Structural Advantage of Sugar Beet \(\alpha\)-Glucosidase to Stabilize the Michaelis Complex with Long-chain Substrate

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The \(\alpha\)-glucosidase from sugar beet (SBG) is an exo-type glycosidase. The enzyme has a pocket-shaped active site, but efficiently hydrolyzes longer maltooligosaccharides and soluble starch due to lower \(K_m\) and higher \(k_{cat}/K_m\) for such substrates. To obtain structural insights into the mechanism governing its unique substrate specificity, a series of acarviosyl-maltooligosaccharides was employed for steady-state kinetic and structural analyses. The acarviosyl-maltooligosaccharides have a longer maltooligosaccharide moiety compared with the maltose moiety of acarbose, which is known to be the transition state analog of \(\alpha\)-glycosidases. The clear correlation obtained between \(K_i\) of the acarviosyl-maltooligosaccharides and \(\log(K_m/k_{cat})\) for hydrolysis of maltooligosaccharides suggests that the acarviosyl-maltooligosaccharides are transition state mimics. The crystal structure of the enzyme bound with acarviosyl-maltohexaose reveals that substrate binding at a distance from the active site is maintained largely by van der Waals interactions, with the four glucose residues at the reducing terminus from the active site pocket. The four glucose residues at the reducing terminus from the active-site pocket.

The enzyme seems to ingeniously use the self-stabilizing property of the substrate to form a stable ES complex.

Glucans of various types are widely distributed in nature. Each type of glucan adopts a unique conformation, which is dependent on the type of glycosidic linkage in the molecule. For the effective degradation of a variety of glucans, various subsites are present in glycoside hydrolases (1). Most endo-type glycoside hydrolases such as \(\alpha\)-amylase, dextranase, and cellulase contain cleft-shaped subsites (2–5), which, in \(\beta\)-amylase, extend from a pocket-shaped active site (6). Cellobiohydrolase, a cellulose-hydrolyzing enzyme, binds the \(\beta\)-1,4–glucan chain within a tunnel-shaped subsite (7). In the case of Coprinopsis cinerea cellobiohydrolase, the conformation of the tunnel-shaped subsite has been observed to change from an open to closed conformation in response to substrate binding (8). Such structures of enzyme subsites facilitate the loosening of the packed conformation of glucans via multiple interactions and contribute to the effective degradation of these carbohydrate polymers.

\(\alpha\)-Glucosidase is an exo-type enzyme, which catalyzes the hydrolysis of \(\alpha\)-glucosidic linkage at the non-reducing termini of substrate molecules. In addition to other exo-type glycosidases, \(\alpha\)-glucosidase has a pocket-shaped active site. A majority of \(\alpha\)-glycosidases exhibits preference for disaccharides and trisaccharides as substrates (9, 10). In contrast, several \(\alpha\)-glycosidases belonging to glycoside hydrolase family 31 (GH31) (11) are known to display specificity for substrates with a high degree of polymerization (DP) (12–14). Among them, the \(\alpha\)-glucosidase from sugar beet exhibits the highest specificity for long-chain maltooligosaccharides and soluble starch due to lower \(K_m\) and higher \(k_{cat}/K_m\) (15). The crystal structure of SBG in a complex with the pseudo-tetrasaccharide inhibitor, acarbose (AC4), was determined for structural analysis of its substrate specificity (16). The overall structure of SBG was found to be substantially similar to that of the other GH31 \(\alpha\)-glucosidases of known structure, and comprises a catalytic domain with \((\beta/\alpha)_8\)-barrel fold, and the N- and C-terminal domains with \(\beta\)-sandwich structures. SBG has a pocket-shaped active site, also found in other related \(\alpha\)-glucosidases (9, 10, 17–19). This pocket is formed by loops that exist between the \(\beta\)-strands and the \(\alpha\)-helices of the catalytic domain as well as a long loop (des-
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The loops that follow the third and fourth β-strands of the catalytic domain contain short insertions named subdomains b1 and b2, respectively. The structural study (16) demonstrated that Phe236 and Asn237 on the N-loop play a role in the specificity of SBG for long-chain substrates; however, a complete understanding is yet to be achieved, particularly because of the binding of substrates at subsites remote from the active site. In the present study, to comprehend the binding of long-chain substrates to SBG, a series of unique long-chain inhibitors, acarviosyl-maltoooligosaccharides (AC5-AC10, where the numeral represents DP), was employed as the ligands for structural resolution of the resulting complexes. The aforementioned studies provided important clues toward understanding the reason behind the specificities of SBG and the other GH31 enzymes for longer substrates; however, a complete understanding is yet to be achieved, particularly because of the binding of substrates at subsites remote from the active site.

In the current study, the potential of the various acarviosyl-maltoooligosaccharides for the inhibition of SBG was evaluated, and the crystal structures of SBG bound with AC5–AC8 were determined. The structures of the complexes of SBG with acarviosyl-maltoooligosaccharides elucidated the mechanism of substrate binding at subsites remote from the active site pocket. The specificity of SBG for long-chain substrates is likely due to the structure of remote subsites, which accommodate the stable single-helical conformation of long-chain amylose.

**EXPERIMENTAL PROCEDURES**

**Materials**—The substrates for kinetic analysis included a series of maltooooligosaccharides ranging from maltose (Glc₂) to maltoheptaose (Glc₇) (Nihon Shokuhin Kako, Tokyo, Japan), amylose EX-I (Glc₁₈, average DP of 18; Hayashibara, Okayama, Japan), and soluble starch (Nacalai Tesque, Kyoto, Japan). The concentration of non-reducing termini of the soluble starch was estimated as 0.136 μmol/mg using the Smith degradations method (22). A series of acarviosyl-maltoooligosaccharides ranging from acarviosyl-maltooctaose (AC5) to acarviosyl-maltooctaose (AC10) were enzymatically synthesized and purified by HPLC as previously reported (21). The purified acarviosyl-maltoooligosaccharides were evaporated to dryness and dissolved in water, and their concentrations were determined on the basis of absorbance at 214 nm. For the nomenclature of the sugar rings of acarviosyl-maltoooligosaccharides, the valienamine residue at the non-reducing end was termed as ring A, the 4-amino-4,6-dideoxy α-d-glucose residue as ring B, and the glucose residues in the maltoooligosacharyl moiety from the non-reducing toward the reducing side, as rings C, D, E, and so forth (Fig. 1).

**Enzyme Purification and Preparation**—Purification of native SBG from sugar beet seeds and preparation of endoglycosidase-F3-treated native SBG for crystallization were reported previously (16).

Heterologous expression of mutant SBGs in Pichia pastoris and their purification were carried out according to a previous report (20). The expression vector for each mutant enzyme was constructed by PCR using the PrimeSTAR mutagenesis basal kit (Takara Bio, Otsu, Japan), pGAPZαA vector carrying a SBG gene as the template, and primers 5′-AGGGACGTCATT-GTATGGATCCCAAC-3′ and 5′-CAAGTTAGCCCTCCAT-TGAACTAGC-3′ for Leu240→Ala mutation, 5′-CATATT-GCTATCAATATTTCTGAGGCC-3′ and 5′-ATTGATAGCC-ATAATGGGATGATTGCAAG-3′ for Lys493→Ala mutation, 5′-GGCCGTGCCTCCAAATAGACACT-3′ and 5′-TATGGACGGCCCTCCAGAATTATT-3′ for Val501→Arg mutation, 5′-GGCCGTGCCTCCAAATAGACACT-3′ and 5′-TATGGACGGCCCTCCAGAATTATT-3′ for Val501→Ala mutation, and 5′-CGTGTAGCTAATAATAGCAAGACT-3′ and 5′-ATTGACCCCGCTCCAGAATTATT-3′ for Pro502→Ala mutation with the underlined nucleotides indicating the mutated codons.

**Crystal Structure Analyses**—Co-crystallizations of endoglycosidase F3-treated native SBG individually with AC5-AC8 were performed by the hanging-drop vapor diffusion method at 25 °C, with the following composition of the drops: 3 μl of the enzyme (4.2 mg/ml), 3 μl of reservoir solution (50 mM sodium acetate buffer (pH 4.5), 50 mM ammonium sulfate, and 16–18% PEG monomethyl ether 2000), and 1 μl of the ligand (33.3 mM AC5, 15.8 mM AC6, 7.79 mM AC7, or 4.44 mM AC8). X-ray diffraction data were collected on beamline BL41XU at SPring-8 (Hyogo, Japan) in the same manner as described previously (16). All diffraction data sets were indexed, integrated, scaled, and merged using XDS (23). Crystals of AC5 and AC8 complex belonged to the space group. Complex structures of AC5 and AC8 were determined by a molecular replacement method with phenix.autormr (24, 25) using the SBG-AC4 complex (Protein Data Bank code 3W37) as a search model and those of AC6 and AC7 were determined by rigid body refinement with phenix.refine (26) using the SBG-AC4 complex model. After several cycles of manual model corrections with Coot (27) and refinement with REFMAC5 (28) and phenix.refine, the refinement converged. The coordinates and structure factors have been deposited in the Protein Data Bank with codes 3WEL, 3WEM, 3WEN, and 3WEO for the AC5, AC6, AC7, and AC8 complexes, respectively. Data collection and refinement statistics are summarized in Table 1.
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**TABLE 1**

Data collection and refinement statistics for data sets of SBG complex forms.

| Crystal            | AC5 complex | AC6 complex | AC7 complex | AC8 complex |
|--------------------|-------------|-------------|-------------|-------------|
| Crystal AC5 complex| P2₁,2₁,2₁   | P2₁,2₁,2₁   | P2₁,2₁,2₁   | P2₁,2₁,2₁   |
| Space group        |             |             |             |             |
| Unit cell parameters| (a, b, c) (A) | (a, b, c) (A) | (a, b, c) (A) | (a, b, c) (A) |
| (84.7, 97.9, 106.8) | (97.4, 139.4, 149.4) | (97.4, 138.9, 149.0) | (86.2, 99.2, 107.4) |
| Resolution range (A) | 38.9–1.84 (1.95–1.84) | 44.4–2.59 (2.75–2.59) | 42.2–2.59 (2.57–2.59) | 43.0–1.45 (1.54–1.45) |
| No. of unique reflections | 77,140 (11,929) | 31,809 (4,936) | 31,018 (4,862) | 162,274 (25,946) |
| Rfactor, Reff (%)  | 98.8 (95.8) | 99.4 (97.3) | 97.4 (95.5) | 99.5 (99.3) |
| (I/σ(I))%           | 9.16 (2.01) | 16.87 (3.14) | 15.04 (3.22) | 8.02 (2.46) |
| Multiplicity*        | 3.55 (3.55) | 5.57 (5.49) | 5.61 (5.50) | 3.66 (3.61) |
| Refinement           |             |             |             |             |
| Rwork (%)          | 0.1724      | 0.2038      | 0.1993      | 0.1234      |
| Rfree (%)          | 0.1975      | 0.2374      | 0.2451      | 0.1499      |
| No. of protein atoms | 6726        | 6606        | 6606        | 6824        |
| No. of water molecules | 604         | 216         | 175         | 1027        |
| No. of sugar residues of N-glycans | 6          | 5           | 5           | 6           |
| Root mean square deviation values from ideal | Bond lengths (A) | 0.007 | 0.005 | 0.003 | 0.008 |
| Bond angles (°)     | 1.140       | 1.182       | 0.799       | 1.308 |
| Ramachandran plot analysis | Favored region (%) | 96.8 | 97.6 | 96.1 | 97.5 |
| Allowed region (%)  | 2.3         | 3.7         | 3.7         | 2.4         |
| Outlier region (%)  | 0.2         | 0.1         | 0.2         | 0.1         |

* Values in parentheses are for the highest-resolution shell.

**RESULTS**

**SBG Inhibition by Acarviosyl-maltooligosaccharides**—The type of inhibition and the inhibition constants of AC4–AC10 for the hydrolysis of native SBG, which was prepared from sugar beet seeds, were determined using 1/[S] versus 1/v plots with Glc₇ as a substrate. The 1/[S] versus 1/v plots of all the acarviosyl-maltooligosaccharides showed linear correlation and interacted with each other on the y axis, indicating that the acarviosyl-maltooligosaccharides are competitive inhibitors of SBG. AC4 inhibited SBG with $K_i$ of 15.4 ± 3.5 μM, and the $K_i$ values decreased with increasing DP of the inhibitor (Table 2). In particular, a greater difference was found between the $K_i$ of AC4 and AC5 compared with the other acarviosyl-maltooligosaccharides. The plots of log $K_i$ for AC4–AC7 against log($K_{m}/k_{cat}$) for the hydrolysis of Glc₇–Glc₇ as well as log $K_i$ for AC4–AC7 against log $K_{m}$ for Glc₇–Glc₇ showed linear correlation with correlation coefficients of $r = 0.964$ (slope = 1.44) and $r = 0.985$ (slope = 1.59), respectively (Fig. 2). These results suggest that the acarviosyl-maltooligosaccharides mimic both the transition and ground states.

**Crystal Structures of Complexes of SBG with Acarviosyl-maltooligosaccharides**—SBG was co-crystallized individually with the acarviosyl-maltooligosaccharides AC5, AC6, AC7, and AC8, and the structures of the corresponding complexes were determined at 1.8, 2.6, 2.6, and 1.5 Å, respectively (Table 1). Analysis of the structures of all these complexes revealed that SBG existed as a monomer in each asymmetric unit and had three N-glycans at Asn404, Asn728, and Asn823 (Fig. 3A), as well as the previous crystal structures of SBG (16). The overall structures obtained in the present study were almost identical to the ligand-free and AC4-complex structures, and the root mean square deviations between every pair, as estimated by the Dali pairwise server (30), was within 0.5 Å. Co-crystals of sufficient size for structure determination could not be obtained for SBG with AC9 or AC10.

**Biochemical Assays—α-Glucosidase activity, protein concentration, and the effects of pH were measured as described previously (20). The type of inhibition and inhibition constants ($K_i$) of AC4–AC10 for native SBG were determined using 1/[S] versus 1/v plots. The hydrolysis velocities for Glc₋ (0.2, 0.24, 0.3, 0.4, 0.6, and 1.2 mM) in the presence of AC4 (6, 8, and 10 μM), AC5 (2, 4, and 6 μM), AC6 (0.5, 1, and 2 μM), AC7 (0.5, 1, and 2 μM), AC8 (0.5, 1, and 2 μM), AC9 (0.5, 1, and 2 μM), or AC10 (0.5, 1, and 2 μM) were measured under standard reaction conditions (20). The values of apparent $K_m$ for Glc₋ were obtained from the $x$ axis intercept of 1/[S] versus 1/v plots in the presence of the inhibitor, and the values of $K_i$ (μM) were calculated from the equation,

$$K_{m}^{app} = K_m(1 + [I]/K_i) \quad (\text{Eq. 1})$$

where $K_{m}^{app}$ is the apparent $K_m$ in the presence of the inhibitor, $K_m$ is the actual $K_m$ in the absence of inhibitor, and [I], the concentration of the inhibitor. The values of mean ± S.D. for $K_i$ at three inhibitor concentrations were calculated.

For the determination of kinetic parameters ($k_{cat}$, $K_m$, and $K_{cat}/K_m$), the initial rates for eight substrate concentrations were measured, and the kinetic parameters $k_{cat}$ (s⁻¹) and $K_m$ (mM) were determined from [S] versus v plots by fitting to Michaelis-Menten equation. The enzyme concentrations used were 0.388–0.778 (native SBG), 1.45–2.90 (L240A), 1.25–2.50 (K493A), 0.944–1.89 (V501A), 1.11–2.22 (V501R), or 1.41–2.81 nm (P502A).

The correlations between log $K_i$ for AC4–AC7 and log($K_{m}/k_{cat}$) for the hydrolysis of Glc₋–Glc₋ or between log $K_i$ for AC4–AC7 and log $K_{m}$ for Glc₋–Glc₋ were analyzed for evaluating the transition state mimicry of the acarviosyl-maltooligosaccharides. The correlations were derived on the basis of the equation $K_i = dK_{TS} = dK_{non}(K_m/k_{cat})$, where $d$ and $k_{non}$ represent proportionality and non-enzymatic reaction rate constants, respectively (29).
The electron density corresponding to acarviosyl-maltoligosaccharides was observed only at the active site of each structure (Fig. 3B). The electron density of the reducing glucose residue of AC7 (ring G) was not completely observed. This might be because of the kinetically weak affinity at subsite +2 of AC7 (ring G) was not completely observed. This might be because of the kinetically weak affinity at subsite +2 of AC7 (ring G) was not completely observed. This might be because of the kinetically weak affinity at subsite +2 of AC7 (ring G) was not completely observed. The conformations of rings A–D were extended, and these rings, particularly the acarviosine unit, were tightly bound to the enzyme through several hydrogen bonds. In contrast, the conformations of rings D–H were similar to that of the native helical conformation through intramolecular hydrogen bonds.

The conformations of rings A–C were extended, and these rings, particularly the acarviosine unit, were tightly bound to the enzyme through several hydrogen bonds. In contrast, the conformations of rings D–H were similar to that of the native helical conformation through intramolecular hydrogen bonds.

Rings C, D, and E were bound to the N-loop and subdomain b2 (Fig. 4B). Phe236, Asn237, and Leu240 on the N-loop contacted rings D and E, and directed the subsequent glucose residues (rings F–H) toward subdomain b2. Phe236 and Asn237 established interactions with ring D, as shown in our previous report (16, 20), whereas the side chain of Leu240 extended van der Waals contact with ring E. Ring E also bound the enzyme through hydrogen bonds between the O6 and O5 atoms and the hydroxy group of Ser497 in subdomain b2. Rings F, G, and H were located at a region of subdomain b2 spanning Lys493 to Pro502. The conformation of this region was tightly packed by 10 hydrogen bonds between the side chains and backbone, and this region interacted with rings F, G, and H via hydrogen bonds through the backbone and van der Waals contact through the side chains. Ring F interacted with the backbone carbonyls of Ser497 and Gly498 via a water molecule. Three hydrogen bonds were observed between ring H (O2 and axially oriented O1) and the backbone carbonyls of Arg500 and Gly498. Val501 was significantly close to rings F and G, and Lys493 and Pro502 were found in the vicinity of ring H. The multiple sequence alignment indicated that the region from Lys493 to Pro502 was highly conserved but Val501 of SBG was atypical among plant α-glucosidases (Fig. 4C).

**Site-directed Mutagenesis**—The structures of these complexes suggested that the side chains of Leu240, Lys493, Val501, and Pro502 are likely to be involved in substrate binding. The contributions of these residues to substrate binding were assessed using the mutant enzymes, L240A (Leu240 → Ala), K493A (Lys493 → Ala), V501A (Val501 → Ala), and P502A (Pro502 → Ala), which were generated using site-directed mutagenesis. In addition, the characterization of V501R (Val501 → Arg) was also performed to determine the functional role of the conserved arginine residue in the other plant α-glucosidases (Fig. 4C).

The conformations of rings A–C were extended, and these rings, particularly the acarviosine unit, were tightly bound to the enzyme through several hydrogen bonds. In contrast, the conformations of rings D–H were similar to that of the native helical conformation through intramolecular hydrogen bonds with adjacent glucose residues (Fig. 4A). All glucose residues had cis-orientation and were connected by O2′–O3 hydrogen bonds, for instance, O2 (ring D)–O3 (ring E); only the rings F and G were trans-oriented, and two hydrogen bonds, O6 (ring F)–O3 (ring G) and O5 (ring F)–O3 (ring G), were observed.
Both K493A and P502A exhibited almost the same Km values for all the substrates as rSBG, even though their kcat values decreased. The kcat/Km values for soluble starch were 80- (K493A) and 88-fold (P502A) higher than each for Glc2, namely the substrate specificities of the mutant enzymes were almost identical to rSBG.

L240A showed that its kcat for all the substrates equaled 59–72% that of rSBG. In contrast, the extent of decrease in kcat/Km values was dependent on the DP of the substrates. For instance, kcat/Km for Glc2–Glc4 substrates equaled 51–56% that of rSBG, whereas for Glc5–Glc7, the values equaled only 23%. Reduction in kcat/Km was also observed for Glc18 and soluble starch (21% of rSBG values for both substrates). This reduction in kcat/Km was associated with an increase in Km. Significantly higher Km of L240A was observed for substrates with DP of more than 4; for instance, the Km for Glc5–Glc7 was 2.6–2.8-fold that of rSBG, whereas for Glc3–Glc7, it was 1.1–1.3-fold. These results suggest that the substitutions of Val501 caused a reduction in affinity at subsite +4, which is in contradiction with the structure-based analysis, which revealed that Val501 is located close to rings F and G, occupying subsites +5 and +6.

DISCUSSION

In the current study, acarviosyl-maltooligosaccharides were employed for clarifying the mode of substrate binding at sites remote from the active site pocket. The Ki values of the acarviosyl-maltooligosaccharides AC5–AC10 for SBG were significantly lower compared with AC4, indicating that these acarviosyl-maltooligosaccharides are more effective inhibitors of SBG than AC4. The clear correlation observed between log Ki for AC4–AC7 and log(Km/kcat) for the hydrolysis of Glc4–Glc7 suggests that the inhibitors are transition state analogs (29). Relative to the substrate, the tighter binding of the acarviosyl-maltooligosaccharides to the active site results in a value of Ki, which is 3 orders of magnitude lower than Km (upon equating Km with Ks); this could be considered a consequence of the valienamine unit, which is considered to mimic the glycosyl cation-like transition state. The reduction in Ki with an increase in DP can be accounted for by a decrease in the dissociation constant of the maltooligosaccharide unit from the enzyme, because the correlation of log Ki versus log Km is similar to that of log Ki versus log(Km/kcat). In the hydrolysis reaction, the increase in kcat/Km with an increase in the DP of substrates is due to the decrease in Km for these substrates. The
values of $k_{\text{cat}}$ are almost unaltered, but $K_m$ decreases with an increase in the DP of maltooligosaccharides from 2 to 7 (Table 2). In other words, maltose binding at subsites -1 and +1 provides sufficient binding energy for lowering the activation energy of SBG, and the binding energy at the subsequent subsites +2, +3, and so forth are chiefly employed for decreasing the dissociation constant. This kinetic behavior is very similar to the inhibitory behavior of the acarviosyl-maltooligosaccharides; therefore, the binding of the acarviosyl inhibitors could be considered to represent the binding mode of the substrates, and hence, serves as an adequate probe for characterization of the remote substrate-binding site. The observed slope (1.44) of the correlation between log $K_i$ and log($K_m$/$k_{\text{cat}}$) as opposed to the expected slope (1.0) suggests a less than ideal mimicry of the transition state by the analog (31). The optimal mimicry of the transition state by an inhibitor should result in a 10$^{-12}$-fold or lower $K_i$, which is described as $K_{TS}$ in the case of transition state analog, compared with the $K_m$, considering the equation $K_{TS} = k_{non}/k_{cat}$ and the reported values of $K_{non}/k_{cat}$ (29, 32). The evaluated $K_i$ of the acarviosyl-maltooligosaccharides, of the range $(2.5 - 8.8) \times 10^{-3}$-fold that of the $K_m$ for the substrate, indicates a lesser degree of mimicry despite the tighter binding of acarviosyl-maltooligosaccharides to the active site compared with the substrate. The slightly higher values of $K_i$ of acarviosyl-maltooligosaccharides compared with the theoretical values are attributable to imperfect mimicry by the valienamine unit.

Crystal structure analyses of SBG bound with the acarviosyl-maltooligosaccharides revealed the molecular basis of substrate binding at a site distant from the active site pocket. The N-loop and the region spanning Lys$^{493}$ to Pro$^{502}$ in subdomain b2 are involved in the binding of the maltooligosaccharide part of the acarviosyl-maltooligosaccharides, even though Lys$^{493}$ and Pro$^{502}$ have little contribution to decreasing $K_m$ values for all the substrates (Table 3). In a previous study from our group (16), the role of Ser$^{497}$ (in subdomain b2) in governing the affinity at subsite +4 was demonstrated through site-directed mutagenesis. In agreement with the previous study, the structures of complexes with acarviosyl-maltooligosaccharides revealed the interaction of Ser$^{497}$ with O5 and O6 of ring E through hydrogen bonds. The glucose residues of the maltooligosaccharide part were primarily observed to make contact with remote subsites through van der Waals interactions. The aliphatic side chains of Leu$^{240}$ and Val$^{501}$ significantly interacted with rings E and F/G, respectively. Therefore, the mutant enzyme L240A had a substantially reduced affinity at subsite +4. In contrast, the kinetic characteristics of V501A are in conflict with its structural attribute. Structural analysis suggested that Val$^{501}$ likely contributes to the affinity at subsites +5/+6; however, the mutant V501A exhibited reduced affinity at subsite +4. This contradiction could be explained by the effect of the Val$^{501}$ mutation on Ser$^{497}$. As shown in our previous report (16), the mobility of the side chain of Ser$^{497}$ is high, resulting in its observation as a dual conformation of the AC4 complex structure. The side chain of Val$^{501}$ is located close to the hydroxy group of Ser$^{497}$, and the mutation (Val$^{501}$ → Ala) may increase the mobility of the hydroxy group of Ser$^{497}$; such increased mobility is likely to diminish the interaction of the hydroxy group with the substrate, leading to lowered affinity at subsite +4. Furthermore, V501R showed the same substrate specificity as V501A, indicating that an arginine residue at the Val$^{501}$ position, which is observed in other plant α-glucosidases, is not enough to increase affinity at subsite +4. It is possible that Val$^{501}$ of SBG is one of the key residues to achieve its
enormous specificity for long-chain substrates as compared with other plant enzymes.

The higher specificity of SBG for longer substrates, which is responsible for the lower dissociation constant of substrates with high DP from the enzyme, appears to be rationalized by the conformation of rings D–H. Of the rings A–H bound to SBG, rings A–C at the non-reducing terminus are extended and the energy gained by binding with the extended substrate contributes to decreasing the activation energy of the hydrolysis reaction. In contrast, rings D–H remain in the stable conformation, as also observed in cycloamyloses and the helical structures of V-amylose (33). SBG is unlikely to obtain the binding energy required for lowering the activation energy from the binding of rings D–H. The binding of substrates in a stable conformation appears to be advantageous compared with a strained conformation insofar as the decrease in dissociation constant is concerned, providing additional energy is unnecessary. As mentioned above, the higher specificity for longer substrates is responsible for the lower dissociation constant of the substrates with high DP from the enzyme. The subsite structure suitable for native substrate conformation is likely to result in lower $K_m$ for the substrates with high DP without the extra energy. Moreover, SBG is likely to ingeniously use the self-stabilizing property of amylose and soluble starch to form stable $E_S$ complexes with these long-chain substrates.

The high specificity of SBG for amylose and soluble starch is also attributable to the band-flip, which is found between rings F and G in structures of AC7 and AC8 complexes. The band-flip observed in the helical structures of the larger cycloamyloses and V-amylose has been generally accepted to be responsible for relieving the strain induced in the macrocycle (34). A likely hypothesis is that AC8 forms the band-flip to alleviate the strain caused, and the structure of SBG is fit to flip. However, the subsite structure of SBG has also been considered to provoke the band-flip; thereby, relieving the strain in long-chain helical substrates leading to the formation of a stable $E_S$ complex. In the latter case, the subsite structure likely allows SBG to handle $\alpha$-1,6-branched structures in the substrate at the $trans$-oriented point. In the structures of complexes with acarviosyl-maltooligosaccharides, the hydroxy groups of C6 atoms of the rings A–F are close to the enzyme surface, and $\alpha$-1,6-branched structures are unlikely to be accommodated there. However, the $trans$-oriented bond results in the O6 atoms of rings G and H facing outward, which would allow the accommodation of $\alpha$-1,6-branched structures. The extremely higher specificity of SBG for soluble starch (branched substrate) than Glc18 (linear substrate) may be attributable to this mode of substrate binding.

The current study has highlighted the utility of acarviosyl-maltooligosaccharides and provided structural insights into the mechanism that governs the high specificity of SBG for substrates with high DP. The acarviosyl-maltooligosaccharides proved to be useful as inhibitors of SBG. Moreover, kinetic analysis has demonstrated that the binding of the series of acarviosyl inhibitors represents the binding mode of the substrates. Analyses of the structure of the acarviosyl-maltooligosaccharide complexes revealed that subsites remote from the active-site pocket of the enzyme complement the native structure of amylose, which adopts a helical conformation through intramolecular hydrogen bonds. This mode of binding likely leads to lower $K_m$ and higher specificity for longer substrates. The study of acarviosyl-maltooligosaccharides is likely to prove useful for the study of other $\alpha$-glycosidases as well. For example, glucoamylase shows notable specificity for longer malto-

![TABLE 3](image-url)
ligosaccharides, but the crystal structure of the enzyme has been solved with acarbose (35). The use of acarviosyl-maltoooligosaccharides is likely to clarify the mode of substrate binding at subsites far from the active site of glucoamylase.

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