



Title	Structural Elucidation of Dextran Degradation Mechanism by Streptococcus mutans Dextranase Belonging to Glycoside Hydrolase Family 66
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Citation	Journal of Biological Chemistry, 287(24), 19916-19926 https://doi.org/10.1074/jbc.M112.342444
Issue Date	2012-06-08
Doc URL	https://hdl.handle.net/2115/51763
Rights	This research was originally published in Journal of Biological Chemistry. Nobuhiro Suzuki; Young-Min Kim; Zui Fujimoto; Mitsuru Momma; Masayuki Okuyama; Haruhide Mori; Kazumi Funane; Atsuo Kimura. Structural Elucidation of Dextran Degradation Mechanism by Streptococcus mutans Dextranase Belonging to Glycoside Hydrolase Family 66. Journal of Biological Chemistry. 2012; 287:19916-19926. © the American Society for Biochemistry and Molecular Biology.
Type	journal article
File Information	JBC287-24_19916-19926.pdf



Structural elucidation of dextran degradation mechanism by *Streptococcus mutans* dextranase belonging to glycoside hydrolase family 66*

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*Running title: *Crystal structure of Streptococcus mutans GH-66 dextranase*

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Keywords: dextranase, crystal structure, catalytic mechanism

Background: Dextranase hydrolyzes α -1,6-linkages of dextran producing isomaltooligosaccharides.

Result: Crystal structure of *Streptococcus mutans* dextranase belonging to the glycoside hydrolase family 66 was determined.

Conclusion: The enzyme structures complexed with isomaltotriose and suicide substrate revealed the enzyme's catalytically important residues.

Significance: This is the first structural report for a GH-66 enzyme elucidating the enzyme's catalytic machinery.

SUMMARY

Dextranase is an enzyme that hydrolyzes dextran α -1,6 linkages. *Streptococcus mutans* dextranase (SmDex) belongs to glycoside hydrolase family 66, producing isomaltooligosaccharides of various sizes, and consisting of at least five amino acid sequence

regions. The crystal structure of the conserved fragment from Gln-100 to Ile-732 of SmDex, devoid of its N and C-terminal variable regions, was determined at 1.6 Å resolution and found to contain three structural domains. Domain N possessed an immunoglobulin-like β -sandwich fold, domain A the enzyme's catalytic module, comprising a $(\beta/\alpha)_8$ -barrel, and domain C formed a β -sandwich structure containing two Greek key motifs. Two ligand complex structures were also determined and, in the enzyme/isomaltotriose complex structure, the bound isomaltooligosaccharide with four glucose moieties was observed in the catalytic glycone cleft and considered to be the transglycosylation product of the enzyme, indicating the presence of four subsites -4 to -1 in the catalytic cleft. The complexed structure with 4',5'-epoxypentyl- α -D-glucopyranoside, a

suicide substrate of the enzyme, revealed that the epoxide ring reacted to form a covalent bond with the Asp-385 sidechain. These structures collectively indicated that Asp-385 was the catalytic nucleophile and Glu-453 the acid/base of the double displacement mechanism, in which the enzyme showed a retaining catalytic character. This is the first structural report for a GH-66 enzyme elucidating the enzyme's catalytic machinery. (215 words)

Introduction

Streptococcus mutans is a Gram-positive bacterium that has been implicated as a major cariogenic bacteria (1, 2), which metabolizes sugars, including sucrose, glucose, fructose and lactose, to lactic acid. With *S. mutans* accumulation on tooth surfaces as dental plaque, the lactic acid concentration increases and lowers the oral cavity pH, which leads to demineralization of tooth enamel, the origin of dental caries. Extracellular glucans, synthesized from sucrose by glucosyltransferase enzymatic activities, are known to enhance *S. mutans* biofilm formation. Glucosyltransferases synthesize both water-soluble glucan, such as dextrans with linear α -1,6-linked glucose units, and water-insoluble glucans, which are sticky polysaccharides composed of glucose units, predominantly in α -1,3-linkage, and with various degrees of branching and associated with bacterial cell adhesion to the tooth surface.

Endodextranase (EC 3.2.1.11; 6- α -D-glucan-6-glucanohydrolase) from *S. mutans* (SmDex) is an enzyme that hydrolyzes α -1,6-linkages of dextran (1, 3, 4) and produces isomaltooligosaccharides (IGs) of various sizes; it has been shown to modify glucan structure by controlling the amount and content of extracellular glucans (5, 6). SmDex belongs to the glycoside hydrolase family (GH)-66 according to the CAZy database (<http://www.cazy.org/>) (7). GH-66 enzymes consist of dextranases and cycloisomaltooligosaccharide

glucanotransferases (CITase; EC 2.4.1.248), enzymes found mainly in bacteria. Both enzymes utilize dextran as a substrate, but CITases synthesize cycloisomaltooligosaccharide (cyclic saccharides linked by α -1,6-glucosyl bonds) from dextran by intramolecular transglycosylation (8–10). The *dex* gene containing an open reading frame of 2553 bp encoding SmDex enzyme, was first cloned from *S. mutans* Ingbritt and its nucleotide and amino acid (aa) sequences were determined (11). The expressed protein is composed of 850 aa residues with a molecular mass of 94.5 kDa, but the formation of protease-associated multiple isoforms has been reported; similar observations have also been also reported regarding many other GH-66 dextranases of native and recombinant forms (12). According to amino acid sequence analysis of GH-66 enzymes, SmDex has been divided into five regions: a signal peptide sequence (N-terminal 24 aa), an N-terminal variable region (Ser-25–Asn-99), a conserved region (Gln-100–Ala-615), a glucan binding site (Leu-616–Ile-732) and a C-terminal variable region (Asn-733–Asp-850) (13–15). CITase has an extra-long insertion of ~90 aa inside the dextranase conserved region (16).

Biochemical studies using site-directed mutagenesis based on amino acid sequence comparison with other glucosyltransferases have revealed that Asp-385 is essential for the catalytic reaction (14). On the other hand, Asp-270 of CITase from *Bacillus circulans* T3040 (17) and Asp-243 of endodextranase from *Thermotoga lettingae* TMO (18), both corresponding to Asp-385 in SmDex, have been implicated as catalytic residues. Here, we recently conducted mutational analyses on two GH-66 enzymes, CITase and dextranase from *Paenibacillus* sp., and described three amino acid residues essential for catalysis (19, 20). The detailed catalytic mechanism of this enzyme family has, however, not been elucidated due to the lack of the determination of a GH-66 enzyme's three dimensional structure. We have

recently produced a truncation mutant of SmDex, which is devoid of its N and C-terminal variable regions (SmDexTM; bearing Gln-100 to Ile-732) (21). The recombinant full-size SmDex protein is proteolytically degraded to more than seven polypeptides (23–70 kDa) during long storage. SmDexTM does not accept any further protease-degradation and shows enhanced substrate hydrolysis, suitable for further biochemical analysis and industrial applications. In this article, the crystal structure of SmDexTM is introduced, representing the first described crystal structure of a GH-66 family enzyme as well as insights into other family structures in relation to their enzymatic activities. The binary complex structures were also determined in the presence of IGs and of a covalently bonded structure from a suicide substrate, which allowed elucidation of the enzymatic reaction mechanism together with the substrate recognition site.

EXPERIMENTAL PROCEDURES

Expression and crystallization of SmDex—SmDexTM originated from *S. mutans* ATCC 25175 (American Type Culture Collection, Manassas, VA, USA) and was expressed, purified and crystallized as previously reported (21, 22). The SmDex gene lacking the N-terminal 99 and C-terminal 118 residues was cloned into pET28 (Novagen Inc., Madison, WI, USA) and overexpressed in *Escherichia coli* BL21 (DE3) cells (Agilent Technologies Inc., Santa Clara, CA, USA). The expressed protein was purified by Ni²⁺-charged Hi-TrapTM Chelating HP column chromatography (GE Healthcare, Ltd., Buckinghamshire, UK) and anion exchange chromatography using a Hi-TrapTM Q-SepharoseTM HP (GE Healthcare). The resulting protein was concentrated to 3.6 mg ml⁻¹ and crystallized by the sitting-drop vapor diffusion method using an equal amount of precipitant solution composed of 30% polyethylene glycol monomethyl ether 2000 (w/v, Hampton Research, Aliso Viejo, CA, USA), 0.1 M phosphate-citrate buffer at pH 4.2

(Hampton Research) and 293 K. Selenomethionine (SeMet)-labeled SmDexTM was expressed in LeMaster medium (23) using the methionine-auxotrophic strain *E. coli* B834(DE3) and crystallized under the same conditions as for the native enzyme.

Data collection and structure determination—Diffraction experiments for the native and SeMet-substituted crystals were conducted at the protein microcrystallography beamline, BL-17A, of the Photon Factory (PF), High Energy Accelerator Research Organization, Tsukuba, Japan. Native diffraction data to 1.60 Å resolution (space group *P*2₁) were collected using an ADSC-Q270 CCD detector (Area Detector Systems Corp., Poway, CA, USA). Crystals were cryocooled in a nitrogen gas stream to 95 K. While complexed structural analyses of the enzyme with isomaltotriose (IG3; Seikagaku Corp., Tokyo, Japan) or 4',5'-epoxypentyl- α -D-glucopyranoside (E5G) (24, 25), SmDexTM crystals were soaked into a drop containing 5% (w/v, 99 mM) IG3 or 1 mM E5G in the precipitant solution for 10 min or 1 h, respectively, before the diffraction experiment. Diffraction data of the IG3 complex to 1.90 Å resolution were collected at 100 K, using R-axis VII imaging plate area detectors and CuK α radiation generated by a rotating-anode generator MicroMax007 (Rigaku Corp., Akishima, Japan). The E5G complex data were collected at the beamline BL-5A, PF. All data were integrated and scaled using the DENZO and SCALEPACK programs in the HKL2000 program suite (26). Crystal structure was determined by the multiwavelength anomalous dispersion method using SeMet-labeled crystals (27), and 12 selenium atom positions were determined and initial phases calculated using SOLVE/RESOLVE program (28, 29). The solution was subjected to the auto-modeling ARP/wARP program (30) settled in the CCP4 program suite (31). Manual model building and molecular refinement were performed using the Coot (32) and REFMAC5 programs (33, 34).

For the analyses of ligand-binding structures, structural determination was conducted using the ligand-free structure as the starting model and the bound ligand observed in the electron density difference map. Data collection and refinement statistics are shown in Table 1. Stereochemistry of the models was analyzed with the Rampage program (35) and structural drawings prepared using the PyMol program (DeLano Scientific LLC, Palo Alto, CA, USA).

RESULTS

Overall structure of SmDexTM—The crystal structure of SmDexTM was determined by the multiwavelength anomalous dispersion method using SeMet derivative data and successively, the native and two ligand complex structures with IG3 (SmDexTM/IG) or E5G (SmDexTM/E5G) determined. Structural refinement statistics are summarized in Table 1 and the quality and accuracy of the final structures were further demonstrated as more than 98% of their residues fell within the common regions of the Ramachandran stereochemistry plot. The recombinant SmDexTM molecule was composed of a single polypeptide chain of 643 aa (98–740), where the N-terminal Met-98 and Asp-99 and the C-terminal ⁷³³LEHHHHH⁷⁴⁰ were derived from the expression vector and purification tag. The N-terminal 4 residues Met-98–Lys-101 and the C-terminal 5 residues His-736–His-740 were not identified due to lack of electron density. The final model consisted of one SmDexTM molecule accompanied with 1 phosphate ion.

The protein formed a multidomain structure comprised of three domains, designated as domains N, A, and C (Fig. 1). Domain N (Asn-100–Asp-210) adopted an immunoglobulin-like β -sandwich fold and its amino acid sequence showed no significant similarity with other domains in glycoside hydrolases, although a number of structures having similar immunoglobulin folds have been observed, including β -galactosidase (domains 2 and 4) (36), β -mannosidase (domains 2 and 4)

(37), endoglucanase (PKD domain) (38), and bacterial sialidases (immunoglobulin-like linker domain) (39). The topology of domain N resembled a C2-type immunoglobulin fold, consisting of seven antiparallel β -strands forming 3-stranded and 4-stranded β -sheets (Fig. 1B) (40, 41): a short loop split the 5th β -strand into two strands N β 5 and N β 5' and, accordingly, the 2nd strand was divided into N β 2 and N β 2'.

Domain A (Asp-211–Lys-593) was mainly composed of a $(\beta/\alpha)_8$ -barrel, which is a catalytic domain in many glycoside hydrolases. Two catalytic amino acid residues, which were detected by the SmDexTM/IG complex structure described below, were located on the concave surface formed by the central β -barrel C-terminal side. A structural homology search, using the Dali server (42), revealed that the domain A structure was similar to the catalytic domains of many GH-13 subfamilies, especially subfamily 20, such as in *Thermoactinomyces vulgaris* R-47 α -amylases 1 (TVAI, PDB code 2D0H, subfamily unknown) (43) and 2 (TVAIL, PDB code 3A6O, subfamily 20) (44) and maltogenic amylase (PDB code 1GVI, subfamily 20) (45) with Z-scores of 18.2, 18.7, and 18.6, and root-mean-square (rms) differences of 3.6, 3.7, and 3.5 Å, respectively. There were five relatively large looped regions in this domain, ranging from 23 to 50 residues in length, and the 50-residue long loop 3 associated with loop 2 to form a small subdomain structure (Fig. 1, dark green), which was similarly positioned in comparison to the domain B of α -amylases, forming one sidewall of the catalytic cleft.

Domain C (Val-594–Ile-732) adopted an antiparallel β -sandwich structure, consisting of 10 β -strands but basically belonged to two 4-stranded Greek key motifs, which are found in many GHs. Sequence and deletion analyses of SmDex by Morisaki et al. indicated this domain as a dextran binding domain (15), but the deduced beginning of the domain Leu-616 was different from Val-594 in the actual domain structure observed here.

The domain arrangement of SmDex resembled some GH-13 proteins, including TVAI (43), which consists of domain N with an immunoglobulin fold, catalytic domain A with a $(\beta/\alpha)_8$ -barrel and domain B, which possesses a structure dissimilar from loop 3 of SmDex, and the domain C with the Greek key motifs. The relative arrangement of domains N and C was, however, different between these enzymes, when the catalytic domains were superimposed (Fig. 2).

Crystal structure of SmDexTM complexed with isomaltotriose—IG3 was used for enzyme-substrate/product complex analysis as a means to elucidate the enzyme's catalytic mechanism. In the electron density map, bound sugars were observed at two positions, in the catalytic cleft and on domain N (Fig. 1). In the catalytic cleft, the modeled ligand was composed of four glucose moieties and appeared as a tetraisomaltooligosaccharide (IG4, Figs. 3A and 3B), the product of a reversed or transglycosylation reaction by the enzyme. The other bound isomaltooligosaccharide showed two glucose moieties that appeared to be an isomaltose in the gap between domain N of an enzyme molecule and domain C of an adjacent molecule in the crystal (Fig. 3C). The two domains contributed to the binding through a few hydrogen bonds and hydrophobic interactions, but no aromatic sidechain was involved. Taking these facts into account, the latter ligand appeared to be a crystal packing artifact.

The overall structure of the SmDexTM/IG complex was almost identical to that of ligand-free SmDexTM with an rms difference of 0.73 Å, implying that ligand binding had little effect upon the overall structure. The main differences in the backbone structures were confined to the solvent-exposed loops in domains A and C, and four residues (Ile-249–Lys-252) in loop 2 of domain A were invisible in the SmDexTM/IG complex's electron density map.

The catalytic cleft was located at the center surface of the $(\beta/\alpha)_8$ -barrel. The enzyme subsites were named according to Davies et al. (46) and the transglycosylated ligand occupied subsites –4 to –1 from the non-reducing end to the reducing end, with the glucose moieties in subsites –4 to –1 designated as Glc–4 to Glc–1. All glucose moieties were in the relaxed chair conformation with the glucose moiety Glc–1 in the β -anomeric conformation and the O1 atom in the proximity of the Asp-385 sidechain, which was the candidate of a catalytic nucleophile. However, dextran has α -1,6-linkages and hydrogen bonds cannot be formed with the scissile bond of the natural substrate. The Asp-385 O δ 2 atom was also close to the Glc–1 C1 atom with a distance of 3.1 Å, implying its role as a nucleophile. The other acidic residue, Glu-453, formed a hydrogen bond with the Glc–1 O2 atom via the O ϵ 2 atom. When Glc–1 adopts an α -anomeric conformation, as in natural substrates, the Glu-453 O ϵ 1 atom would be situated within hydrogen bonding distance of the Glc–1 O1 atom and could provide hydrogen in the catalytic bond, suggesting that it would act as an acid/base in the catalytic reaction. The O3 and O4 atoms of Glc–1 hydrogen-bonded to the Ala-559 mainchain O atom and the Tyr-257 O η atom, respectively, and Glc–1 was recognized by 5 direct hydrogen bonds.

Glc–2 was also found to participate in 5 direct hydrogen bonds with three tyrosine residues, Tyr-257, Tyr-260 and Tyr-307, and aspartic acid Asp-258. Asp-258 recognized two oxygen atoms O2 and O3 of Glc–2, playing an important role in substrate recognition at subsite –2. Besides the hydrogen bonds, subsite –2 was built by the hydrophobic residues of Trp-277, Trp-280, and Met-309, and Glc–2 was strictly recognized by the protein. In contrast, Glc–3 showed no direct hydrogen bonding to the protein and Glc–4 exhibited one hydrogen bond between its O2 atom and the sidechain of Thr-563. These two glucose moieties were embedded onto the two aromatic residues of Trp-280 and Tyr-560, appearing to be loosely

recognized with their electron density, especially for Glc-4, being relatively weak. The average *B*-factors of 6 cyclic atoms of the glucose rings were 51.6, 45.7, 56.7, and 64.8 Å² for Glc-1 to 50

5 Glc-4, respectively, which also implied that subsites -1 and -2 showed strong glucose binding.

Crystal structure of SmDexTM complexed 55

10 with E5G—The SmDexTM/E5G complex structure was determined to label and visually identify the enzyme's nucleophile residue. The resulting overall structure superimposed well with the ligand-free SmDexTM and 60 SmDexTM/IG complex structures, with rms differences of 0.20 and 0.65 Å, respectively. The electron density at the active site accommodated a single E5G moiety and the E5G density was connected to the Asp-385 Oδ2 atom, revealing 65 that E5G was covalently bound to the enzyme. The E5G glucose moiety was located at subsite -2, identical to Glc-2 of the SmDexTM/IG complex structure, and recognized in the same manner by Tyr-257, Asp-258, Tyr-260, and 70 Tyr-307 (Fig 3D). The E5G alkyl chain, consisting of the 1'-4'-C atoms, occupied the similar positions of C6, C5, O5 and C1 atoms of the Glc-1 of the IG3 complex, respectively, but was slightly shifted towards Asp-385 (Fig 3E). 75 This was probably due to the covalent linkage between the E5G 4'-C atom and the Asp-385 Oδ2 atom after opening of the epoxide ring formed by 4' and 5'-C's and epoxy oxygen.

35 DISCUSSION

In this study, the crystal structure of SmDexTM was determined, yielding the first example of a fully described GH-66 protein. The length of GH-66 proteins (including precursor 85 forms) varies from 536 to 1686 aa residues. The 633 aa sequence used in this study covered the entire conserved region of GH-66 proteins and thus, the SmDexTM crystal structure obtained could be considered a structural representative 90 of this family. Although CITases have a carbohydrate-binding module family (CBM)-35

that splits the catalytic domain A into two regions in their primary structures (Fig. 4) (10, 16), the GH-66 protein minimal component comprises three domains N, A, and C. In addition, the larger GH-66 proteins have C-terminal variable regions and are expected to contain different domain structures, as can be deduced from the Conserved Domain Database (47). These proteins include CBM-6 or CBM-35 in *Paenibacillus* sp. dextranase (48), CITase (10), CBM-4/9, or CBM-61 in *Paenibacillus* sp. dextranase, and CBM-2 or a fibronectin type III fold in *Catenulisporea acidiphila* dextranase (49). The roles of these C-terminal domains remain unclear, but CBMs generally are involved in binding complicated substrates and in assisting catalysis in the catalytic domain, and they are assumed to be dextran-binding modules. In addition, some GH-66 enzymes possess regions in their C-terminus for cell wall association, and include sorting signals with LPXTG cell wall anchor motifs in SmDex (13) and S-layer homology (SLH) domains, which associate noncovalently with cell walls (50).

SmDex, as well as the other GH-66 dextranases and CITases, are retaining enzymes, targeting substrates with α-anomeric conformations and releasing an α-anomeric product, and thus their catalytic mechanism is supposed to be a double displacement mechanism (51). There have been reports suggesting that Asp-385 of SmDex (14) and the corresponding residues in other GH-66 enzymes are the nucleophiles (17, 18). Here, the SmDexTM/IG complex structure revealed that the Asp-385 Oδ2 atom was located close to the Glc-1 C1 atom, with a distance of 3.1 Å. In addition, the SmDexTM structure when complexed with E5G, a widely used suicide substrate employed to label the nucleophile of the retaining glycosidases (52), showed that the epoxy ring opened up and the 4'-C atom of the alkyl chain moiety formed a covalent bond with the Asp-385 Oδ2 atom. Structural evidence obtained here visually proved that Asp-385 is the nucleophile of SmDex. On the other hand,

there has been no report identifying the catalytic acid/base entity in GH-66 proteins. The SmDexTM/IG complex structure here showed that the C-1 hydroxyl group of Glc-1 took β -anomeric conformation and that the distance between the Glu-453 O ϵ 1 atom and the Glc-1 C1 atom was 3.9 Å. However, the distance between the Glc-1 O1 and Glu-453 O ϵ 1 atoms would be within hydrogen-bonding distance assuming the Glc-1 O1 atom was in α -anomeric conformation. Furthermore, in the ligand-free SmDexTM structure, the Glu-453 O ϵ 1 and Asp-385 O δ 2 atoms were apart by 6.0 Å, a reasonable distance between the two catalytic residues of a retaining type glycosidase (51). Taking these observations into account, Glu-453 was identified as this enzyme's catalytic acid/base.

In addition to structural analyses, a separate mutational approach has been utilized to clarify the catalytic residues of GH-66 enzymes, using *Paenibacillus* sp. 598K CITase (PsCITase) (20) and *Paenibacillus* sp. dextranase (PsDex) (19). Three acidic residues Asp-144, Asp-269, and Glu-341 are predicted to be the catalytically important residues for PsCITase; similarly, Asp-189, Asp-340, and Glu-412 are important for PsDex. When a chemical rescue reaction is applied to D340G or E412Q mutants of PsDex by using α -isomaltotetraosyl fluoride with sodium azide NaN₃, the D340G and E412Q mutants formed β and α -isomaltotetraosyl azides, implying that Asp-340 and Glu-412 are a nucleophile and an acid/base-catalyst respectively. PsCITase Asp-269 and PsDex Asp-340 correspond to Asp-385 of SmDex, and PsCITase Glu-341 and PsDex Glu-412 correspond to Glu-453 of SmDex. Biochemical analyses and structural observations agree that aspartic acid is the catalytic nucleophile and glutamic acid the catalytic acid/base of the GH-66 enzymes.

Also from mutational analyses, another aspartic acid, Asp-144 of PsCITase and Asp-189 of PsDex, is essential for catalysis. They correspond to Asp-258 of SmDex, which formed

two hydrogen bonds to the two oxygen atoms of Glc-2. This indicated that accurate substrate binding at subsite -2 was essential for the enzymatic activity, in addition to the catalytic residues directly involved in the hydrolysis.

The catalytic nucleophile of SmDex Asp-385 was located at the end of strand A β 4 and the acid/base catalyst Glu-453 at the end of strand A β 6. Many GHs have the $(\beta/\alpha)_8$ -barrel as a catalytic domain, but SmDex was similar to the clan GH-D enzymes in that the catalytic nucleophile was positioned at the end of 4th β -strand and the catalytic acid/base on the loop adjacent to the end of 6th β -strand of the $(\beta/\alpha)_8$ -barrel (Fig. 5A). The difference here was that GH-D enzymes possess two catalytic aspartate residues, while SmDex had glutamate Glu-453 for the acid/base catalyst. According to the CAZy database, three families, GH-27, GH-31, and GH-36, are listed in clan GH-D. SmDex and GH-D proteins show similar domain arrangements, such that the 3-D structures of GH-27 enzymes are composed of two domains corresponding to domains A and C in SmDex, with an exception of β -arabinopyranosidase from *Streptomyces avermitilis*, which contains extra two domains, domains III and IV (CBM-13) (53). Similarly, domains A and C are also conserved in GH-31 and GH-36 proteins, with GH-31 possessing two more domains—e.g., domain N and a distal C-terminal domain in α -xylosidases (PDB codes, 1WE5 and 2XVG) (54, 55) —while α -galactosidases in GH-36 (PDB codes, 2XN0 and 2YFO) (56, 57) have one extra domain similar to the domain N in GH-31. Enzymes in GH-13 also have a $(\beta/\alpha)_8$ -barrel domain A and antiparallel β -domain C, but GH-13 is grouped in clan GH-H and the positions of the catalytic residues are different from SmDex. The catalytic nucleophile is the aspartic acid located at the end of the $(\beta/\alpha)_8$ -barrel 4th β -strand and the proton donor the glutamic acid located at the 5th β -strand end (Fig. 5B) (44, 58).

Most enzymes in the clan GH-D are exo-type enzymes, working from

oligosaccharide chain ends, and their active sites form a pocket-type structure but, in contrast, an isomaltodextranase belonging to GH-27 is considered to be an endo-type exception, acting

5 within oligosaccharide chains (59). SmDex is an endo-type enzyme, which possesses a catalytic cleft positioned across the surface of the (β/α)₈-fold. The cleft was surrounded by the loops of the (β/α)₈-barrel, such that the loops 2, 55
10 3 and 4 formed one sidewall and loops 6 and 7 the other (Fig. 6). In the SmDexTM/IG structure, four glucose moieties were observed in the glycone side of the cleft and the bound ligand appeared similar to IG4, which was assumed to 60
15 be the product of the reverse or transglycosylation enzyme reaction, as the protein used was a wild-type protein and a relatively high IG3 concentration was employed in the soaking experiment. Furthermore, 65
20 continuous electron density was observed around the Glc-4 O6 atom, implying that the bound ligand might be isomaltooligosaccharide with a degree of polymerization of five or greater. As Glc-4 was located at the catalytic 70
25 cleft end, subsite -5 did not exist and the remaining glucoses might have been disordered. Among the four subsites, Glc-2 showed the strongest electron density, compared with the other glucose moieties, and had the lowest 75
30 *B*-factor. In addition, the SmDexTM/E5G complex structure demonstrated that the E5G glucose moiety occupied subsite -2. These observations suggested that it was necessary for the substrate to be captured by the enzyme at the 80
35 subsite -2 for a catalytic reaction to be possible. Amino acid sequence alignment with GH-66 proteins showed that five amino acids, which hydrogen bond with Glc-1 and Glc-2 of the bound IG molecule (Tyr-257, Asp-258, Tyr-307, 85
40 Asp-385 and Glu-453), are strictly conserved in GH-66 enzymes, while Tyr-260 is nevertheless conserved in streptococcal dextranases (Fig. 4). This implied that other GH-66 enzymes can be expected to recognize dextran in a manner 90
45 almost identical to SmDex. In contrast, amino acid residues contributing hydrophobic

interactions with Glc-3 and Glc-4 are less conserved and represent regions that might influence an enzyme's product specificity.

On the other hand, no ligand was observed in the aglycone side of the catalytic cleft. The glycone side was covered by protruding loops 2 and 7, while the aglycone side of the cleft was open and wide (Fig. 6). Dextran shows high solubility and appears to have flexible structure, such that the wide cleft might preferably take in such an unstructured substrate. The aglycone side of the cleft mainly consisted of loops 3, 4, and 6 with some aromatic residues arranged at the loops, and that also appear to help with substrate uptake. Subsites of the aglycone side remained unclear, but judging from the position of Glc-1, subsite +1 appeared to be surrounded by the hydrophobic residues of Ile-387, Val-434, and Trp-455, but Glc+1 did not seem to be specifically recognized.

Original SmDex contains a C-terminal variable region after domain C, and a N-terminal variable region before domain N. In a previous paper, recombinant full-size SmDex protein (95.4 kDa) was expressed but was also proteolytically degraded to form a shorter, truncated isoform of 89.8 kDa (21). When series of truncation mutants, with deleted C-terminal and/or N-terminal variable regions, were constructed and examined, SmDexTM was found to be devoid of its N and C-terminal variable regions, proteinase-resistant, and also displayed the enhanced substrate hydrolysis compared to full-size SmDex protein. Here, SmDexTM structure revealed that Asn-102, the N-terminal residue, was located in the proximal region of the A α 8 head, rather close to the catalytic cleft's glycone side, and the C-terminus His-735 was positioned in the opposite side. Although the structure of these regions remains unclear, it cannot be denied that, as a pro enzyme, the N-terminal variable region might approach the catalytic cleft's aglycone side and the C-terminal variable region might approach the glycone side and thus both might hinder

pro-SmDex from accessing the substrate. Another possibility is that the unstructured N and C-terminal variable regions might cause aggregation of the enzyme, leading to decreased hydrolytic activity.

Morisaki et al. have reported that SmDex domain C is necessary for dextran binding in deletion-mutant studies (15), but here, no apparent dextran-binding site was found in domain C of the SmDexTM/IG structure. This domain widely contacts the catalytic domain and

appears to contribute to its stabilization. In particular, an elongated loop between the β -strands C β 3 and C β 4 was observed here to be in contact with the catalytic domain's loops 6 and 7, which were involved in the formation of the catalytic cleft, and appeared to hold their relative positions. Therefore, amino acid deletion in domain C might result in conformational changes in the catalytic cleft, causing reduction of dextran binding.

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Acknowledgments—We would like to thank the beamline researchers and staff at the PF.

FOOTNOTES

*This work was supported, in part, by the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry.

The atomic coordinates and structure factors (codes [3VMN](#), [3VMO](#), and [3VMP](#)) have been deposited in the Protein Data Bank Japan (<http://www.pdb.org/>).

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³ Supported by a grant-in-aid for JSPS (Japan Society for the Promotion of Science) Postdoctoral Fellowship for Foreign Researchers (17-05467).

⁴ Abbreviations used are: CBM, carbohydrate-binding module family; CITase, cycloisomaltooligosaccharide glucanotransferase; E5G, 4',5'-epoxypentyl α -D-glucopyranoside; GH, glycoside hydrolase family; IG, isomaltooligosaccharide; IG3, isomaltotriose; IG4, isomaltotetraose; PsCITase, *Paenibacillus* sp. 598K CITase; PsDex, *Paenibacillus* sp. Dextranase; rms, root mean square; SeMet, Selenomethionine; SmDex, *Streptococcus mutans* endodextranase; SmDexTM, N and C-terminal truncated mutant of *Streptococcus mutans* endodextranase; TVAI, *Thermoactinomyces vulgaris* R-47 α -amylases 1; TVAII, *Thermoactinomyces vulgaris* R-47 α -amylases 2

FIGURE LEGENDS

FIGURE 1. Structure of SmDexTM. *A*, stereoview of SmDexTM/IG complex ribbon model. Each domain in different colors; domains N, A, and C, blue, green, and orange, respectively; loop 3 in domain A, dark green; two catalytic residues, red; bound IG3-derivative molecules, gray. *B*, Topological diagram of SmDexTM; α -helices, 3_{10} -helices and β -strands, as filled cylinders, shaded cylinders, and filled arrows, respectively.

FIGURE 2. Superimposed models of SmDexTM (green) and TVAI (blue). TVAI domain structure (43) similar to SmDex, from PDB code 1JI1; two catalytic residues of SmDexTM indicated.

FIGURE 3. Bound ligand structures in SmDexTM. *A*, stereoview of SmDexTM/IG catalytic cleft; bound IG, gray stick model; two catalytic residues, pale red; estimated hydrogen bonds, cyan break lines. *B*, $2F_{\text{obs}} - F_{\text{calc}}$ electron density map of the bound IG3 derivative in the catalytic cleft; contour level, 1σ . *C*, a 2nd IG3 derivative found between domains N and C of the different molecules in the SmDexTM/IG complex structure; electron density map around the ligand, 0.5σ contour level. *D*, E5G molecule covalently bonded to Asp-385 in SmDexTM/E5G catalytic cleft; E5G, teal stick model; electron density map around the ligand, 1σ contour level. *E*, superimposition of IG3 derivative and E5G molecules bound in the catalytic cleft of the SmDexTM/IG and SmDexTM/E5G complex structures.

FIGURE 4. Amino acid sequence alignment of GH-66 proteins. Amino acid number, the secondary structural elements, and domain color for SmDex; residues involved in hydrogen bonding with sugar molecules at subsites -1 and -2, black closed circles; catalytic residues, asterisks; conserved residues, boxed; putative CBM-35 domains in CITases, yellow boxes; LPXTG motif, black box. In order, proteins are dextranase from *S. mutans* ATCC 25175 dextranase (SmDex, GenBank accession number AEB70967), dextranase from *S. rattii* ATCC196645 (SrDex, AEJ54248), dextranase from *S. salivarius* M-33 (SsDex, BAA06127), CITase from *B. circulans* T-3040 (BcCIT1, BAA09604), CITase from *B. circulans* U-155 (BcCIT2, BAA13595), dextranase from *Paenibacillus* sp. Dex70-1B (PspDex, AAQ91301) and dextranase from *T. lettingae* TMO (TIDex, ABV33789). Alignment was prepared using the ESPript program (60).

FIGURE 5. Superimposed model of the catalytic sites of SmDexTM on the related structures. *A*, comparison with GH-D proteins; SmDexTM, green; α -galactosidase from *Trichoderma reesei* (PDB

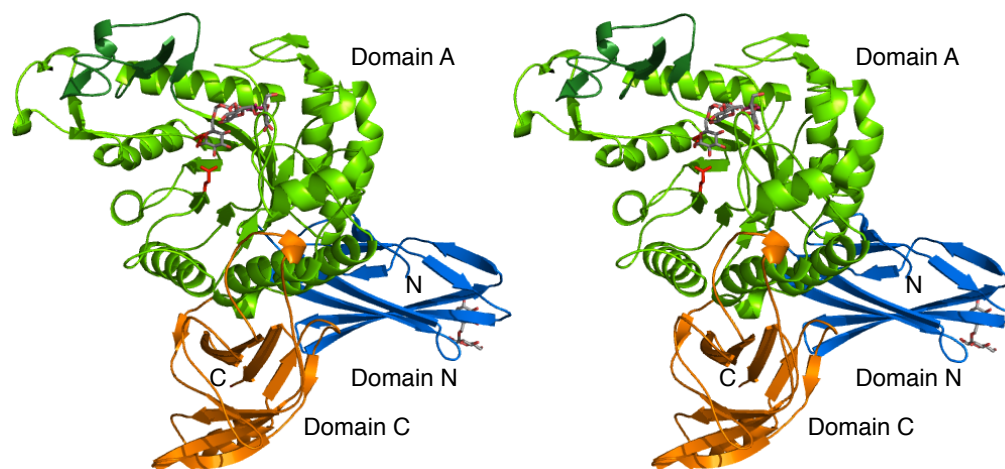
code, 1SZN) belonging to GH-27 (61), orange; α -xylosidase from *E. coli* (PDB code, 1XSJ) belonging to GH-31 (62), dark olive; α -galactosidase from *Lactobacillus acidophilus* NCFM (PDB code, 2XN0) belonging to GH-36, yellow. Nucleophiles Asp-132, Asp-416, and Asp-479, general acids Asp-226, Asp-482, and Asp-549 of these proteins and the corresponding residues of SmDex Asp-385 and Glu-453, stick models. *B*, comparison with TVAII (blue; PDB code, 3A6O) (44), whose catalytic domain showed highest structural homology by Dali-search; catalytically important residues of enzymes, stick models.

FIGURE 6. Surface model of the SmDexTM/IG complex structure. *A*, overall structure; domains N, A, and C, blue, green, and orange, respectively *B*, close-up view of catalytic cleft; bound IG3 derivative, two catalytic residues, and hydrophobic residues forming cleft, stick models.

TABLE 1. Data collection and refinement statistics

	SmDexTM		SeMet SmDexTM			SmDexTM/IG	SmDexTM/E5G
PDB code	3VMN					3VMO	3VMP
Data collection	PF-BL17A		PF-BL17A			MicroMax	PF-BL5A
Wavelength (Å)	0.97000	0.97888 (Se-peak)	0.97931 (Se-edge)	0.98400 (low remote)	0.96400 (high remote)	1.54178	1.00000
Space group	$P2_1$		$P2_1$			$P2_1$	$P2_1$
Unit cell (Å, °)	$a = 53.2$, $b = 89.7$, $c = 63.3$, $\beta = 102.3$		$a = 56.6$, $b = 90.1$, $c = 63.3$, $\beta = 99.7$			$a = 57.0$, $b = 90.3$, $c = 63.0$, $\beta = 100.3$	$a = 53.4$, $b = 90.0$, $c = 62.8$, $\beta = 102.3$
Resolution (Å)	50.0 - 1.60 (1.66 - 1.60)	50.0 - 1.8 (1.86-1.80)	50.0 - 1.8 (1.86-1.80)	50.0 - 1.8 (1.86-1.80)	50.0 - 1.8 (1.86-1.80)	50.0 - 1.90 (1.97 - 1.90)	50.0 - 1.90 (1.97 - 1.90)
No. reflections	571,898	423,407	421,508	431,201	393,582	476,322	310,988
Unique reflections	76,683 (7,641)	57,046 (5,676)	57,460 (5,362)	57,508 (5,685)	56,018 (4,483)	49,514 (4,954)	45,454 (4,112)
R_{merge}	0.063 (0.175)	0.078 (0.195)	0.078 (0.289)	0.060 (0.196)	0.073 (0.361)	0.057 (0.408)	0.085 (0.267)
Completeness (%)	100.0 (100.0)	100.0 (99.9)	99.3 (93.6)	99.9 (99.5)	96.9 (78.3)	99.7 (100.0)	98.3 (89.7)
Redundancy	7.5 (7.5)	7.4 (7.3)	7.3 (5.9)	7.5 (6.9)	7.4 (6.1)	9.6 (9.4)	6.9 (5.6)
$I/\sigma(I)$	39.2 (11.5)	38.7 (11.3)	33.9 (4.7)	37.9 (8.6)	30.3 (2.7)	46.1 (8.4)	28.9 (5.4)
Refinement							
Resolution (Å)	61.8 - 1.6					29.2 - 1.90	61.4 - 1.90
R / R_{free}	0.151 / 0.182					0.187 / 0.237	0.196 / 0.238
No. of reflections	72,799					46,979	43,068
No. of atoms	5,619					5,333	5,408
No. of protein atoms	5,029					4,985	5,012
No. of ions	1 PO_4^{2-}					1 PO_4^{2-}	1 PO_4^{2-}
No. of ligand atoms	-					68 (6 sugar residues)	42 (1 E5G)
No. of water molecules	587					275	373
Average B factor (Å ²)	12.7					35.1	26.5
protein atoms	11.6					34.7	26.0
ions	12.9					68.9	33.4
ligands	-					51.2	41.1
water molecules	22.4					37.4	33.1
Rms deviations in bond length (Å)	0.019					0.018	0.017
Rms deviations in bond angles (°)	1.823					1.570	1.538
Ramachandran plot (%) (favored/allowed/disallo wed)	98.4 / 1.6 / 0					96.8 / 3.0 / 0.2	97.8 / 2.2 / 0

A



B

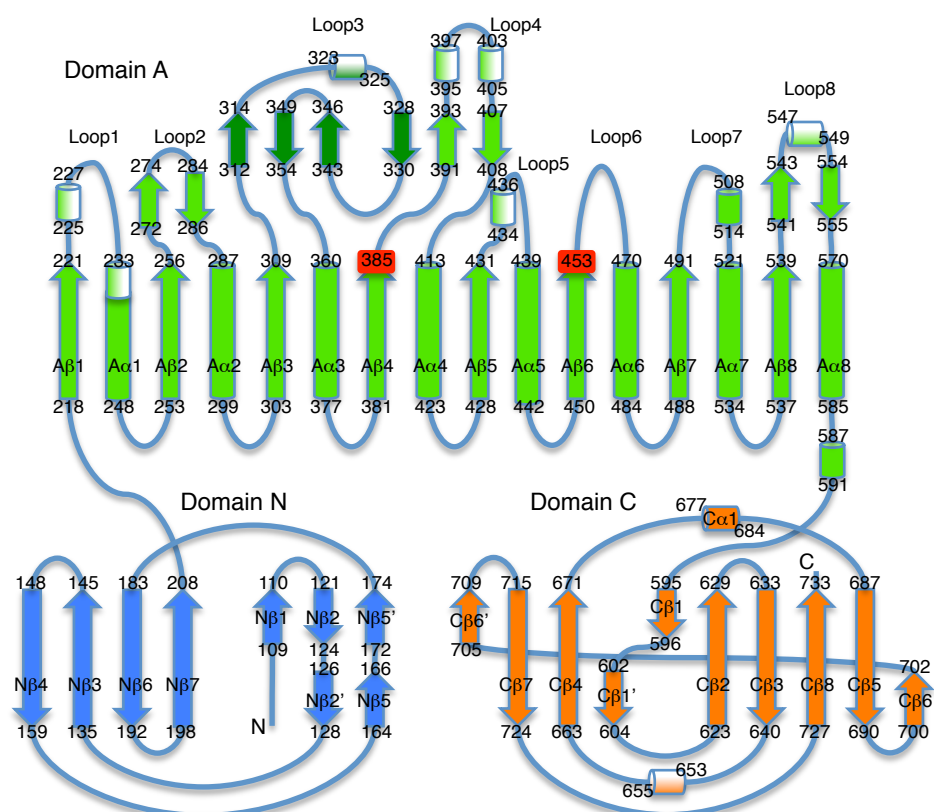


Fig. 1 Suzuki et al.

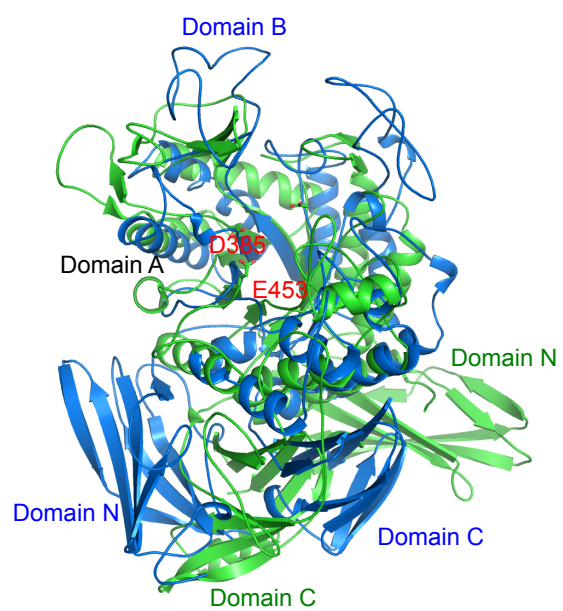


Fig. 2 Suzuki et al.

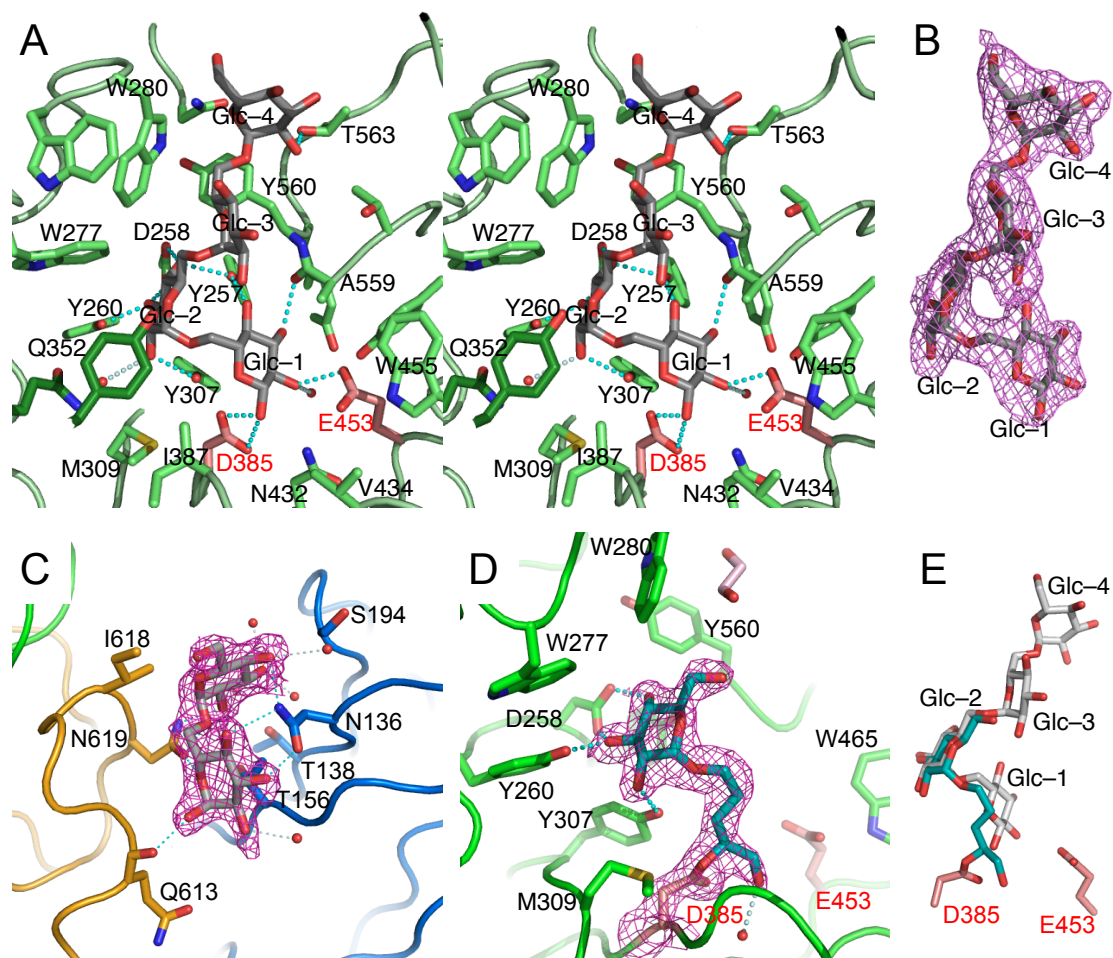


Fig. 3 Suzuki et al.



Fig.4 Suzuki et al.

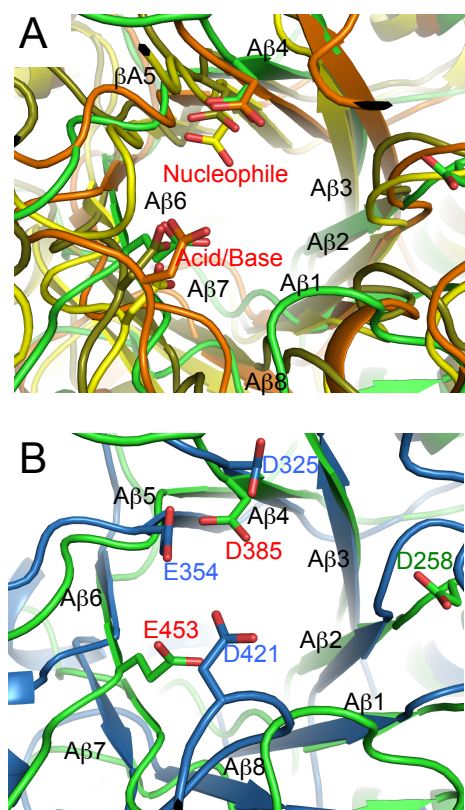


Fig. 5 Suzuki et al.

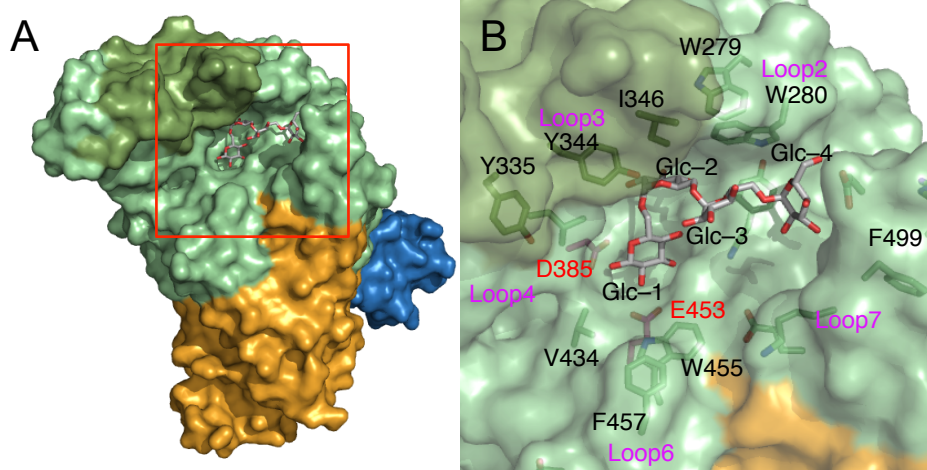


Fig. 6 Suzuki et al.