch (subsite -1), *N*-acetyl group (subsite -2) and C-4 epimers (subsite -2) are indicated by boxes. Lower panel: Partial amino acid sequence alignments of EngBF and EngCP in the putative regions involved in substrate recognition at the three positions. Deletions present in EngCP are boxed.

*p*NP and Glcβ1-3GalNAcα1-*p*NP than EngBF. These interpretations may be valuable for altering the substrate specificity of endo-αs by targeting the regions via engineering approaches.

Conclusion.

The crystal structure of native EngBF protein was determined at 2.25 Å resolution. EngBF comprises multiple domains that surround a central catalytic domain. The catalytic domain of EngBF resembles that of the GH13 α -amylase family. Automated docking analysis was performed to predict the interactions between the enzyme and substrate. The catalytic residues, the substrate-binding manner and the important residues involved in substrate recognition were predicted. The putative interactions were verified by mutational study. It was concluded that Asp 789, Glu822 and Asp682 function as nucleophile, acid/base catalyst and third conserved residue, respectively. Structural basis underlying the substrate specificities of other GH101 endo- α s was also provided.

We thank the staff of the Photon Factory for the X-ray data collection. This work was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

REFERENCES

- 1) A. Variki, R. Cummings, J. Esko, H. Freeze, G. Hart and J.D. Marth: *Essentials of Glycobiology*, Cold Spring Harbor Laboratory, Cold Spring Harbor, pp. 1-653 (1999).
- 2) I. Brockhausen: Mucin-type *O*-glycans in human colon and

- breast cancer: glycodynamics and functions. *EMBO Rep.*, **7**, 599–604 (2006).
- 3) J.H. Anglin, Jr., M.P. Lerner and R.E. Nordquist: Blood group-like activity released by human mammary carcinoma cells in culture. *Nature*, **269**, 254–255 (1977).
 - 4) G.F. Springer: T and Tn, general carcinoma autoantigens. *Science*, **224**, 1198–1206 (1984).
 - 5) G.F. Springer: T and Tn pancarcinoma markers: autoantigenic adhesion molecules in pathogenesis, prebiopsy carcinoma-detection and long-term breast carcinoma immunotherapy. *Crit. Rev. Oncog.*, **6**, 57–85 (1995).
 - 6) S.J. Danishefsky and J.R. Allen: From the laboratory to the clinic: A retrospective on fully synthetic carbohydrate-based anticancer vaccines. *Angew. Chem. Int. Ed. Engl.*, **39**, 836–863 (2000).
 - 7) H. Ashida, K. Yamamoto, T. Murata, T. Usui and H. Kumagai: Characterization of endo- α -N-acetylgalactosaminidase from *Bacillus* sp. and syntheses of neo-oligosaccharides using its transglycosylation activity. *Arch. Biochem. Biophys.*, **373**, 394–400 (2000).
 - 8) H. Ashida, K. Yamamoto and H. Kumagai: Enzymatic syntheses of T antigen-containing glycolipid mimicry using the transglycosylation activity of endo- α -N-acetylgalactosaminidase. *Carbohydr. Res.*, **330**, 487–493 (2001).
 - 9) L.A. Marcaurelle and C.R. Bertozzi: Recent advances in the chemical synthesis of mucin-like glycoproteins. *Glycobiology*, **12**, 69R–77R (2002).
 - 10) F.G. Hanisch: Design of a MUC1-based cancer vaccine. *Biochem. Soc. Trans.*, **33**, 705–708 (2005).
 - 11) S. DeFrees, Z.G. Wang, R. Xing, A.E. Scott, J. Wang, D. Zopf, D.L. Gouty, E.R. Sjoberg, K. Panneerselvam, E.C. Brinkman-Van der Linden, R.J. Bayer, M.A. Tarp and H. Clausen: GlycoPEGylation of recombinant therapeutic proteins produced in *Escherichia coli*. *Glycobiology*, **16**, 833–843 (2006).
 - 12) K. Amano, Y. Chiba, Y. Kasahara, Y. Kato, M.K. Kaneko, A. Kuno, H. Ito, K. Kobayashi, J. Hirabayashi, Y. Jigami and H. Narimatsu: Engineering of mucin-type human glycoproteins in yeast cells. *Proc. Natl. Acad. Sci. USA*, **105**, 3232–3237 (2008).
 - 13) P. Ruas-Madiedo, M. Gueimonde, M. Fernandez-Garcia, C.G. de los Reyes-Gavilan and A. Margolles: Mucin degradation by *Bifidobacterium* strains isolated from the human intestinal microbiota. *Appl. Environ. Microbiol.*, **74**, 1936–1940 (2008).
 - 14) M. Kitaoka, J. Tian and M. Nishimoto: Novel putative galactose operon involving lacto-N-biose phosphorylase in *Bifidobacterium longum*. *Appl. Environ. Microbiol.*, **71**, 3158–3162 (2005).
 - 15) R. Suzuki, J. Wada, T. Katayama, S. Fushinobu, T. Wakagi, H. Shoun, H. Sugimoto, A. Tanaka, H. Kumagai, H. Ashida, M. Kitaoka and K. Yamamoto: Structural and thermodynamic analyses of solute-binding protein from *Bifidobacterium longum* specific for core 1 disaccharide and lacto-N-biose I. *J. Biol. Chem.*, **283**, 13165–13173 (2008).
 - 16) M. Nishimoto and M. Kitaoka: Identification of N-acetylhexosamine 1-kinase in the complete lacto-N-biose I/galacto-N-biose metabolic pathway in *Bifidobacterium longum*. *Appl. Environ. Microbiol.*, **73**, 6444–6449 (2007).
 - 17) K. Fujita, F. Oura, N. Nagamine, T. Katayama, J. Hiratake, K. Sakata, H. Kumagai and K. Yamamoto: Identification and molecular cloning of a novel glycoside hydrolase family of core 1 type O-glycan-specific endo- α -N-acetylgalactosaminidase from *Bifidobacterium longum*. *J. Biol. Chem.*, **280**, 37415–37422 (2005).
 - 18) T. Katayama, K. Fujita and K. Yamamoto: Novel bifidobacterial glycosidases acting on sugar chains of mucin glycoproteins. *J. Biosci. Bioeng.*, **99**, 457–465 (2005).
 - 19) T. Katayama, J. Wada, K. Fujita, M. Kiyohara, H. Ashida and K. Yamamoto: Functions of glycosidases isolated from *Bifidobacteria*. *J. Appl. Glycosci.*, **55**, 101–109 (2008).
 - 20) H. Ashida, R. Maki, H. Ozawa, Y. Tani, M. Kiyohara, M. Fujita, A. Imamura, H. Ishida, M. Kiso and K. Yamamoto: Characterization of two different endo- α -N-acetylgalactosaminidases from probiotic and pathogenic enterobacteria, *Bifidobacterium longum* and *Clostridium perfringens*. *Glycobiology*, **18**, 727–734 (2008).
 - 21) D. Koutsoulis, D. Landry and E.P. Guthrie: Novel endo- α -N-acetylgalactosaminidases with broader substrate specificity. *Glycobiology*, **18**, 799–805 (2008).
 - 22) H.M. Goda, K. Ushigusa, H. Ito, N. Okino, H. Narimatsu and M. Ito: Molecular cloning, expression and characterization of a novel endo- α -N-acetylgalactosaminidase from *Enterococcus faecalis*. *Biochem. Biophys. Res. Commun.*, **375**, 541–546 (2008).
 - 23) M.E. Caines, H. Zhu, M. Vuckovic, L.M. Willis, S.G. Withers, W.W. Wakarchuk and N.C. Strynadka: The structural basis for T-antigen hydrolysis by *Streptococcus pneumoniae*: A target for structure-based vaccine design. *J. Biol. Chem.*, **283**, 31279–31283 (2008).
 - 24) S. Kamitori, S. Kondo, K. Okuyama, T. Yokota, Y. Shimura, T. Tonozuka and Y. Sakano: Crystal structure of *Thermoactinomyces vulgaris* R-47 α -amylase II (TVaII) hydrolyzing cyclodextrins and pullulan at 2.6 Å resolution. *J. Mol. Biol.*, **287**, 907–921 (1999).
 - 25) E.A. MacGregor, S. Janecek and B. Svensson: Relationship of sequence and structure to specificity in the α -amylase family of enzymes. *Biochim. Biophys. Acta*, **1546**, 1–20 (2001).
 - 26) G.M. Morris, D.S. Goodsell, R.S. Halliday, R. Huey, W.E. Hart, R.K. Belew and A.J. Olson: Automated docking using a Lamarckian Genetic Algorithm and an empirical binding free energy function. *J. Comput. Chem.*, **19**, 1639–1662 (1998).
 - 27) A.M. Brzozowski and G.J. Davies: Structure of the *Aspergillus oryzae* α -amylase complexed with the inhibitor acarbose at 2.0 Å resolution. *Biochemistry*, **36**, 10837–10845 (1997).
 - 28) Y. Matsuura: A possible mechanism of catalysis involving the essential residues in the enzymes of α -amylase family. *Biologia (Bratislava)*, **57**, 21–27 (2002).
 - 29) Y. Matsuura, M. Kusunoki, W. Date, S. Harada, S. Bando, N. Tanaka and M. Kakudo: Low resolution crystal structures of Taka-amylase A and its complexes with inhibitors. *J. Biochem. (Tokyo)*, **86**, 1773–1783 (1979).
 - 30) Y. Matsuura, M. Kusunoki, W. Harada and M. Kakudo: Structure and possible catalytic residues of Taka-amylase A. *J. Biochem. (Tokyo)*, **95**, 697–702 (1984).
 - 31) I. Matsui, S. Yoneda, K. Ishikawa, S. Miyairi, S. Fukui, H. Umeyama and K. Honda: Roles of the aromatic residues conserved in the active center of *Saccharomycopsis* α -amylase for transglycosylation and hydrolysis activity. *Biochemistry*, **33**, 451–458 (1994).
 - 32) N.K. Vyas: Atomic features of protein-carbohydrate interactions. *Curr. Opin. Struct. Biol.*, **1**, 732–740 (1991).

ビフィズス菌由来 endo- α -N-acetylgalactosaminidase の X 線結晶構造解析

鈴木龍一郎¹, 片山高嶺², 伏信進矢¹, 北岡本光³
熊谷英彦², 若木高善¹, 祥雲弘文¹, 芦田 久⁴
山本憲二⁴

¹ 東京大学大学院農学生命科学研究科応用生命工学専攻
(113-8657 東京都文京区弥生 1-1-1)

² 石川県立大学生物資源工学研究所
(921-8836 石川県石川郡野々市町末松 1-308)

³ 独立行政法人農業・食品産業技術総合研究機構
食品総合研究所

(305-8642 つくば市観音台 2-1-12)

⁴ 京都大学大学院生命科学研究科
(606-8502 京都市左京区北白川追分町)

Endo- α -N-acetylgalactosaminidase (endo- α) は、ムチン型 O-glycan のコア 1 構造 (Ga β 1-3GalNAc α 1-Ser/Thr) の O-グリコシド α 結合を加水分解する酵素であり、glycoside hydrolase (GH) family 101 に分類されている。本酵素はその糖転移活性により、様々な複合糖鎖の合成に利用できることから、構造機能相関を解明することが求められている。Bifidobacterium longum JCM1217 株由来の endo- α (EngBF) はすでに詳細な機能解析が行われている。EngBF は、ムチン型コア 1 糖鎖を特異的に認識してコアタンパクからガラクト-N-ビオース (GNB, Gal β 1-3GalNAc) を無傷で遊離する。本発表では、2.25 Å 分解能で決定した EngBF の結晶構造を報告する。EngBF の触媒ドメインは GH13 α -アミラーゼファミリーの TIM バレルフォールドと類似していた。 α -アミラーゼファミリーとの構造比較により、EngBF の触媒残基は Asp789 (求核種) および Glu822 (酸塩基触媒) であることが明らかになった。さらに、EngBF の基質認識の構造的基盤を GNB のドッキング解析および変異導入実験によって予想し、Clostridium perfringens strain 13 由来 endo- α (EngCP) と比較した。EngBF と EngCP の異なる基質特異性は、基質結合ポケットを形成する領域におけるアミノ酸配列の違いに帰結された。我々の結果は、ムチン型 O-glycan を intact な状態で遊離する endoglycosidase の反応機構と基質認識機構に関する知見を提供するものである。

〔質問〕 東京農工大 農学 農学 殿塚

1) TVAII と立体構造上相同性を有するとのことですが、TVAII は、溶液中はダイマーで存在します。本酵素が、溶液中はオリゴマーで存在する可能性がありますか。また特徴的な結晶のパッキングについて、酵素のオリゴマー形成などに関連しているということは考えられますか。

2) 本酵素は、多くのドメインから構成されていますが、各ドメインについて、個別に立体構造の相同性の検索を行ったとき、どのようなものと相同性がありますか。

〔答〕

1) ゲルろ過カラムでの溶出位置から、オリゴマーで存在する可能性は低いと考えています。本酵素は結晶中では特徴的なパッキングをとっていますが、パッキング界面における分子間の相互作用は少なく、結晶も壊れやすいものでしたので、偶然の産物であると思われ、オリゴマー形成と関連しているとは考えられません。それゆえ、本酵素は溶液中でモノマーで存在すると考えております。

2) DALI サーバーを用いて各ドメインについて、個別に立体構造の相同性の検索を行ったところ、Z score が高いものはほとんど得られませんでした。触媒ドメインにおいてランキングが高いものは全てアミラーゼファミリーに属する酵素でした。それ以外の β -strand rich domains においては、主に CBM が上位として得られました。

〔質問〕

大阪市大 理 伊藤

EngBF 分子構造を構成するドメインのうち、ムチン型糖鎖の結合するペプチド部分を認識あるいは相互作用するような領域に関する特徴はあるのでしょうか。

〔答〕

ペプチド部分と相互作用する領域に関する考察はありませんが、ムチンにはコアペプチドを完全に覆うくらいの多くの糖鎖が付加していますので、触媒ドメイン周辺に位置している複数の CBM と類似したドメインが、複数の結合価でこれらの糖鎖と結合するのではないかと考えています。その結果、触媒ドメインは基質であるムチン型糖鎖に効率よくアクセスできるのではないかと考えられます。

〔質問〕

日大・生資科・農化 袴田

ドッキングモデルを作成する際に、ホモ二糖である基質の還元末端、非還元末端の配向をどのように決定したのでしょうか？

〔答〕

以前の研究から、本酵素の触媒活性に重要な三つのアミノ酸残基 (Asp682, Asp789, Glu822) が推定されております。本酵素が加水分解反応を触媒するのは、還元末端側の N-アセチルガラクトサミンとセリンもしくはトレオニンとのグリコシド結合であります。それゆえ、これら三つのアミノ酸残基が還元末端側の N-アセチルガラクトサミンのアノマー炭素の近辺に位置するように配向を決定しました。