



Title	Molecular mechanism for the substrate specificity of <i>Arthrobacter globiformis</i> M6 α -glucosidase CmmB, belonging to glycoside hydrolase family 13 subfamily 30
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1 Molecular mechanism for the substrate specificity of *Arthrobacter globiformis* M6 α -
2 glucosidase CmmB, belonging to glycoside hydrolase family 13 subfamily 30

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Abstract

α -Glucosidase hydrolyzes α -D-glucosides to produce α -D-glucose. Glycoside hydrolase family 13 (GH13) contains α -glucosidases together with various amylolytic enzymes. GH13 α -glucosidases fall into several distinct subfamilies, and subfamily 30 (GH13_30) includes numerous *Actinomycetes* α -glucosidases. GH13_30 α -glucosidase CmmB from *Arthrobacter globiformis*, specific to maltooligosaccharides, is involved in the intracellular metabolism of cyclobis-(1 $\rightarrow$ 6)- α -maltosyl. Herein, the function and structure of CmmB were investigated to advance understanding of the structure–function relationship. CmmB showed the highest $k_{\text{cat}}/K_{\text{m}}$ for maltose of maltooligosaccharides, and $k_{\text{cat}}/K_{\text{m}}$ drastically decreased with increasing substrate chain-length. The crystal structures of CmmB in complex with a pseudodisaccharide (acarviosin) and pseudotetrasaccharide (acarbose) were determined at resolutions of 1.60 and 1.70 Å, respectively. The overall structure of CmmB was typical of a GH13 α -glucosidase. Most of the structure of the $\beta\rightarrow\alpha$ loop 7 of the catalytic (β/α)₈-barrel domain was not determined in the acarbose complex, but the C-terminal side of this loop was modeled in the acarviosin complex. For binding to acarviosin, this loop took on a closed conformation, and I360 and R364 on this loop formed subsite +1 together with H224 and W280. Alanine substitution of these residues indicated that R364 was essential for the catalysis through a hydrogen bond with the O6 of the D-glucose residue in subsite +1. The $\beta\rightarrow\alpha$ loop 7 is flexible upon binding to substrates, but I360 and R364 cannot participate in binding to maltooligosaccharides longer than maltose. The loss of interactions with these residues was concluded to result in the low preference for maltotriose and longer maltooligosaccharides.

Keywords: α -glucosidase; glycoside hydrolase family 13; α -amylase;

37 maltooligosaccharide

38

39 Abbreviations

40 BLAG, *Bifidobacterium longum* α -glucosidase; CmmB, *Arthrobacter globiformis* M6 α -

41 glucosidase; GH, glycoside hydrolase; GH13_30, GH family 13 subfamily 30; HaG,

42 *Halomonas* sp. H11 α -glucosidase; pNPG, *p*-nitrophenyl α -D-glucopyranoside; RMSD,

43 root mean square deviation; SmDG, *Streptococcus mutans* dextran glucosidase

1. Introduction

α -Glucosidase (EC 3.2.1.20) hydrolyzes the α -D-glucosidic linkage at the non-reducing end of substrates, including maltooligosaccharides and sucrose, to produce α -D-glucose (Okuyama et al., 2016). α -Glucosidase is involved in a starch degradation pathway together with α -amylase (EC 3.2.1.1) and debranching enzymes (EC 3.2.1.41 and EC 3.2.1.68). In addition, α -glucosidase catalyzes transglucosylation, in which a D-glucosyl residue at the non-reducing end of substrates is transferred to the hydroxy group of an acceptor molecule, such as a sugar or alcohol (Takaku, 1988; Konishi & Shindo, 1997; Ojima et al., 2012a). Transglucosylation is used industrially in the production of functional oligosaccharides, such as isomaltooligosaccharides and nigerooligosaccharides from starch.

According to the sequence-based classification of carbohydrate-active enzymes (Drula et al., 2022), most α -glucosidases are classified into the glycoside hydrolase (GH) families 13 and 31. GH family 13 is one of the largest GH families, including various amylolytic enzymes, such as α -amylase, cyclodextrin glucanotransferase (EC 2.4.1.19), and branching enzyme (EC 2.4.2.18). GH family 13 enzymes can be further divided into 47 subfamilies using phylogenetic analysis, and α -glucosidases are found in the subfamilies 17, 23, 30, 31, 38, 40, and 44 (Stam et al., 2006; Brown et al., 2023). GH family 13 enzymes have three common domains: domain A, a catalytic domain displaying a $(\beta/\alpha)_8$ -barrel fold; domain B, a long loop connecting the third β -strand and the α -helix of domain A ($\beta \rightarrow \alpha$ loop 3); and domain C, a domain formed by two antiparallel β -sheets that follows domain A (MacGregor et al., 2001). The classical conserved regions I, II, III, and IV of GH family 13 enzymes, which include the essential residues for catalysis, are located at the C-termini of the third, fourth, fifth, and seventh β -strands of domain A,

respectively. In addition, GH family 13 α -glucosidases have a protruding $\beta \rightarrow \alpha$ loop 8 in domain A, which includes two α -helices, and this loop is called domain B' and forms part of the pocket-shaped active site together with domain B and the $\beta \rightarrow \alpha$ loop 4 (Watanabe et al., 1997; Hondoh et al., 2008; Shirai et al., 2008; Yamamoto et al., 2010; Møller et al., 2012; Shen et al., 2015; Auiewiriyankul et al., 2018; Miyazaki & Park, 2020).

The GH family 13 subfamily 30 (GH13_30) is composed of *Actinomyces* α -glucosidases, including *Arthrobacter globiformis* M6 α -glucosidase CmmB (Mori et al., 2008 & 2011), *Bifidobacterium adolescentis* α -glucosidase BAD_0452 (van den Broek et al., 2003), *Bifidobacterium breve* α -glucosidase Agl3 (Kelly et al., 2016), *Bifidobacterium longum* α -glucosidase BLAG (Kim et al., 2017), *Nesterenkonia* sp. α -amylase AmyF (Solat & Shafiei, 2021), and *Thermomonospora curvata* α -glucosidase AglA (Janda et al., 2000). *A. globiformis* M6 produces a cyclic tetrasaccharide, cyclobis-(1 $\rightarrow$ 6)- α -maltosyl, from starch and maltooligosaccharides by the concerted actions of 6- α -maltosyltransferase (Mukai et al., 2005 & 2006). CmmB is highly specific to the α -(1 $\rightarrow$ 4)-glucosidic linkage (Mori et al., 2008), and is predicted to be involved in the intracellular degradation of cyclobis-(1 $\rightarrow$ 6)- α -maltosyl together with the hydrolase that splits the α -(1 $\rightarrow$ 6)-linkage in this cyclic tetrasaccharide (Mori et al., 2008 & 2011). The substrate specificity of CmmB has only been examined using a single concentration of substrates (Mori et al., 2008), and thus further analysis is required for better understanding of this enzyme. GH13_30 includes several thousands of protein sequences, but only a few members of this subfamily have been characterized, and no three-dimensional structures have been elucidated. CmmB shows 42–50% of amino acid sequence identity with the characterized GH13_30 enzymes: sequence identity with *B. adolescentis* BAD_0452, *B. breve* Agl3, *B. longum* BLAG, *Nesterenkonia* sp. AmyF, and *T. curvata* AglA was 42%,

44%, 45%, 48%, and 50%, respectively. In the present study, we analyzed in detail the substrate specificity of CmmB, the crystal structure in complex with competitive inhibitors of transition state analogs, and the functions of enzymes with mutations at residues in subsite +1 to better understand the structure–function relationships of CmmB.

2. Materials and methods

2.1. Substrates

Isomaltose, panose, *p*-nitrophenyl α -D-glucopyranoside (pNPG), and sucrose were purchased from Tokyo Chemical Industry (Tokyo, Japan), Hayashibara (Okayama, Japan), Fujifilm Wako Pure Chemical (Osaka, Japan), and Kanto Chemical (Tokyo, Japan), respectively. Maltose, maltotriose, and maltotetraose were kindly provided by the Research Institute of Nihon Shokuhin Kako (Tokyo, Japan). Kojibiose was prepared by the reverse phosphorolysis of kojibiose phosphorylase (Yamamoto et al., 2011). Nigerose was prepared by the transglucosylation towards maltose using *Acremonium strictum* α -glucosidase (Yamamoto et al., 2004). Glc(α 1-4)Man, Glc(α 1-4)GlcNAc, and Glc(α 1-4)Xyl were prepared by the reverse phosphorolysis of maltose phosphorylase (Gao et al., 2019).

2.2. Preparation of expression plasmids

The *cmmB* gene (GenBank number, BAI67603.1; the codons were optimized for expression in *Escherichia coli*) was synthesized by Eurofins Genomics (Tokyo, Japan), and amplified by PCR using the primers (5'-AAGAAGGAGATATACATATGTCGCATACGATCGAACGCC-3' and 5'-CAGTGGTGGTGGTGGTGGTGCTCGAGGGCTGCAACCCACGATGAAG-3') and

116 Primestar HS DNA polymerase (Takara Bio, Kusatsu, Japan). The amplified DNA was
117 inserted into pET-23a (Novagene, Darmstadt, Germany) using an In-Fusion HD cloning
118 kit (Takara Bio). The inserted DNA and flanking regions were sequenced using an
119 Applied Biosystems 3130 genetic analyzer (Life Technologies, Carlsbad, CA, USA).
120 Site-directed mutagenesis was introduced using overlap extension PCR (Ho et al., 1989),
121 and the mutated gene was inserted into pET-23a.

123 2.3. Preparation of CmmB variants

124 Transformants of *E. coli* BL21 (DE3) harboring the expression plasmid for each
125 CmmB variant were cultured in 1 L of LB medium containing 100 µg/mL ampicillin at
126 37°C until the A_{600} value reached 0.5. Recombinant CmmB was produced by the
127 induction culture with vigorous shaking at 18°C for 24 h in the presence of 0.1 mM
128 isopropyl β-D-thiogalactopyranoside. Bacterial cells were harvested by centrifugation
129 (9600 ×g, 4°C, 10 min), and disrupted in 20 mM imidazole-HCl buffer (pH 7.0)
130 containing 0.5 M NaCl by sonication. Cell-free extract was obtained by centrifugation
131 (9600 ×g, 4°C, 10 min). Recombinant enzyme was purified from the extract by Ni affinity
132 column chromatography using a Chelating Sepharose Fast Flow column (Cytiva, Uppsala,
133 Sweden; 2.6 cm i.d. × 3.5 cm) equilibrated with 20 mM imidazole-HCl buffer (pH 7.0)
134 containing 0.5 M NaCl. Adsorbed protein was eluted using a linear gradient of 20–500
135 mM imidazole (elution volume, 200 mL) after the elution of non-adsorbed protein with
136 the equilibration buffer. The collected sample in the purification of the wild-type enzyme
137 was concentrated to approximately 10 mL by ultrafiltration using a Vivaspin YM-30
138 concentrator (Sartorius, Göttingen, Germany) and subjected to gel filtration column
139 chromatography using a Toyopearl HW-55S column (Tosoh, Tokyo, Japan; 2.6 cm i.d. ×

100 cm) equilibrated with 10 mM HEPES-NaOH buffer (pH 7.0). The collected fractions of the mutant enzymes were dialyzed against 10 mM HEPES-NaOH buffer (pH 7.0). The concentration of the wild-type enzyme was determined by amino acid analysis using an amino acid analyzer L-8900 (Hitachi High-Technologies, Tokyo, Japan) after complete hydrolysis of the purified enzyme in 6 M HCl at 110°C for 24 h. The concentrations of the mutant enzymes were determined using the specific coefficient of A_{280} for 1 mg/mL of the wild-type enzyme = 1.98.

2.4. Estimation of the molecular mass of CmmB by gel filtration column chromatography

To estimate molecular mass of CmmB under nondenaturing conditions, gel filtration column chromatography was performed under following conditions: column, Asahipac GF-510 HQ (7.5 mm i.d. × 300 mm, Shodex, Tokyo, Japan); elution, 10 mM sodium phosphate buffer (pH 7.0) containing 0.2 M NaCl; flow rate, 0.5 mL/min; detection, A_{280} . The Gel Filtration Standard (Bio-Rad, Hercules, CA, USA), containing a standard set of proteins, was used to estimate the molecular mass of the sample from elution volume.

2.5. Enzyme assay

2.5.1. Standard enzyme assay

A reaction mixture (50 μ L) containing enzyme, 2 mM pNPG, 42 mM sodium phosphate buffer (pH 7.6), and 0.2 mg/mL bovine serum albumin was incubated at 37°C for 10 min. The reaction was stopped by the addition of 100 μ L of 1 M Na_2CO_3 , and the A_{400} value of the liberated *p*-nitrophenol ($\epsilon_{1 \text{ mM}, 400 \text{ nm}} = 5.56$) was measured. Enzyme activity (1 U) was defined as the amount of enzyme hydrolyzing 1 μ mol of pNPG in 1 min under these conditions.

2.5.2. Optimum pH and temperature

Optimum pH and temperature of CmmB variants were determined from reaction velocity to 2 mM pNPG at various pH and temperatures, respectively. Reaction pH was changed by using 80 mM modified Britton-Robinson buffer (pH of mixture of acetate, phosphate, and glycine was adjusted using 6 M NaOH) as the reaction buffer.

2.5.3. Kinetic parameters

The reaction velocity for various concentrations (0.2–20 mM) of the following substrates was measured using a standard assay: maltose, maltotriose, maltotetraose, Glc(α 1-4)Man, Glc(α 1-4)GlcNAc, Glc(α 1-4)Xyl, kojibiose, nigerose, isomaltose, sucrose, and pNPG. The liberated D-glucose was measured after stopping the reaction by the addition of 100 μ L of 2 M Tris-HCl buffer (pH 7.0). The D-glucose quantification kit, Glucose CII Test Wako (Fujifilm Wako Pure Chemical), was used. Kinetic parameters were determined by fitting the Michaelis-Menten equation to the obtained reaction rates. Grafit version 7.0.2 (Erithacus Software, East Grinstead, UK) was used for non-linear regression. $\Delta\Delta G$ values for the mutagenesis were calculated from the following equation:

$$\Delta\Delta G = -RT \{ \ln(k_{\text{cat}}/K_{\text{m}})_{\text{mutant}} - \ln(k_{\text{cat}}/K_{\text{m}})_{\text{wild type}} \}$$

R, gas constant; T, the absolute temperature

The inhibition constants for the pseudodisaccharide acarviosin and the pseudotetrasaccharide acarbose were determined from the reaction rates for the pNPG hydrolysis in the absence and presence of inhibitors. The reaction rates for pNPG (0.2–2 mM) were measured in the presence of 0, 0.313, and 1.25 μ M acarviosin (Toronto Research Chemicals, Toronto, ON, Canada) or 0, 2.5, and 5 μ M acarbose (Carbosynth,

Compton, Berkshire, UK). The rate equation for the competitive inhibition was fitted to the reaction rates obtained using Grafit version 7.0.2.

2.6. Crystallization and data collection

Crystals of CmmB in complex with acarbose and acarviosin were prepared by co-crystallization using the sitting-drop vapor-diffusion method. Appropriate crystals of CmmB complexed with acarbose were obtained within 2 months at 20°C with a drop (1.5 μ L) containing 4.7 mg/mL CmmB, 5 mM acarbose, 0.1 M lithium acetate, 10% (w/v) polyethylene glycol 3400 (MP Biomedicals, Solon, OH, USA), and 5 mM HEPES-NaOH buffer (pH 7.0). Crystals of the acarviosin complex appeared within 3 weeks at 20°C in a drop containing 6.5 mg/mL CmmB, 5 mM acarviosin, 0.5 M lithium chloride, 10% (w/v) polyethylene glycol 6000 (Fujifilm Wako Pure Chemical), and 55 mM HEPES-NaOH buffer (pH 7.0).

For X-ray diffraction experiments, the crystals were directly picked up from the crystallization solution and flash-cooled under a nitrogen stream at 100 K or in liquid nitrogen. Diffraction data of the acarbose and acarviosin complexes were collected on beamline BL-45XU at SPring-8 (Hyogo, Japan) and beamline BL-5A at Photon Factory (Tsukuba, Japan), respectively. The data sets were indexed, integrated, scaled, and merged using the XDS program suite (Kabsch, 2010). The asymmetric units of the acarbose and acarviosin complexes contained two and one molecules, respectively. Matthews coefficients (Matthew, 1968) of 1.99 $\text{\AA}^3/\text{Da}$ and 2.18 $\text{\AA}^3/\text{Da}$, and estimated solvent contents of 38.1% and 43.6%, were calculated for the acarbose and acarviosin complexes, respectively. All data collection statistics are summarized in Table 1.

2.7. Structure solution and refinement

The structures of CmmB in complex with acarbose and acarviosin were determined by the molecular replacement method with the program AutoMR in the Phenix program package (McCoy et al., 2007; Adams et al., 2010). A model structure of CmmB constructed with Colabfold (Mirdita et al., 2022) and the structure of CmmB in complex with acarbose (the monomer, chain A, without ligand) were used as structural models to solve the structure of the complexes with acarbose and acarviosin, respectively. Several rounds of refinement were performed using the program Phenix.refine in the Phenix program suite. Protein structure model was alternated with manual fitting and rebuilding based on $2F_o - F_c$ and $F_o - F_c$ electron densities using COOT (Emsley & Cowtan, 2004). Water molecules and ligands were built based on $2F_o - F_c$ and $F_o - F_c$ electron densities. The final refinement statistics and geometry defined by MolProbity (Chen et al., 2010) are shown in Table 1. The atomic coordinates and structure factors were deposited in the Protein Data Bank (<http://wwpdb.org/>; codes 8YIE and 8YIF). All structure figures were generated by PyMol ver. 2.5.0 (Schrödinger, LLC, New York, NY, USA).

3. Results

3.1. Substrate specificity of CmmB

To investigate the substrate recognition mechanism of CmmB, the substrate specificity of CmmB was investigated using the kinetic parameters for various α -D-glucosides, including α -(1 $\rightarrow$ 4)-D-glucosyl heterobioses. Recombinant CmmB, which had a Leu-Glu-His-His-His-His-His extension at the C-terminus of the complete CmmB sequence, was produced in *E. coli* BL21(DE3), and purified to homogeneity (Fig. S1). Molecular mass of CmmB was estimated to be 57 kDa in the SDS-PAGE analysis, which

was close to the theoretical value from the amino acid sequence (64 kDa). In the gel filtration column chromatography, CmmB is eluted in two peaks of 105 kDa and 46 kDa (Fig. S2), suggesting that CmmB partly formed dimer. From the peak area, dimer ratio was determined to be 37% (w/w). The purified enzyme showed 9.33 U/mg of specific activity toward 2 mM pNPG. In the reactions with 0.2–8 mM pNPG, the reaction rates for pNP and D-glucose production were almost identical, indicating that this enzyme predominantly catalyzed hydrolysis under the analytical conditions used. The $k_{\text{cat}}/K_{\text{m}}$ toward pNPG was the highest of all the tested substrates (Table 2). CmmB showed the highest $k_{\text{cat}}/K_{\text{m}}$ toward maltose of all the glucobioses (structures of substrates tested are shown in Fig. S3), and the $k_{\text{cat}}/K_{\text{m}}$ values toward kojibiose, nigerose, and isomaltose were 0.0874%, 3.09%, and 0.0189% of the value toward maltose, respectively. Sucrose was a better substrate than the glucobioses, other than maltose, in terms of the $k_{\text{cat}}/K_{\text{m}}$ value. The $k_{\text{cat}}/K_{\text{m}}$ values toward Glc(α 1-4)Man, Glc(α 1-4)GlcNAc, and Glc(α 1-4)Xyl were 17.9%, 0.421%, and 1.22% of that toward maltose, respectively. The k_{cat} value toward Glc(α 1-4)Man was comparable to that toward maltose, but the K_{m} value toward this disaccharide was 4.92-fold higher than toward maltose. Saturation curves for Glc(α 1-4)GlcNAc and Glc(α 1-4)Xyl were not obtained under the analytical conditions used (1–20 mM). CmmB also hydrolyzed maltooligosaccharides that were longer than maltose, but the $k_{\text{cat}}/K_{\text{m}}$ value severely decreased with an increasing degree of polymerization. The $k_{\text{cat}}/K_{\text{m}}$ values for maltotriose and maltotetraose were 5.86% and 0.416% of that for maltose, respectively. Although CmmB showed a much lower $k_{\text{cat}}/K_{\text{m}}$ value for isomaltose than for maltose, as described above, CmmB showed a slightly higher $k_{\text{cat}}/K_{\text{m}}$ value for panose than for maltotriose.

3.2. Kinetics of the inhibition of pNPG hydrolysis by acarviosin and acarbose

The inhibition of pNPG hydrolysis by the pseudomaltooligosaccharides, acarviosine and acarbose, which were used as ligands in the X-ray crystallography, was evaluated. Both the pseudomaltooligosaccharides acted as competitive inhibitors, and the K_i values for acarviosin and acarbose were 1.23 ± 0.05 and 2.37 ± 0.06 μM , respectively (Fig. 1). Consistent with the specificity for maltooligosaccharides with a small chain length, acarviosin was a better inhibitor than acarbose. The $\Delta\Delta G$ value calculated from the K_i values toward acarviosin and acarbose [$\Delta\Delta G = -RT \{\ln(1/K_i)_{\text{acarbose}} - \ln(1/K_i)_{\text{acarviosin}}\} = 1.69 \text{ kJ}\cdot\text{mol}^{-1}$] was considerably smaller than the $\Delta\Delta G$ value calculated from the k_{cat}/K_m values toward maltose and maltotetraose [$\Delta\Delta G = -RT \{\ln(k_{\text{cat}}/K_m)_{\text{maltotetraose}} - \ln(k_{\text{cat}}/K_m)_{\text{maltose}}\} = 14.1 \text{ kJ}\cdot\text{mol}^{-1}$].

3.3. Crystal structure of CmmB in complex with acarviosin and acarbose

The structures of the complexes of CmmB with acarviosin and acarbose were solved at 1.60 and 1.70 Å, respectively (Table 1, Fig. 2). The crystal structure of CmmB in complex with acarviosin contained one molecule in the asymmetric unit, while that with acarbose contained two. The acarviosin complex was modeled from A17–H562, and the structure solved was very similar to the model structure constructed with Colabfold (Mirdita et al., 2022) (Fig. S4): the root mean square deviation (RMSD) for the C α of the two structures was 0.476 Å. In the acarbose complex, molecule A was modeled from A17 to A566, and molecule B was modeled from A17 to L561. The structures of the two complexes included regions with poor electron density: in the acarviosin complex, L351–D358, and in the acarbose complex, E348–A369 (molecules A and B), A449–A452 (molecule A), L545–T546 (molecule A), and P544–A549 (molecule B) were disordered.

The structures of the two monomers of the acarbose complex were similar to each other: The RMSD for the C α of these two structures was 0.260 Å. The structure of the acarviosin complex was also similar to that of the two monomers of the acarbose complex: the RMSD values between the acarviosin complex and molecule A of the acarbose complex and between the acarviosin complex and molecule B of the acarbose complex were 0.244 and 0.186 Å, respectively. The overall structure of CmmB was typical of α -glucosidase-type enzymes in GH13, consisting of domains A, B, B', and C (Fig. 2A). A similarity search of the CmmB structure using the Dali server (Holm, 2020) showed that CmmB had high similarity with the GH13_23 α -glucosidases, *Xanthomonas campestris* GH13_23 α -glucosidase XgtA (Z-score, 50.6; Watanabe et al., 2020; PDB entry, 6AAV), *Halomonas* sp. H11 GH13_23 α -glucosidase HaG (Z-score, 50.1; Shen et al., 2015; PDB entry, 3WY1), and *Qipengyuania seohaensis* GH13_23 α -glucosidase QsGH13 (Z-score, 46.8; Zhai et al., 2022; PDB entry, 7VOH). The structure of a large part of the $\beta \rightarrow \alpha$ loop 7 of domain A, E348–A369, was not determined in the acarbose complex, while the C-terminal side of this loop (M359–A369) was modeled in the acarviosin complex (Fig. 2A).

In the substrate binding site of CmmB in complex with acarviosin and acarbose, clear electron density of the ligands was observed in subsites –1 to +1 and –1 to +3, respectively (Fig. 2B and C). The acarviosin residue of acarbose bound to the active site very similarly to acarviosin (Fig. 2D–H). CmmB had the pocket-shaped active site (Fig. 2F and G), and subsite –1 binding to the valienamine residue of acarviosin and acarbose was situated at the bottom of the active site. D221 and E278 were suitably situated to act as a catalytic nucleophile and general acid/base catalyst, respectively. D74, H117, R219, H336, D337, and R428 are located at appropriate positions to form hydrogen bonds with

the valienamine residue in subsite -1. Tyr77 had an aromatic stacking interaction with the valienamine residue. The residues corresponding to Tyr77, H117, R219, H336, and D337 are conserved in most GH family 13 enzymes, and D74 and R428 equivalents are well conserved in GH family 13 α -glucoside acting (exo-type) enzymes. In subsite +1, H224 on the $\beta \rightarrow \alpha$ loop 4 of domain A can form a hydrogen bond with the 2-OH of the 6-deoxy-D-glucosyl residue of the ligands in subsite +1. W280 on the $\beta \rightarrow \alpha$ loop 5 is located in subsites +1 and +2. Trp280 stacked onto the D-glucosyl residue of acarbose in subsite +2. No other interactions were predicted in subsite +2 of the acarbose complex. In subsite +3 of the acarbose complex, V306 had hydrophobic contacts with the D-glucose residue: C β and C γ 1 of V306 were 4.0 Å and 3.5 Å from the C1 and C2 atoms of the D-glucose residue of acarbose, respectively. In the acarviosin complex, the structure of part of $\beta \rightarrow \alpha$ loop 7 of domain A was determined, although temperature factor of this loop was high together with part of $\beta \rightarrow \alpha$ loop 4 and $\beta \rightarrow \alpha$ loop 8 (Fig. S5). This loop made the pocket-shaped active site of the acarviosin complex narrow and deep. I360 and R364 on the $\beta \rightarrow \alpha$ loop 7 were situated in proximity to the 6-deoxy-D-glucosyl residue in subsite +1; these residues were not modeled in the complex with acarbose described above. The C δ of I360 was 4.5 and 4.7 Å from the C5 and C6 atoms of the 6-deoxy-D-glucosyl residue of acarviosin, respectively, and the C γ 2 of this residue was 3.8 Å from the methyl group of acarviosin, and hydrophobic contacts between I360 and this methyl α -D-glucoside residue were assumed. No obvious interaction between R364 and the 6-deoxy-D-glucosyl residue of acarviosin was predicted (the function of this residue is discussed later). As $\beta \rightarrow \alpha$ loop 7 of the acarviosin complex did not have any contact with symmetry mates (Fig. S6), contact with acarviosin was the reason for the stabilization of this loop structure. In the superimposed structures of the complexes, part of the $\beta \rightarrow \alpha$ loop 7, modeled in the

acarviosin complex, clashed onto acarbose in subsites +2 and +3 (Fig. 2H), suggesting that the structure of the $\beta \rightarrow \alpha$ loop 7 of CmmB was flexible and this structure can be altered depending on the chain length of the substrate. The $\beta \rightarrow \alpha$ loop 7 had a closed form when bound to acarviosin, but can convert to an open form to bind longer ligands.

3.4. Functional analysis of the residues forming subsite +1

To investigate the contribution of the residues in subsite +1 to the substrate binding, H224, W280, I360, and R364 were substituted with alanine. The mutated enzymes were prepared in the same fashion as the wild type (Fig. S1). Prepared mutant enzymes showed high activity at pH 7.0–7.6 and 37°C similar to the wild-type enzyme (Fig. S7 and S8). The kinetic parameters for the hydrolysis of pNPG and maltose were determined (Table 3). All the mutant enzymes showed lower $k_{\text{cat}}/K_{\text{m}}$ values toward both the substrates, compared with the wild-type enzyme. In the reaction with pNPG, the k_{cat} value largely decreased through the mutations, while the K_{m} value was only slightly changed. The mutant enzymes showed much lower k_{cat} values for maltose than for pNPG. The W280A, I360A, and R364A mutants showed 2.97–12.1-fold higher K_{m} values for maltose than the wild-type enzyme, while the H224A mutant had a lower K_{m} value. The $\Delta\Delta G$ values of the mutations for the hydrolysis of pNPG and maltose were calculated to be 3.96–11.1 kJ·mol⁻¹ and 3.93–23.8 kJ·mol⁻¹, respectively. The $\Delta\Delta G$ values of the W280A, I360A, and R364A mutations for maltose were higher than for pNPG, while H224A had comparable $\Delta\Delta G$ values for the two substrates. The $\Delta\Delta G$ value for the R364A mutation was the highest in the reactions with both the substrates of all the mutations investigated.

4. Discussion

GH13_30 contains numerous putative α -glucosidases from *Actinomycetes* bacteria, but only limited numbers of these enzymes have been characterized, and no three-dimensional structure has been previously available. In the present study, we investigated the molecular basis for the substrate recognition of CmmB through biochemical and structural analyses. CmmB is highly specific to the α -(1 $\rightarrow$ 4)-linked glucobiose, maltose. Structural analysis of CmmB in complex with acarviosin clearly demonstrated that the structure of the $\beta\rightarrow\alpha$ loop 7 of domain A of CmmB was flexibly altered upon binding to substrates: this loop forms open and closed structures to bind to maltose and longer maltooligosaccharides, respectively. I360 and R364 on the $\beta\rightarrow\alpha$ loop 7 in the closed form were situated in subsite +1 (Fig. 2D and F). H224 and W280 were in subsite +1 regardless of the structure of the $\beta\rightarrow\alpha$ loop 7. Alanine substitution of R364 caused the largest reduction in the $k_{\text{cat}}/K_{\text{m}}$ value toward maltose of all the examined residues (Table 3). The $\Delta\Delta G$ value for the R364A mutation of 23.8 kJ $\cdot$ mol $^{-1}$ was in the range of the values for the loss of a hydrogen bonding interaction between the ligand and a charged amino acid residue (Fersht et al., 1985), suggesting that the charged R364 residue formed a hydrogen bond with maltose. Superimposition of CmmB and HaG in complex with maltose (Shen et al., 2015; PDB entry, 3WY4) suggested that maltose binds to the pocket-shaped active site of CmmB in the manner similar to acarvisin (Fig. 3A). The amino acid residues in subsite -1 of CmmB were spatially conserved with HaG, and H224, W280, I360, and R364 are predicted to form subsite +1 for maltose binding. R364 was suitable for the formation of a hydrogen bond with the O6 of the D-glucose residue in subsite +1. Consistent with this prediction, wild-type CmmB showed a much lower $k_{\text{cat}}/K_{\text{m}}$ value toward Glc(α 1-4)Xyl than toward maltose (Table 2). The $\Delta\Delta G$ value (11.4 kJ $\cdot$ mol $^{-1}$), calculated from the $k_{\text{cat}}/K_{\text{m}}$ values toward these substrates [$\Delta\Delta G = -RT \{ \ln(k_{\text{cat}}/K_{\text{m}})_{\text{Glc}(\alpha 1-4\text{-Xyl})} - \ln(k_{\text{cat}}/K_{\text{m}})_{\text{maltose}} \}$],

$_{4)Xyl} - \ln(k_{cat}/K_m)_{maltose}]$, could be explained by the absence of the hydrogen bond with R364, although this $\Delta\Delta G$ value was lower than for the R364A mutation. I360 of CmmB in the closed form of the $\beta \rightarrow \alpha$ loop 7 was suitably located to have hydrophobic interactions with the D-glucosyl residue of maltose in subsite +1, and the $\Delta\Delta G$ value for the I360A mutation was $11.0 \text{ kJ}\cdot\text{mol}^{-1}$. As the K_m value for maltose was largely increased by this mutation, I360 was thought to have large contribution to the stabilization of the enzyme–substrate complex.

H224 and W280 of CmmB, which are situated within the $\beta \rightarrow \alpha$ loops 4 and 5, respectively, are highly conserved in GH13_30 α -glucosidases, including other characterized enzymes as shown in Fig. 3C. H224 was suitable for the formation of a hydrogen bond with the O2 of the D-glucosyl residue of maltooligosaccharides in subsite +1 (Fig. 2B and C). A similar hydrogen bonding interaction has also been observed in several other GH family 13 enzymes specific for α -(1 $\rightarrow$ 4)-glucans, including α -amylases (Brzozowski & Davies, 1997; Fujimoto et al., 1998; Brayer et al., 2000), cyclodextrin glucanotransferases (Uitdehaag et al., 1999), and α -glucosidases (Auiewiriyankul et al., 2018), with a histidine residue located at four residues from the C-terminal side of the catalytic nucleophile aspartic acid. The H224A mutation in CmmB reduced the k_{cat}/K_m value toward maltose. The $\Delta\Delta G$ value of $3.93 \text{ kJ}\cdot\text{mol}^{-1}$ was in the range for the loss of a neutral hydrogen bond (Fersht, et al. 1985), which suggested that H224 formed a neutral hydrogen bond with the substrate (Table 3). The $\Delta\Delta G$ value calculated from the k_{cat}/K_m values toward maltose and Glc(α 1-4)Man [$\Delta\Delta G = -RT\{\ln(k_{cat}/K_m)_{Glc(\alpha 1-4)Man} - \ln(k_{cat}/K_m)_{maltose}\} = 4.43 \text{ kJ}\cdot\text{mol}^{-1}$] was comparable to the $\Delta\Delta G$ value for the H224A mutation in the hydrolysis of maltose, suggesting that H224 does not form a hydrogen bond with the D-mannose residue of Glc(α 1-4)Man, and the 2-OH of the D-mannose

residue does not cause any steric hindrance upon binding to the active site. The very low $k_{\text{cat}}/K_{\text{m}}$ value toward Glc(α 1-4)GlcNAc presumably resulted from severe steric hindrance between this molecule and the H224 side chain. W280 of CmmB was located in subsites +1 and +2, and the W280A mutation resulted in a large decrease in the $k_{\text{cat}}/K_{\text{m}}$ value toward maltose, indicating that this residue also contributed to the substrate binding in subsite +1 (Table 3). A tryptophan residue, corresponding to W280 of CmmB, is frequently found in other GH family 13 enzymes, including α -amylases (Morishita et al., 1997; Kadziola et al., 1998; Robert et al., 2005), neopullulanases (EC 3.2.1.135; Yokota et al., 2001; Lee et al., 2002; Hondoh et al., 2003), and dextran glucosidases (Hondoh et al., 2008), which have specificity for distinct linkages in the substrates, and this residue is involved in the substrate binding. The W280 residue of CmmB had a stacking interaction with the D-glucosyl residue of acarbose in subsite +2. A similar stacking interaction has been also observed in the enzymes harboring a corresponding tryptophan residue.

A comparison of the structures of the complexes with acarviosin and acarbose showed that the $\beta \rightarrow \alpha$ loop 7 in the closed form disturbed the binding of acarbose (Fig. 2H). Although the structure of the $\beta \rightarrow \alpha$ loop 7 was not visible in the electron density of the acarbose complex, this loop probably has an open flexible conformation to avoid steric hindrance upon binding to maltooligosaccharides longer than maltose. The $\Delta\Delta G$ value calculated from the K_{i} values toward acarviosin and acarbose ($1.69 \text{ kJ}\cdot\text{mol}^{-1}$) was much smaller than the $\Delta\Delta G$ value from the $k_{\text{cat}}/K_{\text{m}}$ values toward maltose and maltotetraose ($14.1 \text{ kJ}\cdot\text{mol}^{-1}$) as described above. Neither of these inhibitors had a hydrogen bond interaction with R364 in subsite +1, and in the acarbose complex, the lack of the hydrophobic interaction with I360 in subsite +1 was expected to be compensated

by the interactions between W280 and the D-glucosyl residue in subsite +2. Thus, the $\Delta\Delta G$ value calculated from the K_i values toward acarviosin and acarbose presumably mainly reflected the energy difference between the open and closed forms of the $\beta \rightarrow \alpha$ loop 7 of CmmB, and the small $\Delta\Delta G$ value implied that the conformational change of this loop did not require a large amount of energy. This result was consistent with the fact that only a few interactions between residues of the $\beta \rightarrow \alpha$ loop 7 and residues of adjacent structures were observed in the acarviosin complex. Additionally, high temperature factor of $\beta \rightarrow \alpha$ loop 7 was observed in CmmB complex with acarviosin (Fig. S5) and low predicted local distance difference value of this loop was shown in the predicted structure of CmmB by Colabfold (Fig. S4). These structural observations suggest that this loop structure was very flexible. As the entrance of the substrate binding site of CmmB with the closed form of the $\beta \rightarrow \alpha$ loop 7 appeared to be too narrow for substrate entrance, it is implied that the $\beta \rightarrow \alpha$ loop 7 of CmmB takes an open conformation before substrate binding. As suggested in the acarviosin complex with CmmB, when maltose binds to the active site, the closed conformation of the $\beta \rightarrow \alpha$ loop 7 is presumably stabilized through the interactions of I360 and R364 with the D-glucose residue of maltose in subsite +1. In the reaction with maltooligosaccharides longer than maltose, the $\beta \rightarrow \alpha$ loop 7 had an open conformation, and I360 and R364 did not participate in the formation of subsite +1. Although some binding affinity is provided through the interaction of W280 with substrates in subsite +2, the lack of the interactions of these residues is presumably the main reason for the low preference of CmmB for maltooligosaccharides longer than maltose (in other words, the high specificity toward maltose).

The multiple alignment shown in Fig. 3C showed that the GH13_30 enzymes have similar length of $\beta \rightarrow \alpha$ loops 7, but the sequence similarity of this loop is low. I360 and

R364 of CmmB are not conserved in this loop: the GH13_30 enzymes other than CmmB possess leucine and glycine at the corresponding position of I360 and R364, respectively. This indicates that only CmmB has the hydrogen bonding interaction between the arginine and the D-glucose residue of maltose in subsite +1 among the characterized GH13_30 enzymes. *B. longum* BLAG also hydrolyzes maltooligosaccharides, but this enzyme shows very low $k_{\text{cat}}/K_{\text{m}}$ towards maltose ($0.1 \text{ s}^{-1}\text{mM}^{-1}$) (Kim et al., 2017). This low $k_{\text{cat}}/K_{\text{m}}$ to maltose might be arisen from lacking the hydrogen bonding interaction, involving the arginine residue, in subsite +1. As BLAG shows very high preference for maltotriose over maltose, this enzyme presumably possesses residues involved in the formation of subsite +2 with high affinity.

The mechanism for the high specificity toward maltose of CmmB is very different from that of HaG (Shen, et al. 2015), which is also specific for maltose over maltooligosaccharides (Ojima et al., 2012b). In HaG, the long $\beta \rightarrow \alpha$ loop 4 of domain A forms a narrow substrate binding site and prevents the binding of maltooligosaccharides longer than maltose (Fig. 3B). A conformational change in this loop was not indicated from the structures of HaG in the substrate-free form, the D-glucose-bound enzyme intermediate, and the maltose complex (Shen, et al. 2015).

It is noteworthy that CmmB and other GH13_30 enzymes, showing high specificity to α -(1 $\rightarrow$ 4)-D-glucosides, has the valine residue next to the catalytic nucleophile, because α -glucosidase-type enzymes in GH13, having valine at the position, show high specificity for α -(1 $\rightarrow$ 6)-glucosides: dextran glucosidase (Saburi et al., 2006; Møller et al., 2012) and oligo-1,6-glucosidase (EC 3.2.1.10; Suzuki et al., 1982; Suzuki & Tomura, 1986) (Fig. 3C). This valine residue in the α -(1 $\rightarrow$ 6)-glucoside hydrolases is a key residue for the specific hydrolysis of α -(1 $\rightarrow$ 6)-glucosides, because the substitution of this valine with

alanine enhances specificity to α -(1 $\rightarrow$ 4)-D-glucosides (Tsujimoto et al., 2007; Saburi et al., 2015). GH13_20 neopullulanase also has valine at this position (Yokota et al., 2001; Lee et al., 2002; Hondoh et al., 2003), although it shows high specificity to α -(1 $\rightarrow$ 4)-D-glucosidic linkage (Imanaka and Kuriki, 1989). Structural comparison of CmmB, *Geobacillus stearothermophilus* neopullulanase (maltotetraose complex; Hondoh et al., 2003; PDB entry, 1J0J), and *Streptococcus mutans* dextran glucosidase (SmDG; isomaltotriose complex, Hondoh et al., 2008; PDB entry, 2ZID) showed that the orientation of the side chain of V222 and V329 of CmmB and neopullulanase, respectively, is similar to each other, but that of V195 of SmDG is different from the others (Fig. 4). The valine residues of CmmB and neopullulanase do not cause any steric hindrance upon binding to maltooligosaccharides (Fig. 4A), while that of SmDG does (Fig. 4B). In SmDG, M198 is predicted to have hydrophobic contact with V195, and V195 presumably cannot take the orientation of the corresponding valine of CmmB and neopullulanase.

5. Conclusion

In this study, the substrate recognition mechanism of the GH13_30 α -glucosidase CmmB was investigated. CmmB showed high specificity toward maltose. Subsite +1 of CmmB for maltose binding was formed by H224 on the $\beta\rightarrow\alpha$ loop 4, W280 on the $\beta\rightarrow\alpha$ loop 5, and I360 and R364 on the $\beta\rightarrow\alpha$ loop 7. Among these residues, R364 was the most essential residue for the hydrolysis of maltose in terms of the $\Delta\Delta G$ value for alanine substitution. From the $\Delta\Delta G$ values for the mutants, $k_{\text{cat}}/K_{\text{m}}$ values for the maltose derivatives, and the structures of the CmmB complexes, R364 was thought to form a charged hydrogen bond with the O6 of the D-glucose residue of maltose in subsite +1. A

structural comparison of the CmmB complexes with acarviosin and acarbose suggested that the structure of the $\beta \rightarrow \alpha$ loop 7 was altered upon binding to maltooligosaccharides. This loop formed closed and open structures to bind to maltose and longer maltooligosaccharides, respectively. As the result of the structural change in the $\beta \rightarrow \alpha$ loop 7, I360 and R364 cannot participate in the formation of subsite +1 to bind maltotriose and longer oligosaccharides. Loss of the interactions between these residues and substrates caused the low preference for maltooligosaccharides longer than maltose.

CRedit authorship contribution statement

Wataru Saburi: Writing – original draft, investigation, project administration, **Takayoshi Tagami:** Writing – review & editing, investigation. **Takuya Usui:** Writing – review & editing, investigation. **Jian Yu:** Writing – review & editing, investigation. **Toyoyuki Ose:** Writing – review & editing. **Min Yao:** Writing – review & editing. **Haruhide Mori:** Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

The data provided in this study will be available from the corresponding author upon reasonable request.

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Figure legends

Fig. 1. Double reciprocal plots for pNPG hydrolysis in the presence of acarviosin and acarbose.

A, Reaction in the presence of acarviosin. Black circles, white circles, and black triangles indicate acarviosin concentrations of 0, 0.313, and 1.25 μ M, respectively. B, Reaction in the presence of acarbose. Black circles, white circles, and black triangles indicate acarbose concentrations of 0, 2.5, and 5 μ M, respectively. The values shown are the average of independent triplicate experiments. Error bars show the standard deviation of the three values.

Fig. 2. Structures of CmmB in complex with acarviosin and acarbose.

A, Overall structures of CmmB complexes with acarviosin (magenta) and acarbose (green). The $\beta \rightarrow \alpha$ loop 7 of the CmmB complex with acarviosin is shown in orange. The partial amino acid sequence of the $\beta \rightarrow \alpha$ loop 7 is shown. The sequences shown in orange and gray indicate the region modeled only in the acarviosin complex and the region disordered in both the complexes, respectively. Q347 and D370 are shown in white sticks. Ligands are shown in stick and ball representation. B and C, $F_o - F_c$ OMIT map of acarviosin and acarbose bound to the substrate binding site, respectively (contoured at 2.5 σ). D and E, Close-up view of the substrate binding site bound to acarviosin and acarbose, respectively. Ligands are shown in stick and ball representation. The subsite numbers are shown. Possible hydrogen bonds are indicated by yellow dotted lines. F and G, Surface representation of the acarviosin and acarbose complex, respectively. The $\beta \rightarrow \alpha$ loop 7, modeled, was shown in orange. H, Superimposition of the acarbose complex onto the acarviosin complex. Left, stick representation ($\beta \rightarrow \alpha$ loop 7 is shown in ribbon representation); right, surface representation of acarviosin complex. The color used are the same as for panels D and E.

Fig. 3. Structural comparison between CmmB and GH family 13 α -glucosidase-type enzymes.

A, Superimposition of HaG maltose complex (green sticks; general acid/base mutant E271Q; PDB entry, 3WY4) onto CmmB acarviosin complex (magenta sticks). R364 and I360 of CmmB are shown in orange sticks. Acarviosin and maltose are shown in ball and stick representation of yellow and pale orange, respectively. B, Comparison of structure of CmmB and HaG. The structures are shown in surface representations. The $\beta \rightarrow \alpha$ loops 4 and 7 are shown in cyan and orange, respectively. Maltose bound to HaG is superimposed onto CmmB. C, Multiple sequence alignment of α -glucosidase-type enzymes from bacterial origins. Multiple sequence alignment was constructed using MAFFT version 7 (Kato et al., 2019), and visualized using ESPript 3.0 (Robert & Gouet, 2014). BAD_0452, *B. adolescentis* ATCC 15703 α -glucosidase (GenBank number, AAL05573.1); Agl3, *B. breve* UCC2003 α -glucosidase (GenBank number, ABE94823.1); BLAG, *B. longum* subsp. *longum* JCM 1217 α -glucosidase (GenBank number, BAJ65782.1); AmyF, *Nesterenkonia* sp. F α -amylase (GenBank number, AWO12117.1); AglA, *T. curvata* α -glucosidase (GenBank number, AAA57313.1); HaG, *Halomonas* sp. H11 α -glucosidase (GenBank number, BAL49684.1); XgtA, *X. campestris* WU-9701 α -glucosidase (GenBank number, AAM42629.1); QsGH13, *Q. seohaensis* SW-135 α -glucosidase (NCBI Reference Sequence number, WP_232725784.1); BspAG13_31A, *Bacillus* sp. AHU2216 α -glucosidase (GenBank number, BBB94188.1); Gsj, *Geobacillus* sp. HTA-462 α -glucosidase (GenBank number, BAE48285.1); BcO16G, *Bacillus cereus* ATCC7064 oligo-1,6-glucosidase (GenBank number, CAA37583.1); SmDG, *S. mutans* ATCC25175 dextran glucosidase (GenBank number, BAE79634.1); LaGH13_31A, *Lactobacillus acidophilus* NCFM glucan 1,6- α -

glucosidase (GenBank number, AAV42157.1). Mutated residues in CmmB are indicated by arrows. Triangles below the alignment show the catalytic residues. The secondary structures of domain A are indicated above the alignment.

Fig. 4. Comparison of the structure of CmmB with dextran glucosidase and neopullulanase.

A, Comparison between CmmB acarviosin complex (magenta, but I360 and R364 are in orange) and *G. stearothermophilus* neopullulanase general acid/base mutant, E357Q, in complex with maltotetraose (green; PDB entry, 1J01). Acarviosin and maltose residue bound to subsites -1 and +1 are shown in stick and ball representation of yellow and pale orange, respectively. B, Comparison between CmmB in complex with acarviosin (the same coloring in panel A) and SmDG general acid/base mutant, E236Q, in complex with isomaltotriose (cyan; PDB entry, 2ZID). The isomaltosyl residue bond to subsites -1 and +1 of SmDG are shown in pale purple.

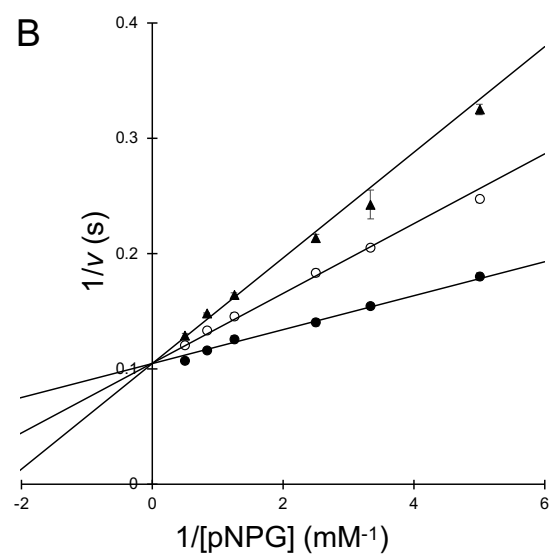
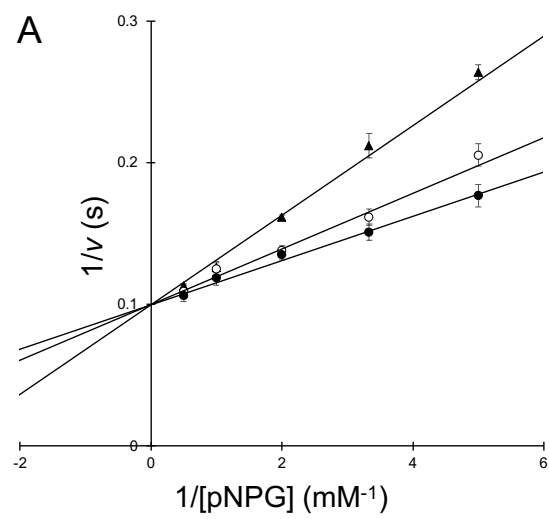


Fig. 1, Saburi et al.

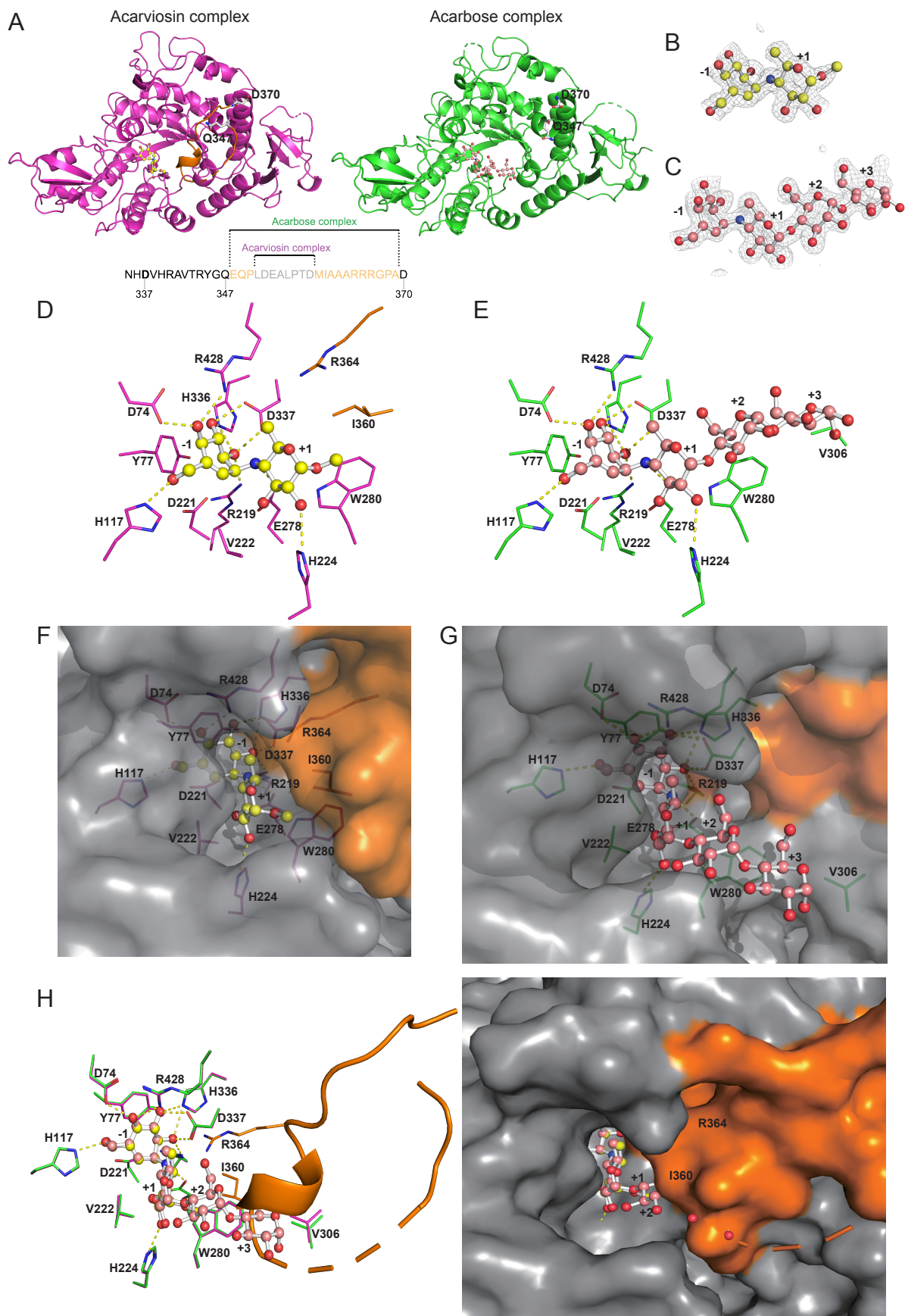


Fig. 2, Saburi et al.

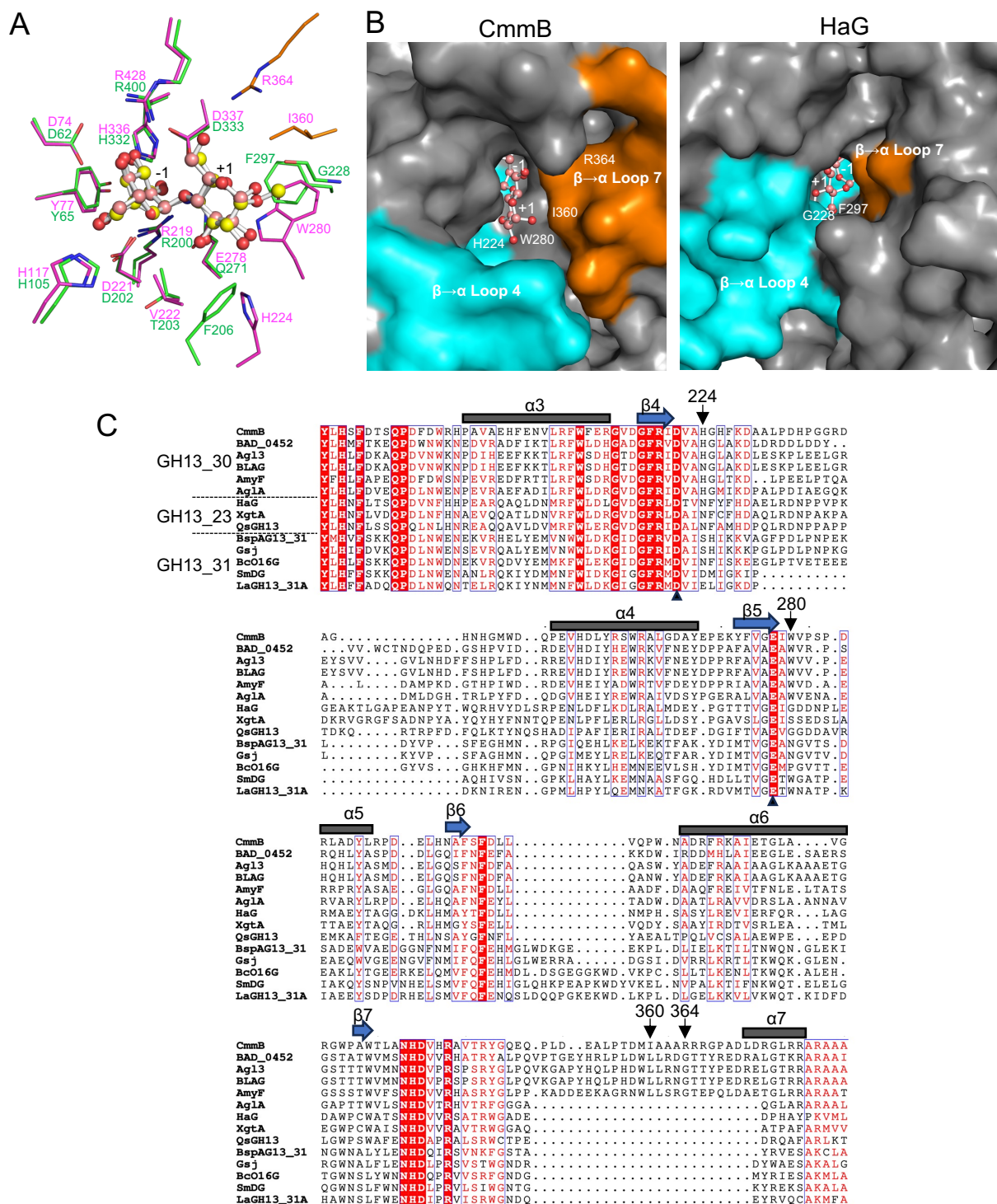


Fig. 3, Saburi et al.

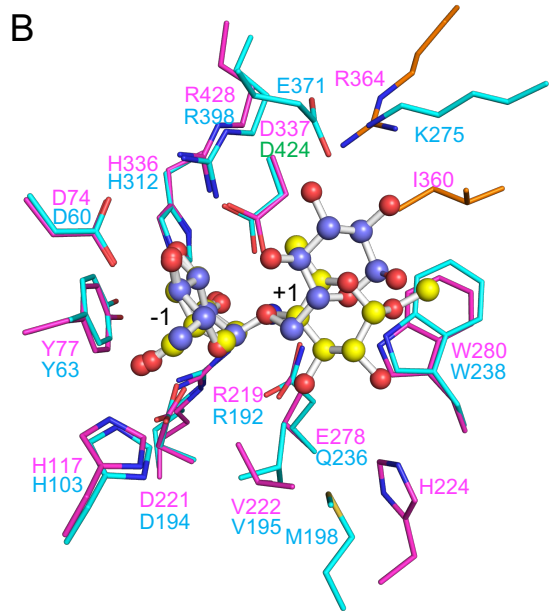
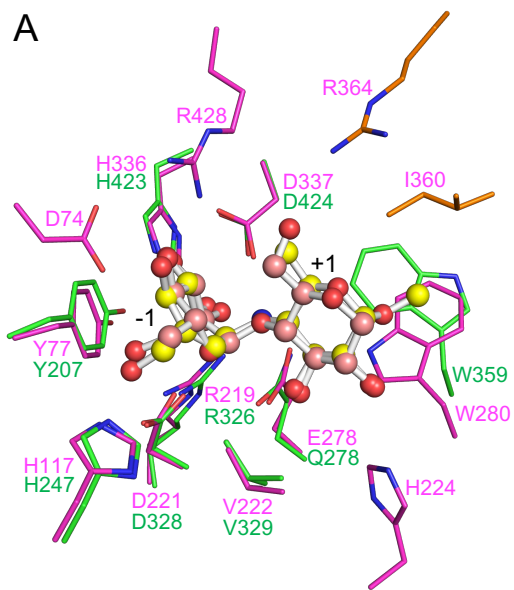


Fig. 4, Saburi et al.

Table 1. Summary of data collection and refinement statistics of X-ray crystallography of CmmB.

		Acarbose complex	Acarviosin complex
PDB entry		8YIE	8YIF
Data collection	Beamline	SPring-8 BL45XU	PF BL-5A
	Space group	$P2_1$	$P2_1$
	Unit cell parameters a, b, c, (Å)	51.4, 66.2, 148	51.2, 73.3, 73.2
	Unit cell parameters α , β , γ (°)	90, 95.7, 90	90, 90.5, 90
	Wavelength (Å)	1.00	1.00
	Resolution range (Å)	50–1.70 (1.76-1.70)	50–1.60 (1.70-1.60)
	Total number of reflections	363,787 (58,259)	468,814 (72,049)
	Number of unique reflections	107,839 (17,080)	70,932 (11,229)
	R _{meas} (%) [*]	10.3 (67.2)	8.91 (73.1)
	$\langle I/\sigma(I) \rangle$	10.0 (2.08)	15.7 (2.78)
	CC _{1/2}	99.7 (72.3)	99.9 (87.3)
	Completeness (%)	99.5 (98.1)	99.6 (98.2)
	Redundancy	3.37 (3.41)	6.61 (6.42)
	Z' **	2	1
Refinement	Number of reflections used	107,830	70,926
	Number of reflections for R _{free}	1,981	1,992
	R _{work} /R _{free} (%)***	16.6/20.5	17.1/19.6
	Number of atoms		
	Macromolecules	8,216	4,232
	Ligand	88	23
	Water	1,053	425
	B-factors (Å ²)		
	Macromolecules	18.4	20.2
	Ligand	26.0	14.1
	Water	27.2	26.8
	RMSD from ideal		
	Bond lengths (Å)	0.007	0.006
	Bond angles (°)	1.20	0.83

Ramachandran

Favored (%)	97.27	97.55
Allowed (%)	2.73	2.26
Outliers (%)	0	0.19

Values in parentheses are for the highest resolution shell.

* $R_{\text{meas}} = \sum_{hkl} \{N(hkl) / [N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ and $N(hkl)$ are the mean intensity of a set of equivalent reflections and the multiplicity, respectively.

****Z', Number of protein molecules in the asymmetric unit.**

*** $R_{\text{work}} = \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$, R_{free} was calculated for 5% randomly selected test sets that were not used in the refinement.

Table 2. Kinetic parameters of CmmB for various substrates.

Substrate	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (s ⁻¹ mM ⁻¹)	Relative $k_{\text{cat}}/K_{\text{m}}$ (%)
pNPG	$(1.09 \pm 0.01) \times 10$	$(2.54 \pm 0.14) \times 10^{-1}$	4.29×10	8.03×10^2
Maltose	$(1.27 \pm 0.03) \times 10$	2.38 ± 0.16	5.34	1.00×10^2
Maltotriose	3.94 ± 0.14	$(1.26 \pm 0.10) \times 10$	3.13×10^{-1}	5.86
Maltotetraose	$(5.67 \pm 0.10) \times 10^{-1}$	$(2.55 \pm 0.07) \times 10$	2.22×10^{-2}	4.16×10^{-1}
Glc(α 1-4)Man	$(1.12 \pm 0.06) \times 10$	$(1.17 \pm 0.10) \times 10$	9.57×10^{-1}	1.79×10
Glc(α 1-4)GlcNAc	N.D.	N.D.	2.25×10^{-2}	4.21×10^{-1}
Glc(α 1-4)Xyl	N.D.	N.D.	6.49×10^{-2}	1.22
Kojibiose	N.D.	N.D.	4.67×10^{-3}	8.74×10^{-2}
Nigerose	N.D.	N.D.	1.65×10^{-1}	3.09
Isomaltose	N.D.	N.D.	1.01×10^{-3}	1.89×10^{-2}
Panose	5.68 ± 0.50	$(1.50 \pm 0.23) \times 10$	3.79×10^{-1}	7.10
Sucrose	$(1.09 \pm 0.05) \times 10$	$(2.03 \pm 0.17) \times 10$	5.37×10^{-1}	1.01×10

Values are average $\pm$ standard deviation from triplicate experiments. N.D., not determined because a saturation curve was not obtained under the analytical conditions. From the slope of the $1/s-1/v$ plot, $k_{\text{cat}}/K_{\text{m}}$ was calculated.

Table 3. Kinetic parameters of CmmB variants for pNPG and maltose.

Substrate	pNPG				Maltose			
	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (s ⁻¹ mM ⁻¹)	$\Delta\Delta G$ (kJmol ⁻¹)	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (s ⁻¹ mM ⁻¹)	$\Delta\Delta G$ (kJmol ⁻¹)
Wild type	$(1.09 \pm 0.01) \times 10$	0.254 ± 0.014	4.29×10	N. A.	$(1.27 \pm 0.03) \times 10$	2.38 ± 0.16	5.34	N. A.
H224A	2.45 ± 0.03	0.275 ± 0.018	8.91	4.05	1.84 ± 0.05	1.59 ± 0.09	1.16	3.93
W280A	$(7.75 \pm 0.03) \times 10^{-1}$	0.286 ± 0.008	2.71	7.11	$(1.43 \pm 0.05) \times 10^{-1}$	7.06 ± 0.71	2.03×10^{-2}	1.44×10
I360A	1.80 ± 0.04	0.195 ± 0.001	9.23	3.96	2.12 ± 0.06	$(2.87 \pm 0.06) \times 10$	7.39×10^{-2}	1.10×10
R364A	$(3.48 \pm 0.10) \times 10^{-1}$	0.615 ± 0.027	5.66×10^{-1}	1.11×10	$(5.46 \pm 0.32) \times 10^{-3}$	$(1.04 \pm 0.04) \times 10$	5.25×10^{-4}	2.38×10

Values are average $\pm$ standard deviation from triplicate experiments. N. A., not applicable. $\Delta\Delta G$ was calculated from the following equation: $\Delta\Delta G = -RT \{ \ln(k_{\text{cat}}/K_{\text{m}})_{\text{mutant}} - \ln(k_{\text{cat}}/K_{\text{m}})_{\text{wild type}} \}$. Data of the wild type is from Table 2.