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Structure and Function of Exo-β-glucosaminidase from *Amycolatopsis orientalis*

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Abstract: We cloned and sequenced the gene encoding exo-β-glucosaminidase (GlcNase) from *Amycolatopsis orientalis*, and found that the gene has an open reading frame of 1032 residues with a calculated molecular mass of 110,557. The GlcNase has been classified as a member of family GH-2. Sequence alignments identified a group of GlcNase-related protein sequences forming a distinct subclass of family GH-2. When mono-*N*-acetylated chitotetraose [$(GlcN)_3$-GlcNAc] was hydrolyzed by the enzyme, the GlcN unit was produced from the nonreducing end together with the transglycosylation products. ^{1}H-NMR spectroscopy revealed that the enzyme is a retaining glycoside hydrolase. The rate of hydrolysis of the disaccharide, GlcN-GlcNAc, was somewhat lower than that of $(GlcN)_2$, suggesting that the *N*-acetyl group of the sugar residue located at (+1) site partly interferes with the catalytic reaction. Based on the time-course of the enzymatic hydrolysis of the completely deacetylated chitotetraose [$(GlcN)_4$], we obtained the values of binding free energy changes of +7.0, −2.9, −1.8, −0.9, −1.0 and −0.5 kcal/mol corresponding, respectively, to subsites (−2) (−1) (+1) (+2) (+3) (+4). Synergism resulting from mixing the *A. orientalis* GlcNase with *Streptomyces* sp. N174 endochitosanase was also observed when chitosan polysaccharide was used as the substrate. To identify the catalytic residue, mutations were introduced into the putative catalytic residues resulting in five mutated enzymes (D469A, D469E, E541D, E541Q and S468N/D469E) which were successfully produced. The four single mutants were devoid of enzymatic activity, indicating that Asp469 and Glu541 are essential for catalysis as predicted from sequence alignment of enzymes belonging to GH-2 family.

Key words: ***Amycolatopsis orientalis*, exo-β-glucosaminidase, chitosanase, subsites, chitooligosaccharides**

Chitin and chitosan are β-1,4-linked polysaccharides of *N*-acetylglucosamine (GlcNAc) and glucosamine (GlcN), respectively, and widely distributed in living organisms, including insects, crustaceans and fungi. In such organisms, chitin- and chitosan-degrading enzymes (chitinases and chitosanases) are directly involved in biological phenomena important for their life, such as moulting, morphogenesis, and aggressive and defensive actions toward the targets.[1)] It is generally accepted that two independent chemical processes catalyzed by endochitinases and exo-β-*N*-acetyl-D-glucosaminidases are involved in chitin degradation.[2,3)] Similar chemical processes are most likely needed for chitosan degradation: endochitosanases randomly hydrolyze chitosan polysaccharide, producing oligosaccharides, which are then hydrolyzed into the monosaccharide units by exo-β-glucosaminidase (GlcNase). For endochitosanases, amino acid sequences have recently been accumulated (http://pages.usherbrooke.ca/rbrzezinski/index.html), and the X-ray crystal structures of several endochitosanases have been reported.[4−6)] However, the structural information on GlcNases is still very limited. GlcNase was first purified from an actinomycete, *Nocardia orientalis* (present name: *Amycolatopsis orientalis*), and characterized by Nanjo *et al.*[7)] The enzyme was found to specifically hydrolyze the β-1,4-glucosaminide linkage of the non-reducing end GlcN residue, producing the monosaccharide unit. After this characterization, several GlcNases were purified from culture filtrates of filamentous fungi; *Trichoderma reesei* PC-3-7,[8)] *Penicillium funiculosum* KY616,[9)] and *Aspergillus oryzae* IAM2660,[10)] and their enzyme functions were characterized. On the other hand, Tanaka *et al.*[11)] reported the primary structure and properties of GlcNase from *Thermococcus kodakaraensis* KOD1. This was the first report on the structure of GlcNase. The enzyme exhibits a very unique structure, which consists of the family GH-35 domain and the family GH-42 domain. Very recently, we cloned, sequenced, and expressed the gene encoding GlcNase from *Amycolatopsis orientalis*, and the enzyme protein produced by the *Streptomyces lividans* system was characterized.[12,13)] We also reported that Asp469 and Glu541 are the catalytic residues.[13)] An exo-β-glucosaminidase possessing a structure similar to that of *A. orientalis* GlcNase was also found in *T. reesei* PC-3-7.[14)] In this review article, all of these findings on *A. orientalis* GlcNase are reviewed, and discussed from the viewpoints of enzymology and glycobiotechnology.

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Abbreviations used are: GlcN, 2-amino-2-deoxy-D-glucopyranose; $(GlcN)_n$, β-1,4-linked oligosaccharide of GlcN with a polymerization degree of *n*; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; $(GlcN)_3$-GlcNAc, mono-*N*-acetylchitotetraose of which the reducing end residue is *N*-acetylated; GlcNase, exo-β-D-glucosaminidase; GH, glycoside hydrolase; ORF, open reading frame; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance.

Cloning of the GlcNase gene and the enzyme production in a heterologous host.

The enzyme protein purified from culture supernatant of *A. orientalis* was partially sequenced, and the N-terminal sequence was determined to be AAGNATPIP GYVNIQ. Further sequencing of a fragment resulting from the GlcNase digestion with trypsin gave an internal sequence of AQ[IL]SQYENVR. PCR primers derived from these sequences allowed us to amplify a fragment of −1.6 kb from the genomic DNA of *A. orientalis*, which was then used as a hybridization probe to clone the full length gene. The GlcNase gene was named *csx*A. The gene encoding GlcNase was subcloned into the shuttle vector pFD666 producing pFD666-*csx*A, which was then transformed into *Streptomyces lividans* TK-24. Activity was observed in the supernatant of the *S. lividans* transformant culture harbouring the plasmid pFD666-*csx*A, whereas no activity was found when the cells were transformed with the vector plasmid alone. The recombinant enzyme (CsxA) was purified from culture supernatant with a final recovery of 48.3% and a 3.7-fold purification by a simple two-step procedure, using SP-Sepharose and hydroxylapatite column chromatography. CsxA was purified to apparent homogeneity, as judged from SDS-PAGE. The GlcNases purified from the native and the heterologous hosts migrated similarly in SDS-PAGE and seemed to have a very similar, if not identical, molecular weight estimated at 103.5 kDa (Fig. 1).

Sequence of the GlcNase-encoding gene csxA.

Sequence analysis of the *csx*A gene reveals an open reading frame of 1032 amino acid residues with a calculated molecular mass of 110,557 Da. A search with the BLAST program (version 2.2.10)[15] revealed that the deduced amino acid sequence of CsxA exhibits similarity to a few hundred protein sequences found in databases (not shown). By similarity, CsxA appears to be a member of family GH-2. As shown in Fig. 1, the segment similar to other family GH-2 members ends at residues 897−899 and is followed by a putative carbohydrate-binding module belonging to family CBM-6, a feature rather unique among family GH-2 members. Two examples of the closest CsxA relatives are also shown in Fig. 1. The amino acid sequence showing the greatest similarity to CsxA is the sequence from the genome of *Streptomyces avermitilis* (SAV1223).[16] This ORF is shorter than CsxA as it does not have a CBM. SAV1223 exhibits 62.5% identity and 86.2% similarity with CsxA in an 893-residue overlap. The recombinant SAV1223 protein has been purified and shown to possess exo-β-glucosaminidase activity.[12] The other close relatives originate from sequenced fungal genomes and are diverse in their modular structure. They all share three modules found in all GH family 2 members: the N-domain or sugar binding domain, the immunoglobulin-like β-sandwich domain and the TIM barrel domain (PFAM database).[17]

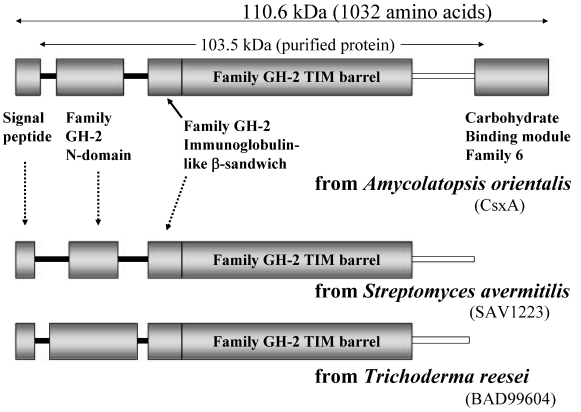

Fig. 1. Modular structure of CsxA and related ORFs.

Wide shaded boxes: modules identified by database searches and alignments. Narrow white boxes: regions of similarity not corresponding to known modules. Narrow black box: low similarity segments. The segment designated by 103.5 kDa of the CsxA sequence was produced in the culture filtrates of *A. orientalis* and *S. lividans* transformant harbouring pFD666-*csx*A.

In family GH-2, the catalytic functions are attributed to a nucleophile which is a glutamate located close to the C-terminus of the seventh β-strand and an acid/base residue which is a glutamate (always preceded by an asparagine) located close to the C-terminus of the fourth β-strand. We first tried to identify the putative catalytic residues in CsxA and related ORFs by alignments with T-Coffee and comparison with residues formally identified in other members of family GH-2.[18−22] We observed however that the way the residues were aligned by this program was very sensitive to the particular subset of sequences included in a given submission. More constant results were obtained by search and alignment in the Superfamily database (version 1.67)[23]. This search identified domain 3 from the 3D structure of human β-glucuronidase (Protein Data Bank entry 1BHG; residues 329−632)[24] as the best template for structure-guided alignment with the TIM-barrel domain sequences from CsxA and related ORFs, and allowed us to define the putative catalytic residues (Fig. 2). This revealed an unexpected trait of the TIM-barrel domains in CsxA and related ORFs: while the putative glutamate nucleophiles seemed to be strictly conserved in all the analyzed GH-2 members, a doublet Ser-Asp instead of Asn-Glu was observed at the location of the putative acid/base residue. This suggests that an aspartate instead of a glutamate could play the role of the catalytic acid/base residue in CsxA. This major difference reinforces the distinct character of the GlcNase-related proteins inside family GH-2. Thus, we proposed that the GlcNases form a distinct subclass of family GH-2.

Mechanism of enzymatic hydrolysis of $(GlcN)_3$-GlcNAc as determined by ^{1}H-NMR spectroscopy.

To examine the catalytic mechanism of CsxA, we determined the anomeric form of the reaction products from the enzymatic hydrolysis of $(GlcN)_3$-GlcNAc using ^{1}H-NMR spectroscopy. The doublet signal derived from the β-form of the GlcN monomer H1 appeared immediately after beginning the enzymatic reaction. However, the signal from the α-form did not appear until 30 min of the reaction time. The result suggests that the enzyme produces the β-form, which is then converted to the α-form by mutarotation. CsxA was found to be a retaining enzyme.

	457	469	536	542
CsAo	HPSVISFHIGSD		WSFNSET	
CsS	HPSVVSFLIGSD		TGFNSET	
CsAn	HSCMLAFLVGSD		FGFASEL	
CsG	HPSVIAFLVGSD		FGFGSEL	
CsH	HPSVLTFLVGSD		FGFGSEL	
CsM	HPSMLTFLVGSD		FGFGSEL	
CsN	HPSILGYLIGSD		FGFGSEL	
MaA	HPSLALWAGGNE		GRFANEF	
MaB	HPSIITWSGNNE		ARFVSEY	
MaH	HPSIIIWSGNNE		ARFASEY	
MaM	HPSIIIWSGNNE		ARLVSEY	
MaT	YPSLVLWNGNNE		PRFVAEF	
MaC	HASLVLWNGGNE		PRFCSEF	
GlC	HPSVVMWSVANE		PIIQSEY	
GlF	HPAVVMWSVANE		PIIQSEY	
GlH	HPAVVMWSVANE		PIIQSEY	
GlM	HPAVVMWSVANE		PIIQSEY	
GlE	HPSVVMWSIANE		PIIITEY	
GlS	HPSVVMWSIANE		PIMITEY	
GlL	HPSVIAWSLFNE		PFVFTEF	
GaC	HPSVLIWSCGNE		PYISCEY	
GaA	HASIVMWSLGNE		PFILCEY	
GaEco	HPSVIIWSLGNE		PLILCEY	
GaEcl	HPSIIIWSLGNE		PLILCEY	
GaL	HPSIIIWSLGNE		PLILCEY	
GaK	HPSIIIWSLGNE		PLILCEY	

Fig. 2. Assignment of putative catalytic residues in CsxA and related ORFs through structure-guided alignment with other GH-2 sequences.

The putative acid/base residues (Asp469) and nucleophile residues (Glu541) are shown in bold. Catalytic residues formally identified by mechanistic studies[18–22] are underlined. Numbering refers to the amino acid sequence of CsxA. Cs, CsxA and related ORFs. CsAo, *Amycolatopsis orientalis* (this work; csxA, AAX62629); CsS, *Streptomyces avermitilis* (NP 822398); CsAn, *Aspergillus nidulans* (AN2824.2); CsG, *Gibberella zeae* (FG02314.1); CsH, *Hypocrea jecorina* (BAD99604); CsM, *Magnaporthe grisea* (MG 05864.4); CsN, *Neurospora crassa* (XP_331434.1); Ma, β-mannosidases from: MaA, *Aspergillus niger* (Q9UUZ3, MANBA_ASPNG); MaB, *Bos taurus* (Q29444, MANBA_BOVIN); MaH: *Homo sapiens* (O00462, MANBA_HUMAN); MaM, *Mus musculus* (Q8K2I4_MOUSE); MaT, *Thermobifida fusca* (Q8KLI9_THEFU); MaC, *Cellulomonas fimi* (Q9XCV4_CELFI); Gl, β-glucuronidases from: GlC, *Canis familiaris* (O18835, BGLR_CANFA); GlF, *Felis silvestris catus* (O97524, BGLR_FELCA); GlH, *Homo sapiens* (P 08236, BGLR_HUMAN); GlM, *Mus musculus* (P12265, BGLR_MOUSE); GlE, *Escherichia coli* (P05804, BGLR_ECOLI); GlS, *Staphylococcus* sp. (patent PCT/US98/19217); GlL, *Lactobacillus gasseri* (Q9AHJ8_9LACO); Ga, β-galactosidases from: GaC, *Clostridium acetobutylicum* (P24131, BGAL_CLOAB); GaA, *Arthrobacter* sp. (Q59140|, GAL_ARTSB); GaEco, *Escherichia coli* (P 00722, BGAL_ECOLI); GaEcl, *Enterobacter cloacae* (Q47077, BGAL_ENTCL); GaL, *Lactococcus lactis* (Q48727; BGAL_LACLA); GaK, *Kluyveromyces lactis* (P00723; BGAL_KLULA).

Enzymatic hydrolysis of (GlcN)₃-GlcNAc as determined by HPLC.

The enzymatic reaction toward $(GlcN)_3$-GlcNAc was monitored by HPLC using detection by UV absorption (220 nm) originating from the *N*-acetyl groups of the substrate and the products. The initial substrate $(GlcN)_3$-GlcNAc was at first degraded into $(GlcN)_2$-GlcNAc, and then into GlcN-GlcNAc, but no *N*-acetylglucosamine monomer was produced at this stage (data not shown). This indicates that CsxA hydrolyzes the substrate from the nonreducing end in an exo-splitting manner. After a longer incubation period, the substrate was finally hydrolyzed into monosaccharides, GlcN and GlcNAc. To evaluate the sugar recognition specificity at (+1) site, we determined the rate of hydrolysis of GlcN-GlcNAc, and compared it with that of $(GlcN)_2$. The rate of the degradation of GlcN-GlcNAc is somewhat lower than that of $(GlcN)_2$. The *N*-acetyl group of the sugar bound to the (+1) site appears to partly interfere with the catalytic reaction. Since CsxA does not act toward $(GlcNAc)_2$, the (−1) site must have absolute specificity for GlcN. The transglycosylation products, whose molecular weights are larger than that of the initial substrate, were also detected in significant amounts. The transglycosylation reaction catalyzed by CsxA would be useful for producing GlcN-containing oligosaccharide derivatives with unique biological functions.

Time-course analysis of the enzymatic hydrolysis of (GlcN)₄.

From the substrate $(GlcN)_4$, CsxA predominantly produced GlcN and $(GlcN)_3$, which was further degraded into GlcN and $(GlcN)_2$ (data not shown). The course of the degradation exhibited a typical case of an exo-splitting enzyme. The transglycosylation product, $(GlcN)_5$, was also produced, together with a lesser amount of $(GlcN)_6$. It appears that $(GlcN)_5$ is produced by the glycosyl transfer of the transition state GlcN to the initial substrate $(GlcN)_4$, as shown in Fig. 3. Similarly, $(GlcN)_6$ appears to be produced by the transfer action to the product $(GlcN)_5$. The reaction time-course quantitatively determined by HPLC was used for the modeling study, which yielded the optimized values of the binding free energies of the individual subsites, (−2), (−1), (+1), (+2), (+3) and (+4), and three rate constants, k_{+1} (cleavage of glycosidic linkage), k_{-1} (transglycosylation), and k_{+2} (hydration). The optimization based on the reaction model shown in Fig. 3 successfully produced the rate constant values and the free energy values of the individual subsites as listed in Table 1. The time-course calculated with these values coincided satisfactorily with the experimental one (data not shown). The affinities for sugar residues were found to be highest at subsites (−1) and (+1), while remote subsites were found to have lesser affinities. The free energy distribution in the substrate binding cleft is very similar to those of glu-

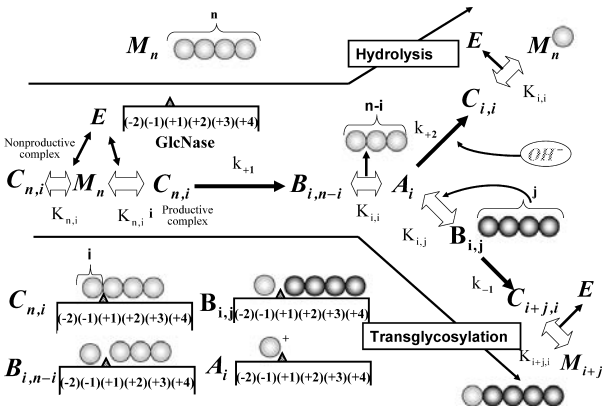

Fig. 3. Reaction model for CsxA-catalyzed hydrolysis and transglycosylation reaction.

E and *Mn* represent the enzyme and the substrate with a polymerization degree of *n*, respectively. Notations of individual complexed states, A_i, $B_{i,j}$ and $C_{n,i}$, are schematically described in the figure. Reaction parameters consist of three rate constants, k_{+1} (bond cleavage process), k_{-1} (bond regeneration process) and k_{+2} (hydration process), and six binding free energy changes of individual subsites, (−2)～(+4). In practical calculation, all of the possible binding modes were taken into consideration.

Table 1. Rate constants and binding free energy values used for the calculation of reaction time-course shown in Fig. 4B.

	Rate constants (s^{-1})			Binding free energy changes (kcal/mol)					
	k_{+1}	k_{-1}	k_{+2}	(−2)	(−1)	(+1)	(+2)	(+3)	(+4)
$(GlcN)_2$	120.0								
$(GlcN)_3$	120.0								
$(GlcN)_4$	60.0	220.0	200.0	+7.0	−2.9	−1.8	−0.9	−1.0	−0.5
$(GlcN)_5$	30.0								
$(GlcN)_6$	25.0								

coamylases possessing similar cleavage specificity.[25−27] The model predicts that shorter substrate chain lengths correspond to higher k_{+1} values. In our previous paper,[12] we reported that the specific activities toward $(GlcN)_2$～$(GlcN)_6$ are similar to each other. The lower k_{+1} values for the longer substrates might be compensated for by the higher affinity to the binding cleft. In fact, by simply assuming additivity, the affinity for $(GlcN)_5$ binding to (−1)～(+4) is higher than that for $(GlcN)_4$ binding to (−1)～(+3) by 0.5 kcal/mol, and $(GlcN)_4$ binding to (−1)～(+3) is stronger than $(GlcN)_3$ binding to (−1)～(+2) by 1.0 kcal/mol.

Synergism between endochitosanase and exo-β-glucosaminidase.

It has been recognized that polysaccharides are enzymatically degraded into monomers by several enzymes with different cleavage specificities.[28] Fukamizo and Kramer[3] reported that endochitinase and β-*N*-acetylglucosaminidase from the moulting fluid of the tobacco hornworm, *Manduca sexta*, concertedly act on the chitin chain, resulting in a synergistic effect on chitin degradation. In the enzymatic degradation of cellulose, synergism has been intensively studied using various types of cellulases from *Trichoderma reesei.*[29,30] Synergism might be found also in chitosan degradation upon mixing the endochitosanase with GlcNase. Thus, we measured the rate of product formation from chitosan by the binary enzyme system consisting of CsxA and *Streptomyces* sp. N174 endochitosanase. In the presence of an excess amount of endochitosanase, the rate of product formation was enhanced by about two-fold of the sum of those obtained by individual enzymes; that is, the synergistic factor was about two (data not shown). By increasing the ratio of CsxA to the endo-splitting enzyme, the synergistic factor was gradually enhanced, and reached maximum (4.1) when the enzyme molar ratio was 2:1 (exo: endo). In the binary chitinase system (exo-β-*N*-acetylglucosaminidase and endochitinase) of the tobacco hornworm, *Manduca sexta*, which is responsible for the destabilization of old cuticle, the greatest synergism of six takes place at a 1:6 (exo: endo) ratio of enzymes, typically found in the moulting fluid secreted from epidermal cells.[3] In the cellulase systems studied thus far, the synergistic factor was reported to be 2−10.[28] Thus, our binary chitosanase system exhibits a moderate synergism, and is efficient for monomer production. In contrast to the binary chitinase system from *Manduca sexta*, a higher amount of the exo-enzyme is required to attain the maximum synergism in our binary chitosanase system. The exo-splitting process might be rate-limiting in the tandem action of the chitosan degradation, whereas the endo-splitting process is rate-limiting in the chitin degradation in insects.[3] An endo-splitting chitosanase was isolated from *A. orientalis*, and enzymatically characterized.[31] The enzyme can hydrolyze the β-1,4-linkage of GlcN-GlcNAc in addition to that of GlcN-GlcN, whereas *Streptomyces* sp. N174 chitosanase hydrolyzes GlcNAc-GlcN in addition to GlcN-GlcN.[32] Thus, some fraction of the products from *A. orientalis* endochitosanase would consist of oligosaccharides possessing a GlcNAc residue at the nonreducing end. Since such oligosaccharides could not be hydrolyzed by CsxA, a lower synergism would be obtained when the endo-splitting chitosanase from *A. orientalis* is used instead of the *Streptomyces* sp. N174 chitosanase. Thus, the binary chitosanase system used in this study is an efficient tool for the industrial production of glucosamine monomer from chitosan.

Catalytic residues.

Sequence alignment of the GH-2 enzymes shown in Fig. 2 revealed that most GH-2 members possess a catalytically important NE diad, which is replaced with a SD diad in the enzymes belonging to the putative GlcNase subclass, including the enzymes from *A. orientalis* and *T. reesei*,[14] and also the SAV1223 protein from *Streptomyces avermitilis*, shown to possess GlcNase activity.[12] The SD diad corresponds to Ser468 and Asp469 in CsxA. A strictly conserved glutamic acid was found at the 541st position of CsxA, and is supposed to be the catalytic nucleophile. Thus, we mutated Ser468, Asp469 and Glu541 residues of CsxA to elucidate their role. We successfully produced and purified the wild type and five mutated enzymes (D469E, D469A, E541D, E541Q and S468N/D469E). The profiles of the CD spectra of D469E and S468N/D469E are different from that of the wild type in the region of 205−215 nm (data not shown). The mutation of Asp469 to glutamic acid would affect the secondary structure of the enzyme. In the spectra for the other mutated enzymes, however, the profiles are basically identical to that of the wild type indicating that the global conformation of the enzyme is not affected by these mutations. Enzymatic activities of these enzyme preparations were determined by quantifying the GlcN liberated from the substrate $(GlcN)_2$. As listed in Table 2, the mutation of Asp 469 to alanine completely abolished the enzymatic activity, while D469E exhibited very low enzymatic activity. Both the E541-mutated enzymes were found to possess very low activity, but the activity of E541D is significantly greater than that of E541Q. These results clearly demonstrated that Asp469 and Glu541 are the catalytic acid/base and the nucleophile, respectively. It is most likely that Asp469 donates the proton to the glycosyl oxygen and the Glu541 carboxylate stabilizes the transition state of the C1 carbon of the −1 sugar residue. The double mutation of Ser468 and Asp469 (SD diad) to Asn and Glu (NE diad), respectively, also abolished the GlcNase activity. In most of the members of family GH-2, which possess endo-β-mannosidase, β-glucuronidase and β-galactosidase activities, the SD diad is replaced with NE.[12] Thus, the double mutant was assayed with the sub-

Table 2. Enzymatic activities of the wild type and mutated exo-β-glucosaminidase from *Amycolatopsis orientalis.*

Enzyme	Activity (U/mg)	Relative activity (%)
Wild type	14.95	100.0
D469E	0.0011	0.007
D469A	n.d.	–
E541D	0.016	0.1
E541Q	0.0019	0.012
S468N/D469E	n.d.	–

n.d., not detected.

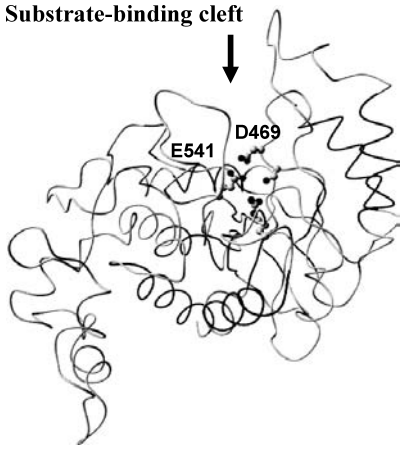

Fig. 4. Structure of the TIM barrel domain of CsxA obtained by homology-modeling calculation (the side view of the barrel fold).

The structure of human β-glucuronidase (Protein Data Bank entry 1BHG; residues 329–632)[24] was used as a template. The molecular model was drawn with the programs YASARA (http://www.yasara.org) and PovRay (http://www.povray.org).

strates, β-galactoside, β-mannoside and β-glucuronide, but no activity was detected toward any of these substrates (data not shown). Some other structural factors might participate in the catalytic activity of the family GH-2 enzymes with the NE diad.

Modeled structure of the Amycolatopsis GlcNase.

Figure 4 shows the three dimensional structure of the TIM barrel domain of CsxA obtained by homology-modeling calculation. The structure of human β-glucuronidase (Protein Data Bank entry 1BHG; residues 329–632)[24] was used as a template. Asp469 and Glu541 are located at the bottom of the substrate binding cleft, and the distance between the two residues is about 3.2 Å. The positioning of the two carboxylates supports the idea that these two residues are catalytic residues. X-ray crystallographic analysis of the enzyme is under progress, and more detailed discussion of the structure and function will become possible in the near future.

CONCLUSION

GlcN occurs in high concentration in the joints. It has been demonstrated that GlcN stimulates the formation of cartilage that is essential for joint repair and is beneficial for arthritis treatment. In this situation, it is highly desirable to produce GlcN efficiently from chitosan polysaccharide. Exo-β-glucosaminidase is one of the most important enzymes for GlcN monomer production from chitinous compounds. For efficiently utilizing the enzyme, it is essential to understand its structure and function. We have successfully cloned, sequenced, and expressed the GlcNase gene from *Amycolatopsis orientalis*, and the recombinant enzyme has been characterized enzymologically. Based on the information, it has become possible to design an optimal condition for GlcN production from chitosan polysaccharide. The enzyme was found to catalyze transglycosylation in addition to hydrolysis. Such an additional activity might be useful for enzymatic synthesis of a novel carbohydrate possessing unique biological functions.

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Amycolatopsis orientalis 由来エキソ-β-グルコサミニダーゼの構造と機能

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Amycolatopsis orientalis 由来エキソ-β-グルコサミニダーゼ (GlcNase) の遺伝子のクローニングを行い，その配列を調べた．その結果，本酵素は 1032 個のアミノ酸からなり，その配列から推定される分子量は，110,557 と算出された．アミノ酸配列に基づいて，本酵素は family GH−2 に属し，その中でその他の GlcNase 遺伝子とともに GlcNase サブクラスを形成していることがわかった．モノアセチル化キトテトラオース［$(GlcN)_3$-GlcNAc］を本酵素で加水分解すると，非還元末端から GlcN 残基が放出され，さらに糖転移反応生成物も得られることがわかった．−1 サイトでは GlcN に対して高い特異性をもつが，+1 サイトではそのような特異性はみられなかった．実験的に得られたキトテトラオース［$(GlcN)_4$］に対する反応の経時変化に基づいて，各サブサイトの親和性 (結合自由エネルギー変化) を推定したところ，−2, −1, +1, +2, +3, +4 のそれぞれに対して，+7, 0, −2.9, −1.8, −0.9, −1.0, −0.5 kcal/mol という値を見積もることができた．本酵素を *Streptomyces* sp. N174 由来エンドキトサナーゼと混合させて，高分子キトサンを基質として反応させたところ，明確な相乗効果を確認することができた．family GH-2 に属するいくつかの酵素とのシーケンスアラインメントおよび部位特異的変異導入によって，触媒残基は Asp469 と Glu541 であることが明らかになった．