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Structure of the Starch-Binding Domain of *Bacillus cereus* β-Amylase

YOON, HYE-JIN, AKIRA HIRATA, MOTOYASU ADACHI, ATSUSHI SEKINE, SHIGERU UTSUMI, AND BUNZO MIKAMI*

Research Institute for Food Science, Kyoto University, Uji, Kyoto 611-0011, Japan

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Abstract The C-terminal starch-binding domain of *Bacillus cereus* β-amylase expressed in *Escherichia coli* was purified and crystallized using the vapor diffusion method. The crystals obtained belong to a space group of $P3_221$ with cell dimensions, a = b = 60.20 Å, c = 64.92 Å, and γ = 120°. The structure was determined by the molecular replacement method and refined at 1.95 Å with R-factors of 0.181. The final model of the starch-binding domain comprised 99 amino acid residues and 108 water molecules. The starch-binding domain had a secondary structure of two 4-stranded antiparallel β-sheets similar to domain E of cyclodextrin glucanotransferase and the C-terminal starch-binding domain of glucoamylase. A comparison of the structures of these starch-binding domains revealed that the separated starch-binding domain of *Bacillus cereus* β-amylase had only one starch-binding site (site 1) in contrast to two sites (site 1 and site 2) reported in the domains of cyclodextrin glucanotransferase and glucoamylase.

Key words: Starch-binding domain, β-amylase, X-ray crystallography, 3D structure

β-Amylase [EC. 3.2.1.2] catalyzes the liberation of β-anomeric maltose from the non-reducing ends of starch and is distributed in plants and microorganisms [1, 2]. The bacterial β-amylases from *Bacillus polymyxa* [3], *Bacillus circulans* [4], *Clostridium thermosulfurogenes* [5], and *Bacillus cereus* [6, 7] have different properties from the plant enzymes in terms of optimum pH, specific activity, isoelectric points, and the ability to digest raw starch, although they exhibit similar molecular weights [2]. The ability to bind and digest raw starch granules only by bacterial β-amylases can be attributed to their C-terminal region, which does not show any similarity to the C-terminal region of plant amylases, yet exhibits a homology with the C-terminal raw starch-binding domain (SBD) reported in glucoamylase, cyclodextrin glucanotransferase (CGTase), and maltotetraose producing α-amylase [6, 8]. X-ray crystallographic and NMR analyses revealed that these SBDs have a similar structure to an 8-stranded Greek key topology with a molecular weight of about 10 kDa, and that two sugar binding sites (site 1 and site 2) exist in the CGTase domain E [9, 10] and in the separated starch-binding domain of glucoamylase [11].

The three-dimensional structure of *Bacillus cereus* β-amylase (BCB) shows that the enzyme is comprised of a $(\beta/\alpha)_8$ core domain and a C-terminal SBD [12]. Out of four bound maltose molecules, three were found in the core domain; however, only one was found in the C-terminal SBD [12]. Since other SBDs, such as the domain E of CGTase and isolated C-terminal domain of glucoamylase, have been reported to have two binding sites for maltose or cyclodextrin [9-11], it has been suggested that the SBD of *Bacillus cereus* β-amylase has an incomplete second binding site or conformational change in the site due to a crystal-packing force [12]. It is necessary to compare the structures of these SBDs at high resolution to elucidate the structural alternation of the second starch-binding site.

This paper describes the structure of the separated SBD of *Bacillus cereus* β-amylase (BCB-SBD) and compares this structure with the SBDs of CGTase and glucoamylase.

Materials and Methods

Expression of the SBD Gene in *Escherichia coli*

The DNA coding of the SBD of *Bacillus cereus* β-amylase (residues 418-516) [12] was amplified by PCR using Primers SBDN (5'-ACCCCTGTTATGCAAACGATTGTAG) corresponding to the N-terminal amino acid sequence of SBD (TPVMQTIV) and the T7 terminator primer. The PCR was conducted on plasmid pEBBA with reagents supplied in kit form (Takara Shuzo Ltd., Kyoto, Japan) in a GeneAmp PCR System 2400 (Perkin-Elmer). The PCR cycle consisted of denaturation at 95°C for 30 s, annealing at 50°C for one min, and an extension at 72°C for 3 min. After 30 cycles, the products were separated by electrophoresis

*Corresponding author
Phone: 81-774-38-3763; Fax: 81-774-38-3764;
E-mail: mikami@soya.food.kyoto-u.ac.jp

on a 1.5% (w/v) agarose gel and purified using glass powder (Takara). The resultant 0.3 kb fragment was blunted using a Blunting Kit (Takara), cut with *Xho*I at the 3' part, and inserted between the filled-in *Nco*I and *Xho*I sites of the expression vector pET21d (Novagen, Madison, WI) to generate pESBD. The expression plasmid pESBD was then transfected into *E. coli* strain HMS174 (DE3). The *E. coli* harboring the expression plasmid was cultured in LB supplemented with carbenicillin (50 μg/ml) at 37°C. At $A_{600\,nm}$ = 0.8, IPTG was added to a final concentration of 1 mM and the cells were cultured at 20°C for 40 h. The cells were then harvested by centrifugation, and disrupted by sonication in a 50 mM Tris buffer (pH 8.0) containing 100 mM NaCl, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride.

Purification of BCB-SBD

BCB-SBD was purified from the supernatant of the cell extract using SP-Sephadex ion-exchange chromatography and gel filtration on Sephacryl S200. Approximately 60 mg of purified protein was obtained from 4 liters of cell broth. The purified BCB-SBD exhibited a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a molecular weight of 11,000. The N- and C-terminal amino acid sequences analyzed by Procise 492 and Procise C 494 sequencers (Applied Biosystem) were identified as MTPVMQTI and XSW, respectively, which were consistent with the cDNA sequence of SBD [12].

Crystallization

BCB-SBD crystals were grown at 18°C using the hanging-drop vapor-diffusion method employing 5 μl of a 4 mg/ml protein solution (0.1 M sodium acetate buffer, pH 4.6) mixed with 5 μl of a precipitant solution (1.5-1.7 M $(NH_4)_2SO_4$ in a 0.1 M acetate buffer, pH 4.6). Hexagonal crystals appeared in one day and grew up to 0.3 mm × 0.3 mm × 0.5 mm over 3 months. These crystals were a trigonal, space group $P3_221$ with a = b = 60.20 Å, c = 64.92 Å, and γ = 120°, and contained one molecule of BCB-SBD in an asymmetric unit.

Data Collection

The BCB-SBD crystal data were collected at a resolution up to 1.91 Å at room temperature, using Cu K_α radiation (λ=1.5418 Å) with a Bruker Hi-Star area detector coupled to a MAC Science M18XHF rotating-anode generator. The collected data were processed with the SADIE and SAINT software packages (Bruker).

Structure Determination

The BCB-SBD structure was determined by the molecular replacement method using the X-PLOR program package [13]. The coordinates of SBD in *Bacillus cereus* β-amylase (residues 418-516 of 1BQZ in PDB) were used as a search model [12]. The rotation search yielded a unique solution that was 2.6σ above the mean employing the data between 15-4.5 Å resolution. A PC refinement of the appropriately rotated search model and an ensuing translation search using the data between a resolution of 15-4.5 Å produced a correct solution at 7.7σ above the mean. Successive rigid body refinements produced an R-factor of 0.47 for the data between a resolution of 15-3.5 Å. Simulated annealing along with positional and B-factor refinements using X-PLOR [13] resulted in R = 0.233 (R_{free} = 0.348) for the 3073 reflections with F>2σ(F) between a resolution of 10.0 and 2.8 Å. The resultant map had a high enough quality to locate each amino acid residue using the graphic program of Turbo-Frodo (Biographics). Visible water molecules with more than 4σ on the |Fo|-|Fc| map were then added to the model. Several cycles of the positional and B-factor refinements followed by model rebuilding were carried out at a resolution of 1.95 Å until no further improvements were added to the model. The final model consisted of 99 amino acid residues and 108 water molecules with R = 0.181 (R_{free} = 0.225) for 8,264 reflections with F>2σ(F) between a resolution of 6.0 and 1.95 Å. The atomic coordinate was deposited with the Protein Data Bank at Brookhaven National Laboratories with PDB code 1CQY.

Structural Comparison

The SBD structures were compared using the Turbo-Frodo program after they were superimposed onto BCB-SBD by the rigid program implemented in Turbo-Frodo. The coordinates of CGTase (PDB code 1CDG) and the separated SBD from glucoamylase (PDB code 1KUL) were taken from the Protein Data Bank at Brookhaven National Laboratory.

Results and Discussion

Quality of the Final Model

The final statistics for the refinement for SBD along with statistics for data collection are shown in Table 1. The final model of SBD contains 99 amino acid residues and 116 water molecules. The model has good geometries and a mean positional error of 0.20 Å, as estimated from a Luzzati plot [14]. A Ramachandran plot of the main-chain conformation angles shows that about 91% lie within the core region, with 100% lying within the allowed region [15, 16]. The average B-factors for the protein and water atoms are 17.3 $Å^2$ and 35.1 $Å^2$, respectively. The average B-factors are low in the strands and high in the loops connecting the strands. The higher average B-factors are found in the loops at residues 444-455, connecting βb1-βb2 (20.6 $Å^2$), at residues 461-466, connecting βa2 and βa3 (23.6 $Å^2$), at residues 474-478, connecting βa3 and βb3 (23.2 $Å^2$), and at residues 487-491, connecting βb3 and βb4a (24.7 $Å^2$). There is one *cis*-peptide bond in the structure between Asn 503 and Pro 504.

Table 1. Statistics of data collection and refinement of BCB-SBD.

Space group	$P3_221$
Cell dimensions	
a, b, c (Å)	60.195, 60,195, 64.922
	$\gamma = 120^\circ$
Data collection	
Measured reflections	66,133
Unique reflections (I>1σI)	10,179
R_{sym} (%)	6.2
Resolution limit (Å)	1.91
Completeness (%)	92.7
Refinement	
Resolution range (Å)	6.0-1.95
Used reflections (|F|>2σF)	8,264 (82.6%)
Residues/waters	99/108
Average B-factor (Å^2)	19.4
r.m.s. deviation	
bond length (Å)	0.007
angle (°)	1.51
R-factor (%)	18.1
Free-R (%)	22.5

Overall structure of BCB-SBD

BCB-SBD is folded into two 4-stranded antiparallel β-sheets (a and b), which form an 8-stranded antiparallel β-barrel (Fig. 1) as has been reported for CGTase [9, 10], and a separated starch-binding domain from glucoamylase [11]. Sheet a consists of four strands, βa1 (residues 418-427), βa2 (residues 459-461), βa3 (residues 466-474), and βa4 (residues 510-515), while sheet b consists of five strands, βb1 (residues 435-441), βb2 (residues 456-458), βb3 (residues 477-486), βb4a (residues 491-496), and βb4b (residues 499-505). BCB-SBD has 31% and 22% identical amino acid residues with the domain E of

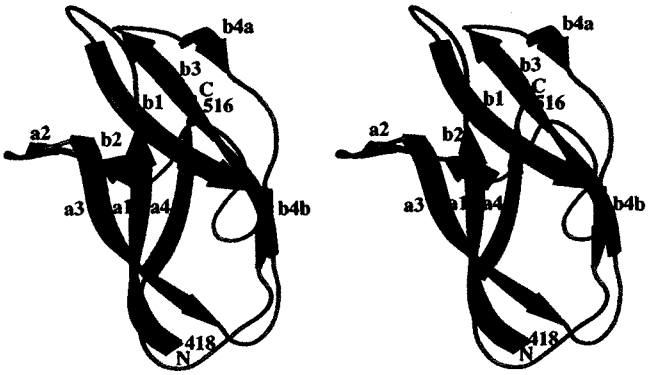

Fig. 1. Schematic representation of the secondary structure of BCB-SBD (stereo view).
The figure was drawn using the Molscript [24] and Raster3D [25] programs. The strands are shown in green (sheet a) and red (sheet b), respectively. The strands in each sheet are numbered.

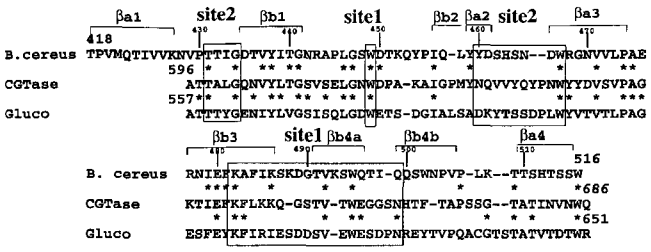

Fig. 2. Alignment of the BCB-SBD sequence with the CGTase E domain [9] and SBD from glucoamylase [11].
The residues of the strands in BCB-SBD are represented as β and numbered. The conserved residues are marked by an asterisk. The regions involved in the starch binding (sites 1 and 2) are boxed.

CGTase [9] and C-terminal SBD of glucoamylase [11, 17], respectively, as shown in Fig. 2. The C-α r.m.s. distances are 0.73 Å for 99 common C-α atoms within 2.4 Å between BCB-SBD and SBD in *B. cereus* β-amylase [12]. C-α distance deviations of greater than 1.5 Å were found at two loops around residues 462-465 and 488-490. The loop between 461-466, connecting βa2 and βa3, has the largest deviation (2.4 Å at residue 464). The large deviation and higher B-factor may indicate the flexibility of this loop in the solution structure. Figure 3 represents the C-α trace of BCB-SBD superimposed onto the domain E of CGTase [9] and separated SBD from glucoamylase [11]. The C-α r.m.s. distance is 0.92 Å for 83 common C-α atoms within 2.4 Å between BCB-SBD and the CGTase domain E. The structures of BCB-SBD and the CGTase domain E are slightly different by deletion of two residues at residues 461-466 near the maltose binding site 2 (loop connecting βa2 and βa3) and also at residues 453, 488, 493, 498, and 503 by one residue insertion, and at 458 and 507 by one residue deletion in the BCB-SBD sequence (Fig. 2). When the BCB-SBD structure is compared with the isolated SBD

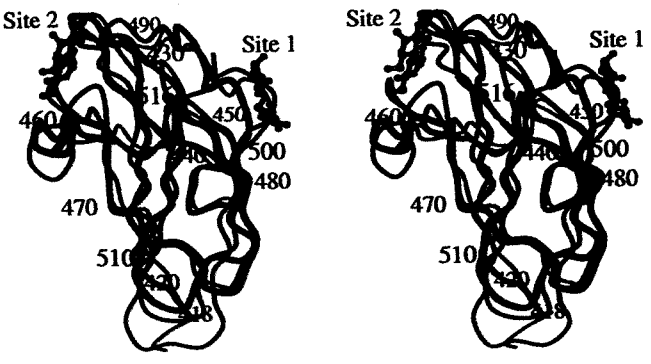

Fig. 3. Stereo diagram of the superpositions of the C-α trace of BCB-SBD (green) with the CGTase E domain (red) and SBD from glucoamylase (blue).
Two bound maltose molecules in the CGTase E domain are drawn with ball-and-sticks (sites 1 and 2). The superpositions were carried out using the RIGID program of Turbo-Frodo. This figure was drawn using the Molscript [24] program.

from glucoamylase [11], the C-α r.m.s. distance increases to 1.35 Å for 77 common C-α atoms within 2.4 Å, thereby suggesting that the BCB-SBD has a closer resemblance to the domain E of CGTase. The most significant differences between BCB-SBD and the SBD of glucoamylase occur at the loop connecting βb4b and βa4 (residues 505–510 in BCB-SBD) with three-residue deletions, and the loop connecting βa2 and βa3 (residues 462–465 in BCB-SBD) with two-residue deletions.

Starch Binding Sites

One maltose binding site was found in the SBD of the BCB complex with maltose [12], which is the same sugar binding site (site 1) as in the CGTase E domain [9] and isolated SBD from glucoamylase [11]. Figure 4(a) shows the binding site 1 of BCB-SBD superimposed onto the SBD in BCB complexed with maltose [12] and onto the CGTase complexed with maltose [9]. In the SBD of BCB complexed with maltose, four residues of Trp 449, Trp 495, Gln 499, and Lys 482 interact with the maltose. Two glucose residues are stacked on each Trp residue and make four hydrogen bonds between the O-ε1 of Gln 499 and the N-ζ of Lys 482. These features of the symmetrical glucose binding sites are very similar to those found in the CGTase E domain [9] except for the substitution of Gln 499 for Asn 676. A comparison of the SBD in BCB and BCB-SBD reveals the rigidness of binding site 1 except for the side-chain conformation change of Gln 499 upon the binding of maltose.

Figure 4(b) represents the BCB-SBD site corresponding to the sugar binding site 2 of the E domain in CGTase with its C-α atoms superimposed onto that of the BCB-SBD. In contrast to the CGTase, no maltose binding was reported in the BCB/maltose complex. In site 2 of the CGTase E domain, one maltose molecule creates hydrogen bonds with the main-chain atoms between Thr 598 O, Ala 599 O, and Gly 601 N, and with the side-chains of Thr 598 O-γ1, Asn 627 O-δ1 and N-δ2, and Gln 628 N-ε2. The former main-chain conformations are well conserved in BCB-SBD (Thr 432, Ile 433 and Gly 434), while the latter side-chains are substituted or deleted in BCB-SBD except for Thr 432. Tyr 633 in CGTase, which is deleted in BCB-SBD, also interacts with bound maltose by stacking its side-chain onto one of the sugar rings. The deletion of two residues, corresponding to Tyr 633 and Pro 634 in CGTase, and the substitution of the loop residues make a conformation change in the hairpin loop connecting βa2 and βa3 between CGTase and BCB-SBD. In BCB-SBD, this loop takes a type III β-turn at residues 462–465, while in CGTase it takes a distorted type III β-turn at residues 628–631 because of the insertion of residues 633 and 634, resulting in a maximum distance deviation of 5.6 Å between His 463 C-α of BCB-SBD and Val 630 C-α of CGTase. In CGTase, Trp 636, which corresponds to Trp

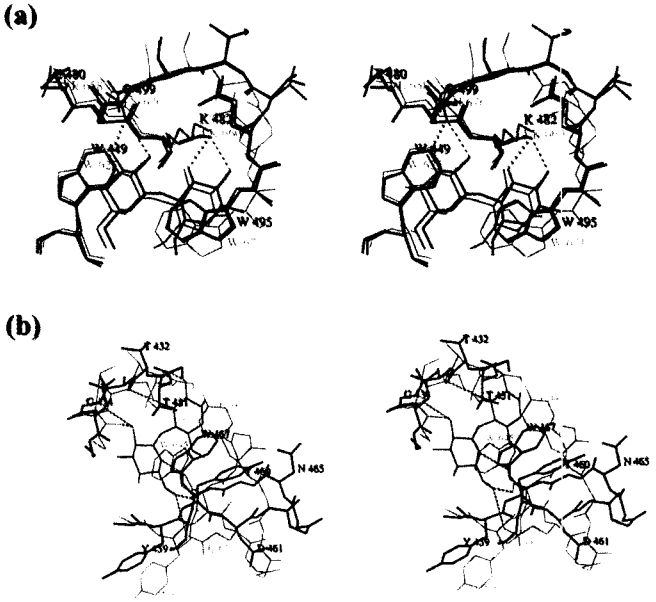

Fig. 4. Stereo diagram of the starch binding site 1 (a) and site 2 (b) of BCB-SBD (green) superimposed onto the SBD in BCB (blue) and CGTase E domain (red).
The figure was drawn with Turbo-Frodo after superimposing with the Turbo-Frodo RIGID program.

467 in BCB-SBD, also interacts with bound maltose. They share almost the same position except for the flip of the side-chain. This is not a difference between BCB-SBD and CGTase, yet is a difference between BCB-SBD and SBD in the β-amylase, because the side-chain has the same conformation as that of CGTase in the SBD of BCB complexed with maltose [12]. The comparison of the site 2 structures of BCB-SBD and CGTase shows that BCB-SBD has an incomplete starch-binding site 2 due to a conformational change.

The two starch-binding sites of SBD from glucoamylase have previously been reported as having the same order of affinity to malto-oligosaccharides [18, 19] and cyclodextrins [18-21]. Furthermore, it has been reported that the two starch binding sites of the CGTase E domain have different functions. Site 1 acts as the main starch recognition, while the other (site 2) is involved in guiding the linear starch chain into the active site [22]. A recent report on the function of the SBD from glucoamylase also suggested that the two binding sites are important in disrupting the surface of the starch granules [23]. The present study of the structure of BCB-SBD revealed that BCB-SBD has an incomplete starch binding site 2 owing to a conformational change due to the deletion and substitution of amino acid residues. Out of three bound maltose molecules in the main domain of BCB, one was found in the core domain 32 Å apart from the active site [12]. This maltose binding site may have the starch-binding function instead of the incomplete starch binding site 2 of the BCB-SBD. In order

to demonstrate the role of the second binding site, the repair of this site is being attempted using protein engineering.

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