Crystal Structure of Recombinant Soybean β-Amylase Complexed with β-Cyclodextrin*

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In order to study the interaction of soybean β-amylase with substrate, we solved the crystal structure of β-cyclodextrin-enzyme complex and compared it with that of α-cyclodextrin-enzyme complex. The enzyme was expressed in Escherichia coli at a high level as a soluble and catalytically active protein. The purified recombinant enzyme had properties nearly identical to those of native soybean β-amylase and formed the same crystals as the native enzyme. The crystal structure of recombinant enzyme complexed with β-cyclodextrin was refined at 2.07 Å resolution with a final crystallographic R value of 15.8% (Rfree = 21.1%). The root mean square deviation in the position of C-α atoms between this recombinant enzyme and the native enzyme was 0.22 Å. These results indicate that the expression system established here is suitable for studying structure-function relationships of β-amylase. The conformation of the bound β-cyclodextrin takes an ellipsoid shape in contrast to the circular shape of the bound α-cyclodextrin. The cyclodextrins shared mainly two glucose binding sites, 3 and 4. The glucose residue 4 was slightly shifted from the maltose binding site. This suggests that the binding site of the cyclodextrins is important for its holding of a cleaved substrate, which enables the multiple attack mechanism of β-amylase.

β-Amylase (α-1,4-glucan maltohydrolase; EC 3.2.1.2) catalyzes the removal of β-anomeric maltose from the nonreducing ends of starch and glycogen. This enzyme is distributed in higher plants and in some microorganisms. The cDNAs from five kinds of plants (soybean (1), barley (2), rye (3), Arabidopsis thaliana (4), and sweet potato (5)) and those of three kinds of bacteria (Bacillus polymixa (6, 7), Bacillus circulans (8), and Clostridium thermosulfurogenes (9)) have been cloned and sequenced. Plant β-amylases are similar to each other in terms of their physicochemical properties, i.e. molecular mass (50–60 kDa), optimum pH, amino acid sequence, and their subunit structure (with the exception of the homotetramer sweet potato β-amylase) (10–12).

cDNAs of β-amylase from barley, sweet potato, and soybean have been expressed in E. coli (1, 5, 13). Yoshigi et al. (14) tried to produce the thermostable barley β-amylase by random and site-directed mutagenesis using the E. coli expression system. The produced 7-fold mutant was more stable than the wild-type recombinant enzyme by 11.6 °C (14). The soybean β-amylase was expressed using pKK233–2 expression vector (1). The catalytic efficiency of the recombinant enzyme, however, was lower than that of the native enzyme.

The crystal structure of native soybean β-amylase complexed with α-cyclodextrin (α-CD) was solved at 2.0 Å (12) by the isomorphous replacement method. Cheong et al. (15) reported the crystal structure of tetrameric sweet potato β-amylase at 2.3 Å resolution. The structural analysis of the soybean maltose-β-amylase complex indicated that Glu186 and Glu380 play important roles in the enzymatic reaction as general acid and base catalysts, respectively (16). This finding is supported by the results of site-directed mutagenesis (17, 18) and affinity labeling (19). In addition, the structures of α-CD-β-amylase and maltose-β-amylase complexes revealed that a flexible loop plays a key role in the reaction.

CDs and maltose competitively inhibit the activity of β-amylase by binding to the active cleft (12, 20). α-CD binds to soybean β-amylase of an open loop form (12), whereas two maltose molecules tandem bind within the active cleft of the enzyme in a closed loop form (16). These maltose binding sites are located on both sides of the catalytic residues and are postulated to be substrate binding sites, subsites 1 and 2 and subsites 3 and 4 (16). The structure of the α-CD-β-amylase complex showed that only one glucose residue in the α-CD binds near subsite 4, where the binding force essentially involves hydrophobic interactions (16). The exact position of this glucose residue is shifted about one-half residue to the side of the reducing end, suggesting the flexibility of subsite 4 against altered positions of glucose residue (16). This flexibility of subsite 4 may elucidate the mechanism of single chain attack of β-amylase on the polymeric substrate (11, 21). β-CD is the cyclic oligosaccharide consisting of seven glucose, while α-CD has six glucose in the ring. The diameter of cavity in β-CD is about 1.2 times as long as that in α-CD, and the bond angle and two torsion angles in the glycosidic links differ slightly between the two CDs (22). Since the K_i value of β-CD (1–2 mM) is roughly 3 times that of α-CD (0.3–0.5 mM) (23–25), it should be clarified whether the glucose residue involved in the binding of α-CD still remains or whether the least favored interactions occur between β-CD and the enzyme.

In this study, the cDNA sequence of soybean β-amylase was cloned and expressed in E. coli. The crystal structure of the recombinant β-amylase complexed with β-CD was analyzed at 2.07 Å to elucidate the flexibility of the substrate binding site.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) D50866. The atomic coordinates and structure factors(1bfn) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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1 The abbreviations used are: CD, cyclodextrin; r.m.s., root mean square; ME, 2-mercaptoethanol.
**EXPERIMENTAL PROCEDURES**

Cloning and Sequencing of Soybean β-Amylase cDNA—Poly(A) RNA was prepared from the developing cotyledons of soybean (*Glycine max* L. cv. Wasesuzumari) using an EXTRACT-A-PLANT RNA isolation kit (CLONTECH) and mRNA purification kit (Amersham Pharmacia Biotech). A cDNA library was constructed in λ ZAPII phage using a ZAP-cDNA synthesis kit (Stratagene) (26). The cDNA ligated with the synthesized oligo(dT) primer (primer N’ 5’-GCCACT-TCCGACGATACATGC) referring to the nucleotide sequence of β-amylase reported for soybean cv. Bonminori (1). Plaque hybridization was performed with 5’-end-labeled primer N in 6 × SSC at 50 °C for 12 h. Nylon membranes (Hybond-N, Amersham) were washed with 6 × SSC at 65 °C. λ ZAPII phages containing full-length cDNA were screened by restriction enzyme (EcoRI and XhoI) analysis, and six clones were selected. The phages containing full-length cDNA were excised with the R408 helper phage, and one (pBSB7) of the resultant plasmids containing the longest cDNA was sequenced by Taq dye cycle sequencing using Applied Biosystems sequencer model 373A.

Construction of an Expression Plasmid for β-Amylase—DNA sequencing coding a mature β-amylase were amplified by polymerase chain reaction using primers N and C (5’-CCGGGATCCACGTTGG-GAAATACCAATCTTCTTC) containing BamHI site. Polymerase chain reaction was conducted on pBSB7 with reagents supplied in kit form (Takara Shuzo Ltd., Kyoto, Japan) in a DNA thermal cycler model 480 (Perkin-Elmer). A polymerase chain reaction cycle consisted of denaturation at 95 °C for 30 s, annealing at 50 °C for 1 min, and extension at 72 °C for 3 min. After 30 cycles, the products were separated by electrophoresis on 1.2% (w/v) agarose gel and purified by using glass microfiber. The resultant 1.5-kilobase pair fragment was blunt-ended with T4 DNA polymerase and ligated to the filled in BamHI site of pBSB7. The blunt 1.5-kilobase pair fragment was inserted into the filled in NcoI site of an expression vector pET21d (Novagen, Madison, WI) to generate pESBA. The cDNA sequence in the pBSB7 was sequenced.

Expression and Detection of β-Amylase from *E. coli*—The expression plasmid pESBA was transformed into *E. coli* strain BL21(DE3), BL21(DE3)pLysS, HMS174(DE3), and HMS174(DE3)pLysS, and pKBSA into JM105, JM107, JM109, MY1184, SOLR, and XLI-Blue. Each *E. coli* strain harboring individual expression plasmid was cultured in LB, TB, 2YT, NZY, and M9 minimal medium (30), each supplemented with ampicillin (50 μg/ml) at 30, 25, 30, and 37 °C for 3, 24, and 40 h. At *A*~600~ = 0.3, 0.8, or 1.5, isopropyl-β-d-thiogalactopyranoside was added to a final concentration of 1 mM. The cells were harvested by centrifugation and disrupted by sonication in 100 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. The cell debris and the supernatant were fractionated by centrifugation. The SDS-polyacrylamide gel electrophoresis analysis of the total cells, the cell debris, and supernatant was performed according to the method of Laemmli (31). The proteins separated in SDS gels and analyzed for the amount of α-amylase activity were pooled and dialyzed against 0.05 M sodium acetate buffer (pH 4.8) containing 1 mM EDTA and 18 mM ME. After dialysis, the protein solution was applied to a CM-Sephadex C-50 column (2 × 25 cm) (33). Proteins were eluted with a pH gradient of 50 mM sodium acetate buffer from 4.5 to 6.0. To investigate the homogeneity of the purified recombinant enzyme, polyacrylamide gel isolectric focusing was performed using Ampholine pH3–10 (Amersham Pharmacia Biotech).

N-terminal Amino Acid Sequence Analysis—The N-terminal amino acid sequence analysis was performed by automatic Edman degradation on an Applied Biosystems model 477A pulse-liquid sequencer system. The purified recombinant protein in buffer solution was dialyzed against distilled water and applied to a polybrene-treated glass membrane.

Crystallization of Recombinant β-Amylase—The purified recombinant enzyme was subjected to crystallization under similar conditions as for native soybean β-amylase (12) by the vapor diffusion method using Linbro multitwell tissue culture plates (34). The crystals were grown at a constant temperature of 4 °C from an initial protein concentration of 10 mg/ml in a drop composed of an equal volume (5 μl each) of a protein solution and a well solution (1 ml) containing 49% ammonium sulfate, 0.1 M sodium acetate buffer, 1 mM EDTA, and 18 mM ME, pH 5.4.

Data Collection—Crystals formed by the hanging drop method were transferred from the drop to 0.1 M pH 5.4 sodium acetate buffer containing 1 mM EDTA and 50% saturated ammonium sulfate for storage. The crystals belong to space group P2~1~21 with unit cell dimensions a = b = 86.03 Å and c = 144.80 Å (Z = 6) and grow to a size of about 0.4 × 0.5 × 0.4 mm. The crystals were soaked in the same buffer containing 5 mM β-CD for 1 h at room temperature before data collection. Data were collected on a RIGAKU R-AXIS IIC imaging plate area detector at the Institute for Chemical Research, Kyoto University. The detector was positioned at a distance of 110 mm from the crystal at a 2θ angle of 7.° 1.5°. The crystal was exposed to x-rays for 15 min in oscillation frame of 1.5°. The crystal diffracted the x-rays to 2.07 Å and was stable during 12 h for 40 oscillation frames. The cell dimensions were refined by the least square method. Data from different frames were integrated separately and then merged together (Rmerge < 6.0% for 152,726 measurements). In the resolution range of 10–2.0 Å, 36,696 of the 53,914 theoretically possible reflections having intensities of more than 2 σ were used for the refinement.

Model Building and Refinement—Structure was refined using the program X-PLOR version 3.1 (35). The initial phases were calculated using the coordinates from the protein structure of native β-amylase complexed with α-CD (12). After rigid body refinement, three amino acids were substituted (as shown in Fig. 1), and atoms in glucose residues 3–5 of β-CD were added to the model. The model was systematically improved throughout iterative cycles containing positional and B-factor refinements. The model was rebuilt using the Turbo-Frodo program (BioGraph) and improved using a stepwise increased resolution of data and the addition of solvent and remaining β-CD atoms. The model was fitted as judged by inspection of electron density maps calculated with both 2Fo − Fc and Fc − Fo coefficients.

RESULTS

Cloning of Soybean β-Amylase cDNA—We screened cDNAs encoding β-amylase from a soybean cDNA library. The first screening gave 200 positive clones from 80,000 plaques. Six phages containing a full-length cDNA were selected from 20 positive clones by restriction enzyme analysis after a second successive screening. The inserts from six positive clones were in the range of 1.6–1.7 kilobase pairs. The longest cDNA was sequenced. The cDNA had an open reading frame with 1488 bp coding a polypeptide composed of 496 amino acids with a calculated molecular mass of 56,069 Da. Comparison of the nucleotide sequence of β-amylase cDNA with that from cv. Bonminori (1) shows that two nucleotide substitutions occur in the coding region, resulting in two replacements of amino acid
residues. The 202nd arginine and 399th lysine were replaced by glycine and arginine, respectively, in Wasesuzumari (Fig. 1).

Expression of Soybean β-Amylase cDNA in E. coli—When β-amylase cDNA was expressed in E. coli at 37 °C, the expression level of active enzyme was only 0.5% and 1% of total E. coli proteins in the cases of pKSBA in JM105 and pESBA in BL21(DE3), respectively. Thus, we tried to establish conditions giving a high level and stable expression by changing E. coli strain, culture temperature, and induction timing and period, as described under “Experimental Procedures.” Although we were unable to induce a high level and stable expression under any conditions in the case of pESBA, we succeeded at doing so in the case of pKSBA under the following conditions: strain JM105 cells harboring pKSBA grown at 37 °C in LB and expression induced at A600 = 0.8 by isopropyl-β-D-thiogalactopyranoside at 20 °C for 40 h. SDS-polyacrylamide gel electrophoresis analysis and specific activity of the extract showed that the expression level was approximately 10% of cellular protein (Fig. 2A, lane 1). Degradation of the expressed protein decreased dramatically compared with that in the case of induction at 37 °C (data not shown), and more than 90% of this protein was recovered in soluble fraction after sonication. The activity of the recombinant enzyme extracted from 1 ml of growth medium was 34.7 units, which was 37.3 times that obtained by Totsuka et al. despite the similar expression conditions (1). In addition, the activity of the purified enzyme was about 3 times as high as that they reported. The cDNA sequence in the pKSBA differed from the cloned cDNA at two positions as a result of the polymerase chain reaction (Fig. 1). One replacement resulted in an amino acid substitution at position 76 (Phe → Leu), as shown in Fig. 1. Since the substitution was regarded as causing no effective change in enzyme properties based on a reasonable specific activity of crude extract, we used pKSBA for further analysis.

Purification and Characterization of Recombinant β-Amylase—E. coli strain JM105 cells harboring expression plasmid pKSBA were used for bench scale preparation of the recombinant enzyme. Seventy g of the wet bacteria were recovered by glycine and arginine, respectively, in Wasesuzumari (Fig. 1). Enzyme Kinetics—A mean positional Rfactor of 0.211, when all observed data upon 318 water molecules, a sulfate ion, and a β-CD molecule. The position of β-CD in active cleft is similar to that of α-CD (12). The five amino acids of the N terminus were missing from the model because of their disorder. The r.m.s. deviations of bond lengths, bond angles, dihedral angles, and improper angles were 0.03 Å, 2.89°, 24.3°, and 1.11°, respectively. The final model contains 490 amino acids (residues 6–495), 318 water molecules, a sulfate ion, and a β-CD; homogenate (5 μg); lane 4, fraction eluted from the CM-Sephadex column (1.5 μg). B, lane 1, native soybean β-amylase; lane 2, purified recombinant β-amylase.

| Parameters                  | Enzyme II | Recombinant |
|-----------------------------|-----------|-------------|
| Apparent K_m (mg/ml)        | 1.94      | 1.90        |
| Apparent V_max (s⁻¹)        | 1260      | 1280        |
| Optimum pH                  | 5.4–6.0   | 5.4–6.0     |
| T_50 (°C)                   | 66.3      | 65.3        |
| K for α-CD (mM)             | 0.70      | 0.68        |
| K for β-CD (mM)             | 1.68      | 1.64        |

Comparison of Structures between the Recombinant Enzyme Complexed with β-CD and the Native Enzyme Complexed with α-CD—To confirm the fidelity of the expression system constructed here, we compared the protein structure of the recombinant enzyme with that of native soybean β-amylase (12). The rigid body fitting indicated an r.m.s. distance of 0.22 Å between

![Fig. 2](image-url)
pairs equivalent C-α atoms. This indicates that the native and recombinant enzyme have the same overall structure. Fig. 3 provides a more detailed comparison. From the plot, three peaks were formed at around Ile$^{102}$, Gly$^{202}$, and Thr$^{342}$. The C-α-C-α distances at Ile$^{102}$, Gly$^{202}$, and Thr$^{342}$ were 1.06, 0.74, and 1.83 Å, respectively. The first region around Ile$^{102}$ is included in the flexible loop having a high B-factor value. The average B value of atoms in residues 96–103 was 40.4Å$^2$. The second is the region around the revised Gly$^{202}$, which was modeled as arginine in the structure of β-amylase complexed with α-CD (12). The displacement resolved the discrepancy observed between the model and electron density during refinement for the crystal structure of the native enzyme. The third region includes Cys$^{343}$. The SH group of the residue in the recombinant β-amylase is reduced, while that in the native enzyme complexed with α-CD is modified by ME to form a mixed disulfide (12). Therefore, these three differences can be discounted, and the structure and enzymatic characteristics described above indicate that the expression system established here is suitable for studies of structure-function relationships of β-amylase by means of x-ray crystallography and protein engineering.

The Structure of Bound β-CD—At the beginning of refine-

![Plot of C-α distances between recombinant β-amylase-β-CD and native β-amylase-α-CD complexes versus residue number. A least-square rigid body fitting was performed by the program of Turbo-Frodo.](image)

![Stereo views of the CD binding sites of soybean β-amylase. A, Fo-Fc omit map contoured at 3 σ for β-CD molecule bound in the active site cleft. B, hydrogen bond interactions of β-amylase with β-CD. The protein residues of each complex were superimposed using the Rigid Program of Turbo-Frodo. Thick lines represent the β-CD structure in the β-CD complex. Thin lines represent the α-CD molecule in the α-CD complex. Residue types and sequence numbers are labeled. The water molecules in the complex with β-CD are indicated by closed circles. Dotted lines represent potential hydrogen bond interactions within 3.2 Å. Protein residues having apparent Van der Waals contact with the β-CD are labeled.](image)
ment, the electron density for sugar units of β-CD was clear and unambiguous in only three glucose residues, 2, 3, and 4, but all seven glucose residues were visible after refinement. Fig. 4 shows an omit map and the structures of β- and α-CDs in the active cleft of the enzyme. While α-CD bound to β-amylase took a flat circular form (12), the conformation of the bound β-CD in the β-amylase complex was found to be a distorted ellipse. The distorted shape of the bound β-CD was dissimilar to the shape observed in the complex of the maltose-binding protein (39) and to that observed in crystalline β-CD hydrate (40). This suggests that the conformation of the bound β-CD changed to fit the enzyme rather than vice versa.

The following parameters (average and S.E.) were found for the seven α-glucose residues in the bound β-CD: glycosidic bridge atom angle, 118.9 ± 0.8°; φ torsion angle (O-4⋯C-1–O-4'⋯C-4'), +166.4 ± 4.5°; ψ torsion angle (C-1⋯O-4'⋯C-4'⋯O-4'), −170.2 ± 4.3°. The S.E. for the φ torsion angle was 1.8 times higher than that of α-CD (14) in the α-CD-β-amylase complex, although that for the ψ angle was almost the same. Moreover, we compared φ and ψ angles in each glycosidic linkage of β-CD with those of α-CD by plotting (Fig. 5). The profiles of the φ and ψ angles were similar to each other except for the ψ angle between glucose residues 4 and 5. The difference of this φ torsion angle was 30°, and for both residues the angle still corresponded to a low energy conformation (41).

Interactions of β-CD with Enzyme—Interactions between the bound β-CD and the recombinant β-amylase are summarized in Table II. There were four direct hydrogen bonds and five water-mediated hydrogen bonds by three water molecules between hydroxyl groups of β-CD and protein. In these interactions, water-mediated hydrogen bonds with H2O1662 were found only in the β-CD complex, although direct hydrogen bonds with O atom of Ala382 and N atom of His300 and water-mediated hydrogen bonds with H2O655 and H2O1702 were also found in the complex of β-amylase with α-CD. Interactions with Van der Waals contacts were similar to those of the α-CD complex (12), and there were the greatest number of contacts between glucose residue 4 and the protein. Leu1883 formed an inclusion complex such as that in the α-CD-β-amylase complex (12).

For comparison of the location of glucose residues, we calculated five r.m.s. distances in a residue in between both CDs after Cα rigid body fitting for the protein. These r.m.s. values were 1.35, 0.84, 0.43, 0.33, and 2.4 Å for residues 1–5, respectively. Residue 4 allowed the best superimposition, and was located one-half residue from subsite 4 in the direction of the reducing end. The r.m.s. values of residues 4–1 gradually increased relative to that from 4 to 5. These results are consistent with the dramatic change of dihedral angle φ between residues 4 and 5 (Fig. 5). This is probably because β-CD forms water-mediated hydrogen bonds with H2O1662, unlike the α-CD complex.

**DISCUSSION**

In general, the expression level of a foreign protein in *E. coli* depends on the kind of promoter, the stabilities of mRNA and the protein product, the culture conditions, and other such factors (42). To obtain a high level expression of recombinant soybean β-amylase, various experimental conditions were studied. The results indicated that the induction temperature and period were the most important factors for high level expression of soybean β-amylase. The system established here provided a recombinant enzyme having properties nearly identical to those of the native enzyme. Although our expression system was similar to that reported by Totsuka *et al.* (1), both the purified recombinant enzyme in this study and the native enzyme exhibited 3 times higher specific activity than that of the recombinant enzyme reported by these previous authors.

As for the reasons why their recombinant enzyme had a much lower specific activity than the native enzyme, they proposed the following three factors: (i) the requirement of a molecular chaperon for correct folding; (ii) the requirement of N-terminal acetylation for activity; and (iii) the microheterogeneity of the native enzyme from soybean seed (1). Comparing our recombinant enzyme with the native enzyme, we found no significant difference in catalytic characteristics or protein structures, indicating that soybean β-amylase does not require N-terminal acetylation and molecular chaperons other than those of *E. coli* for its activity and folding. The cDNA used in this study differs in two positions from that reported by Totsuka *et al.* (1). Gly202 and Arg209 in the cDNA from cv. Wasesuzunari were substi-
tuted with arginine and lysine, respectively, in the cDNA from cv. Bonminori. Gly\textsuperscript{202} and Arg\textsuperscript{399} are conserved in all other plant \( \beta \)-amyloses sequenced so far (12). The latter residue was found to be arginine in the protein sequence of soybean \( \beta \)-amylase (43). The three-dimensional structure of \( \beta \)-amylase complexed with \( \beta \)-CD shows that Gly\textsuperscript{202} is about 10 Å from the catalytic residues near the molecular surface, and the substitution of Gly\textsuperscript{202} for arginine should cause steric hindrance with Tyr\textsuperscript{238} and Asn\textsuperscript{239}. On the other hand, the atoms of N-\( \eta \)-1 and N-\( \eta \)-2 in Arg\textsuperscript{399} (about 20 Å from the active center) form direct hydrogen bonds with O atom in Asn\textsuperscript{189} (2.9 Å), O atom in Met\textsuperscript{441} (2.9 Å), and O atom in Lys\textsuperscript{44} (2.8 Å), and N-\( \epsilon \) atoms form water-mediated hydrogen bonds with O-81 in Asp\textsuperscript{190}. The substitution of Arg\textsuperscript{399} for lysine may distort the protein structure due to a breakdown of the hydrogen network. The two substitutions mentioned above were probably the reason for the higher expression level and specific activity of the recombinant enzyme in the system constructed here relative to those reported by Totsuka \textit{et al.} (1). The substitution at position 202 may have been particularly critical in this regard.

We have determined the structure of \( \beta \)-CD-\( \beta \)-amylase complex and compared it with that of \( \alpha \)-CD complex (12) in order to investigate interactions between \( \beta \)-amylase and its substrates. Our results indicated no significant differences between the two protein structures. Two CD molecules bind to the enzyme with very similar interactions mainly at glucose residues 3 and 4, and the residue 4 was the most readily superimposed. The fact that \( \beta \)-CD has one more glucose residue than \( \alpha \)-CD affects the increase of the \( \phi \) angle between glucose residues 4 and 5. We suggest that the interactions at the glucose residues 3 and 4 are not so much rigid but rather work to hold a substrate in the successive reaction because glucose residue 4 in the \( \alpha \)- and \( \beta \)-CDs is positioned about one-half residue from subsite 4 in the direction of the reducing end.

Generally, amylases cleave substrates by a pathway in which they randomly encounter a substrate chain (12). However, \( \beta \)-amylase and animal \( \alpha \)-amylase degrade polymeric substrates to products sequentially after complexing with the substrates in addition to degrading them randomly. This is commonly called a multiple attack (11, 44, 45) and allows \( \beta \)-amylase to react efficiently. Here, we suggest that the binding site of CDs plays an important role in the action of the enzyme to retain the cleaved polymeric substrate (Fig. 6). This would explain the mechanism of this intriguing reaction of \( \beta \)-amylase as follows; after a polymeric substrate, such as amylase, binds to each subsite of the enzyme from the nonreducing end, the flexible loop immediately closes. In this binding, the terminal maltose unit of the substrate (glucose residues 1 and 2) reverses by torsion in \( \alpha \)-1,4-glycosidic linkage (step 1). The bound substrate is hydrolyzed by the two catalytic residues of Glu\textsuperscript{156} and Glu\textsuperscript{180} in \( \beta \)-amylase (16). The enzymatic reaction produces \( \beta \)-anomeric maltose and a new substrate that is destined to be cleaved in the next step. The flexible loop then opens, resulting in the exposure of the active site to solvent and the departure of the produced \( \beta \)-maltose (step 2). For the next step, shift of the substrate occupying subsites 3 and 4 is required because the terminal maltose unit of the substrate has to reverse before binding to the subsites 1 and 2. The cleaved substrate is released from the subsites and held on the surface of the enzyme for the successive reaction. Furthermore, occu-
tion of the CD-binding site by cleaved substrate fixes the flexible loop in an open conformation (12), which would make it easy to take the substrate in subsites 1 and 2. After all, the holding of the cleaved substrate at the CD binding site leads to a single chain attack (the slipping mechanism) (12) (step 3), whereas the release of the cleaved substrate into solvent leads to a multichain attack. The flexible binding of glucose around subsite 4 by hydrophobic interactions enables the slipping mechanism (16). Leu$^{383}$, which forms an inclusion complex, is to a multichain attack. The flexible binding of glucose around subsite 4 or the residue 4 in CDs via hydrogen bonds. Since the substrate, because the residue seems to block the cleaved sub-

mined in progress.

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