

The Crystal Structure of the Sevenfold Mutant of Barley β -Amylase with Increased Thermostability at 2.5 Å Resolution

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The three-dimensional structure of the sevenfold mutant of barley β -amylase (BBA-7s) with increased thermostability was determined by X-ray crystallography. The enzyme was purified as a single component and crystallized by a hanging drop method in the presence of 14 % PEG 6000. The crystals belong to space group $P4_32_12$ with cell dimensions $a = b = 72.11$ Å, $c = 250.51$ Å. The diffraction data up to 2.5 Å were collected after soaking the crystal in 100 mM maltose with R_{sym} of 8.6 %. The structure was determined by a molecular replacement method using soybean β -amylase (SBA) as a search model and refined to an R -factor of 18.7 %. The final model included 500 amino acid residues, 141 water molecules and three glucose residues, which were located at subsites 1-2 and 4 in the active site. The r.m.s. distance of 485 C α atoms between BBA-7s and SBA was 0.62 Å. Out of the seven mutated amino acids, four (Ser295Ala, Ile297Val, Ser351Pro and Ala376Ser) were substitutions from the common residues with SBA to the thermostable forms. A comparison of the structures of BBA-7s and SBA indicated that the side-chain of Ser376 makes new hydrogen bonds to the main-chain of an adjacent β -strand, and that the side-chains of Val297 reduce an unfavorable interaction between the side-chains of Ala314. The mutation of Ser295Ala breaks the hydrogen bond between Ser295 OG and Tyr195 OH, which seems to be the reason for the unoccupied glucose residue at subsite 3. The tandem mutations at 350-352 including substitutions to two Pro residues suggested the reduction of main-chain entropy in the unfolded structure of this solvent-exposed protruded loop.

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Introduction

β -Amylase (α -1,4-glucan maltohydrolase; EC. 3.2.1.2) catalyzes the liberation of β -anomeric maltose from the non-reducing ends of starch molecules, and is distributed in plants and microorganisms. Since the enzyme was classified in 1924, many investigations have been focused on it (French, 1960; Thoma *et al.*, 1971). The structural features of β -amylase have, however, been clarified only recently. A comparison of the primary structures has shown that the higher plant β -amylases have amino acid sequences of more than 60 % simi-

larity, but that the similarities between plant and microorganism β -amylases are about 30 % (Svensson, 1988). The X-ray crystallographic study of soybean β -amylase (SBA) revealed that the enzyme is composed of a core (α/β)₈-barrel domain that has three long loops (L3, L4 and L5) forming a deep active cleft, and an additional C-terminal loop surrounding the barrel helices (Mikami *et al.*, 1993). The 3D structure of sweet potato β -amylase has shown the same molecular architecture, except for the tetrameric nature of this enzyme (Cheong *et al.*, 1995). Based on the complex of SBA with maltose, it was proposed that two side-chains of Glu residues (Glu186 and Glu380) act as an acid and base pair in the catalytic process (Mikami *et al.*, 1993).

Barley β -amylase (BBA) was cloned and sequenced first by Kreis *et al.* (1987) and by Yoshigi *et al.* (1994a). BBA and SBA are identical in

Abbreviations used: BBA, barley β -amylase; BBA-7s, sevenfold mutant of barley β -amylase; SBA, soybean β -amylase.

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66.5% of their amino acid residues (515 *versus* 495 residues). The C-terminal sequence of BBA is about 30 residues longer than SBA, and has Gly-rich repeats. A similar extended sequence is found in endosperm-specific rye β -amylase (Rorat *et al.*, 1991). Yoshigi *et al.* (1995a) reported that deletion of the C-terminal 54 residues of BBA led to the destabilization of the enzyme, suggesting that the C-terminal loop region stabilizes the enzyme structure. Interestingly, the wild-type BBA and the BBA in barley seeds exhibit multiple forms on PAGE, whereas the deleted mutant exhibits a single band, indicating that the C-terminal Gly-rich region is easily attacked by a contaminated protease in both barely seed and *Escherichia coli* (Yoshigi *et al.*, 1995a).

BBA plays an important role in the saccharifying process of brewing beer. The heat instability of BBA reduces the efficiency of the saccharifying process. In order to enhance the thermostability of BBA, Yoshigi *et al.* (1995b) developed a sevenfold mutant (BBA-7s) that has enhanced thermostability about 11 deg. C higher than the non-mutant enzyme. The mutated residues were Met185 to Leu (Met185Leu), Ser295 to Ala (Ser295Ala), Ile297 to Val (Ile297Val), Ser350 to Pro (Ser350Pro), Ser351 to Pro (Ser351Pro), Gln352 to Asp (Gln352Asp) and Ala376 to Ser (Ala376Ser). Out of the seven mutated amino acids, five were mutated to the corresponding residues found in SBA (Met185Leu, Ser350Pro, and Gln352Asp) or *Clostridium thermosulfurogenes* β -amylase (Ser295Ala and Ile297Val), and two were mutated by the random-mutation method (Ser351Pro and Ala376Ser). Four mutation sites, Ser295Ala, Ile297Val, Ser351Pro and Ala376Ser, were locations for substitutions of amino acids from SBA to the thermostable form. A comparison of the structure of SBA and BBA-7s will serve to elucidate the conformation change favorable to the enhanced thermostability.

Here, we describe the crystallization and structure determination of the complex of the BBA-7s with a substrate analog, maltose, in order to clarify the mechanism of the increased thermostability.

Results

Purification and crystallization of BBA-7s

In order to eliminate the C-terminal heterogeneity of BBA-7s, we prepared a single component of BBA-7s exhibiting a single band on both SDS-PAGE and non-denaturing PAGE. The N-terminal sequence of this component was determined to be Met-Lys-Gly-Asn-Tyr-Val in accord with the N terminus of the reported BBA-7s, which lacks the four N-terminal residues from the original recombinant BBA (Yoshigi *et al.*, 1995b). The molecular mass was determined to be 58,400 Da by matrix-assisted laser desorption ionization mass spectrometry, indicating that it contains the extended C-terminal repeat sequence (Yoshigi *et al.*, 1995b). In hanging

drop vapor-diffusion, the bipyramid crystals appeared at seven days and grew to 1 mm $\times$ 0.3 mm $\times$ 0.3 mm after two months (Figure 1). The crystals belong to space group $P4_32_12$ with cell dimensions $a = b = 72.11$ Å, $c = 250.51$ Å. There is one molecule of BBA in the asymmetric unit of the crystal. The diffraction data to 2.5 Å were collected with an R_{sym} of 8.6% and a completeness of 94.3%. The structure was solved by molecular replacement using SBA coordinates as the search model. The statistics of data collection and refinement are summarized in Table 1.

Quality of the final model

The BBA-7s model comprises amino acid residues from 5 to 504, three glucose residues and 141 water molecules. The C-terminal sequence beyond 504 is not included in the present model because the $2|F_o| - |F_c|$ and $|F_o| - |F_c|$ maps became too thin to trace the correct sequence. Relevant refinement statistics are given in Table 1. From a Luzzati (1952) plot, the mean absolute positional error was estimated to be 0.25 Å. A Ramachandran plot of the main-chain conformation angles showed that about 85.5% lie within the core region, with 100% lying within the allowed region (Ramakrishnan & Ramachandran, 1965; Laskowski *et al.*, 1993). There are three *cis*-peptide bonds in the structure. Two *cis*-peptide positions (between Pro199 and



Figure 1. Crystals of BBA-7s appeared in a hanging drop vapor-diffusion. The approximate size of the maximum is 1 mm $\times$ 0.3 mm $\times$ 0.3 mm.

Table 1. Statistics of data collection and refinement of BBA-7s

Crystal system	Tetragonal
Space group	$P4_32_12$
Unit cell (Å)	$a = b = 72.11, c = 250.51$
Molecules/asym. unit	1
Data collection	
Detector	Siemens Hi-star
Crystal-detector dist. (cm)	20
Used crystal	1
Resolution limit (Å)	2.5
Scan width (deg.)	0.2
Scan speed (deg./min)	0.2
Scan range (deg.)	
ϕ -scan	0-90 (20 22)
ω -scan	0-90 (20 10)
χ -scan	0-50 (20 22, χ 30)
ψ -scan	0-50 (20 22, χ 60)
ω -scan	0-50 (20 22, χ 90)
Measured reflections	122,221
Unique reflections	22,631 ($I > 1\sigma_I$)
Completeness (%)	94.3
R_{sym} (%)	8.6
Final model	5-504 residues, 3 Glc, 141 water
Resolution range (Å)	10.0-2.5
Reflections	18,732 ($ F > 2\sigma_F$)
Completeness (%)	79.9
R -factor (%)	18.7
Free- R (%)	25.6
r.m.s. bond length (Å)	0.014
angle (deg.)	3.24

Gly200, and between Arg418 and Leu419) are conserved in the structure of SBA (Mikami *et al.*, 1994). The third *cis*-peptide position between Pro473 and Lys474 was found only in BBA-7s. The average B -factors were calculated to be 30.9 and 37.7 Å² for protein atoms and solvent atoms, respectively.

Overall structure of BBA-7s

The overall organization of BBA-7s is very similar to that of SBA, except for the C-terminal loop region. Figure 2 shows the ribbon model of BBA-7s including the mutated amino acid residues and the three bound glucose residues. Figure 3 shows a comparison of amino acid sequences for BBA-7s and SBA. The amino acid correspondence for BBA-7s and SBA are 66.3% and 70.9% for the entire region (residues from 5 to 490) and the $(\alpha/\beta)_8$ -barrel core region (residues from 5 to 441), respectively. There is one deletion of four amino acid residues at residue 449, one insertion of one amino acid residue at residue 473 (*cis*-Pro) and an extension of the C-terminal tail from residues 491 to 531 in the BBA-7s sequence. These changes are located only in the C-terminal loop region. The r.m.s. distance between BBA-7s and SBA is 0.62 Å for the entire comparable 482 C α atoms within 2 Å. The r.m.s. distance of 434 C α atoms in the core $(\alpha/\beta)_8$ -barrel (residues from 5 to 440) is 0.58 Å, while that of 48 C α atoms of the C-terminal loop (residues 441 to 500) is 0.81 Å. BBA-7s and SBA share the same secondary structures, except for the insertion of a

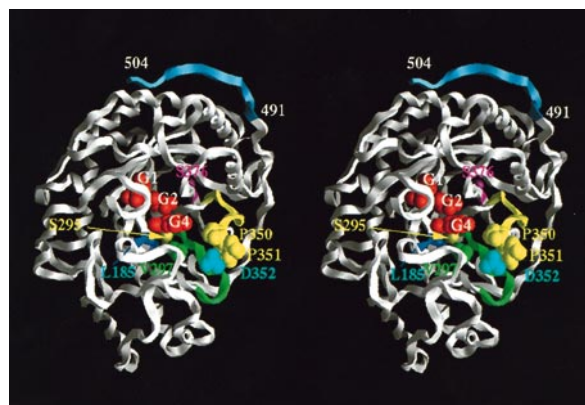
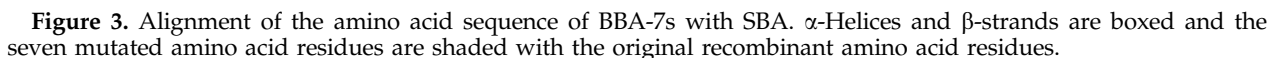


Figure 2. Stereo diagram of the overall structure and the seven mutated sites of BBA-7s. The ribbon model with bound glucose residues (red) and mutated side-chains (yellow, cyan, green, blue and purple) as colored spheres was drawn by GRASP (Nicholls *et al.*, 1991). The main-chain regions of the extended C terminus, L6 and L5 loop 1 are colored blue, yellow and green, respectively.

four-residue helix in SBA. The C-terminal residues beyond 504 cannot be followed because of incomplete electron density, suggesting a disordered main-chain structure of BBA-7s in the C-terminal region. As shown in Figure 2, six mutated residues exist in the loops of the core $(\alpha/\beta)_8$ -barrel (Met185 in L4, Ala295 and Val297 in L5, and Pro350, Pro351 and Asp352 in L6) that surround the active site, and one, Ser376 exists in β 7. Among the seven mutated residues of BBA-7s, four are mutations from SBA residues to thermostable residues (Ser295Ala, Ile297Val, Ser351Pro and Ala376Ser), and three residues are mutations from BBA residues to SBA residues (Met185Leu, Ser350Pro and Gln352Asp). The similarity of the BBA-7s and SBA structures makes it possible to compare the detailed structural differences of the four side-chains (Ala295, Val297, Pro351 and Ser376) that contribute to the thermostability of the enzyme.

Maltose-binding sites

The organization of the active site of BBA-7s, including the closed conformation of the flexible loop (residues 94-100) is quite similar to that of SBA. One maltose molecule and one glucose residue are found in the active site of BBA-7s. Figure 4 shows the $2|F_o| - |F_c|$ map around the model of the glucose residues together with the model of SBA/maltose complex. The glucose-binding sites are identified as subsites 1, 2 and 4 from the maltose-binding sites of SBA (Mikami *et al.*, 1994). The anomer of Glc2 was identified as having the β -configuration, while that of Glc4 seems to have β -configuration as the major anomer. The average B -factors for these glucose residues are: Glc1 31.3 Å², Glc2 48.9 Å² and Glc4 65.7 Å². The invisible glucose residue at subsite 3 together with the



Glc2 are almost compatible with the SBA/maltose complex (Mikami *et al.*, 1994), but a minor difference was found at subsite 4. Glc4 of SBA formed a tight hydrogen bond with NE2 of His300, but the whole sugar ring of BBA-7s moved toward C-6 by about 1 Å.

The mutations of Ser295Ala and Ile297Val are substitutions of BBA (SBA) side-chains by those of thermostable β -amylase from *C. thermosulfurogenes*. The mutated positions of Ser295Ala and Ile297Val are near the binding site of Glc3, whose electron density was invisible in the present analysis. Figure 5 shows a wire model of the BBA-7s/maltose complex around subsite 3 together with that of the SBA/maltose complex. In the SBA/maltose model, OG of Ser297 (Ser295 of BBA) makes a hydrogen bond with OH of Tyr192, which also makes hydrogen bonds to O-6 and O-5 of Glc3. In the BBA-7s/maltose model, the mutated Ala side-chain no longer interacts with Tyr190, which seems to reduce the hydrogen bond interactions of Tyr190 OH with Glc3. This may explain the lack of density for Glc3. Another mutated amino acid residue, Val297, exists near Ala295. In the SBA/maltose model, the distance between OG of Ser297 and CG1 of Ile299 and that between CD1 of Ile299

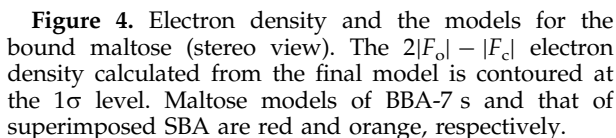


Table 2. Hydrogen bond interactions between BBA-7s and maltose

Glucose atom	Protein atom	Distance in BBA-7s/ maltose (Å)	Distance in SBA/ maltose ^a (Å)	Glucose atom	Protein residues in van der Waals contact (C-C distance <4.5 Å)
Glc1 O-2	Asp99 OD1	3.0	3.5	Glc1 C-1	Leu18, Arg418
Glc1 O-2	Asp99 OD2	2.6	2.7	Glc1 C-2	Leu18, Arg99
Glc1 O-3	His91 NE2	2.9	2.9	Glc1 C-3	Leu18, Trp53, His91, Ala182
Glc1 O-4	His91 NE2	2.7	3.1	Glc1 C-4	Leu18, Asp51, Trp53, His91
Glc1 O-4	Asp51 OD1	2.7	2.6	Glc1 C-6	Met15, Asp51, Ile87
Glc1 O-5	Arg418 NH2	2.7	2.9		
Glc1 O-6	Asp51 OD2	3.0	2.7		
Glc1 O-6	Arg418 NH2	2.8	3.1		
Glc2 O-2	Ala380 O	3.2	2.8	Glc2 C-1	Glu184, Thr340
Glc2 O-3	Val97 O	3.1	3.8	Glc2 C-2	Leu417
Glc2 O-5	Glu184 OE2	3.4	3.5	Glc2 C-4	Ala182
Glc2 O-5	Glu378 OE2	3.1	3.2	Glc2 C-5	Ala182, Glu184
Glc2 O-6	Glu378 OE2	3.0	2.8	Glc2 C-6	Ala182, Glu184, Leu293
Glc2 O-6	Lys293 NZ	3.2	2.8		
Glc4 O-3	His298 NE2	3.2	2.8	Glc4 C-1	Phe198
				Glc4 C-2	Phe198
				Glc4 C-3	Leu381
				Glc4 C-4	Trp229
				Glc4 C-6	Tyr190, Trp196

^a Values from SBA/maltose complex (Mikami *et al.*, 1994).

and CB of Ala314 are 3.8 Å and 3.2 Å, respectively. The latter is about 20 % shorter than the sum of their van der Wals radii (Warne & Morgan, 1978). The corresponding distances in the BBA-7s are 3.9 Å (between CG2 of Val297 and CB of Ala295) and 4.1 Å (between CG2 of Val297 and CB of Ala312), respectively. The interaction between OG of Ser297 and CG1 of Ile299 in SBA is compensated by that between CB of Ala295 and CG2 of Val297 in BBA-7s. In contrast, the distance between CG2 of Val297 and CB of Ala312 in BBA-7s increased by 0.9 Å from the corresponding distance of SBA beyond the experimental error, suggesting that the

mutations of Ile297Val in BBA-7s is effective in reducing the bad contact between CD1 of Ile299 and CB of Ala314 in SBA. The thermal inactivation experiments showed that Ser295Ala and Ile297Val have a ΔT_{50} of 3.2 deg.C and 0.9 deg.C, respectively (Yoshigi *et al.*, 1995b).

Mutated site of Ser351Pro

Three mutated amino acid residues, Ser350Pro, Ser351Pro and Gln352Asp, exist in tandem in loop L6, which protrudes into the solvent and surrounds the entrance of the active site. The mutated residues are located in a helix turn built by Pro351, Asp352 and Ala353. Figure 6 shows the wire model of the loop together with the corresponding model of SBA. Yoshigi *et al.* (1995b) reported that

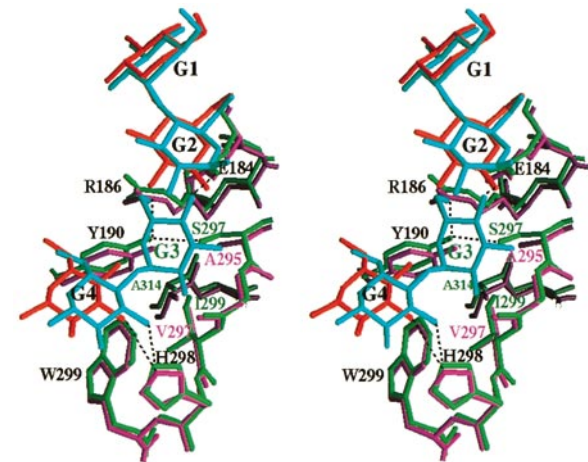


Figure 5. Superposition of the mutated sites of Ser295 and Val297 and the bound maltose from BBA-7s on the corresponding sites from SBA (stereo view). BBA-7s and SBA protein models are colored purple and green, respectively. Maltose models in BBA-7s and SBA are colored red and cyan, respectively. Two molecular models are superimposed by a fitting program implemented in Turbo-Frodo.

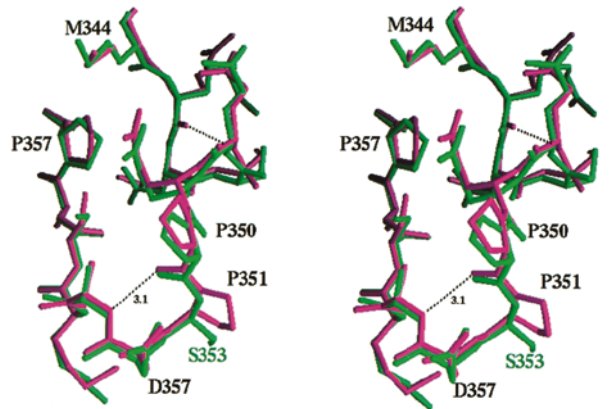


Figure 6. Superposition of the mutated site of Pro350-Asp352 from BBA-7s onto the corresponding site from SBA (stereo view). BBA-7s and SBA models are colored purple and green, respectively. Two molecular models are superimposed by a fitting program implemented in Turbo-Frodo.

the individual mutation of Ser350Pro, Ser351Pro and Gln352Asp increases T_{50} by 2.7, 2.3 and 1.6 deg.C, respectively. Comparison of the main-chain structure of BBA-7s (350Pro-Pro-Asp) and that of SBA (352Pro-Ser-Asp) in this region did not show any significant change in backbone structure. The r.m.s. distance of C^α atoms in L6 (residues 343–357) between BBA-7s and SBA is only 0.29 Å. The main-chain (ϕ, ψ) value of Pro351 ($-59^\circ, -29^\circ$) in BBA-7s is in the β -strand region and near the value of Ser353 ($-62^\circ, -31^\circ$) in SBA. In contrast to Pro351, Pro350 takes (ϕ, ψ) value of ($-51^\circ, 131^\circ$), which is in the α -helix region and belongs to the other cluster of proline conformation angles (MacArthur & Thornton, 1991). In both BBA-7s and SBA, these tandem three residues have higher average B -factors (35.8 and 32.6 Å²) than the overall average values (30.9 and 18.7 Å²).

Mutated site of Ala376Ser

Ser376 exists in the middle of $\beta 7$ of the central β_8 -barrel. As shown in Figure 7, the mutated side-chain of Ser376 has a role in the formation of additional hydrogen bonds. The side-chain of Ser376 points to the interior of the β_8 -barrel. The OG atom of Ser376 makes hydrogen bonds with N and O of Gly413, which is an N-terminal residue of a strand, $\beta 8$. The distances and angles are 2.8 Å and 2.9 Å, and 127° and 150° , for the hydrogen bonds with N and O, respectively. This mutation did not affect the main-chain structure. The r.m.s. distance of C^α atoms in $\beta 7$ and $\beta 8$ between BBA-7s and SBA is 0.34 Å, which is much less than the entire r.m.s. distance of C^α atoms (0.65 Å). These hydrogen bonds reinforce the hydrogen bond network of the central β_8 -barrel structure. This mutation was found by a random mutation method; it is never found in natural β -amylases and it increases T_{50} by 1.0 deg. C (Yoshigi *et al.*, 1995b).

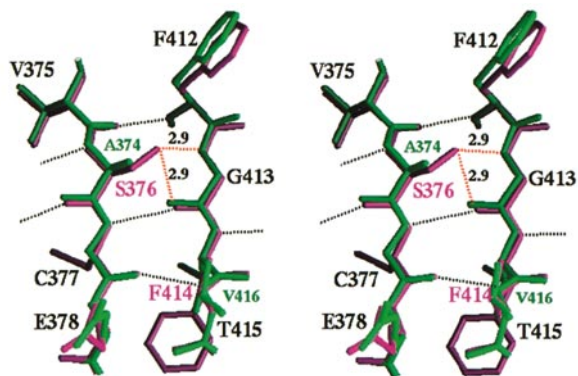


Figure 7. Superposition of the mutated site of Ser376 from BBA-7s on the corresponding site from SBA (stereo view). BBA-7s and SBA models are colored purple and green, respectively. Two molecular models are superimposed by a fitting program implemented in Turbo-Frodo.

Discussion

Strategy of the increased thermostability of BBA-7s

The seven mutated sites in BBA-7s can be classified into four sites. Site 1 is in L5 loop 1 near subsite 3 and contains Ala295 and Val297. Site 2 is in L6 and contains Ser350Pro, Ser351Pro and Gln352Asp. Site 3 is Ser376 in $\beta 7$. Site 4 is Leu185 in L4 loop 1. The sums of the increased ΔT_{50} terms of the single mutation of each site reached 4.1, 6.6, 1.0 and 0.8 deg. C for sites 1, 2, 3 and 4, respectively (Yoshigi *et al.*, 1995b). The change of structure at sites 1, 2 and 3 could be shown by a comparison of the structure of BBA-7s with that of SBA.

The changes of structure suggest that sites 1 and 3 of BBA-7s have increased stability by reducing the conformation energy of the native structure. The mutation of Ile297Val avoids the unfavorable contacts between the two non-mutated side-chains Ile297 and Ala312. Though the exact reason for the increased thermostability achieved by the mutation Ser295Ala is not clear from the present study, one may conclude that the mutation reduces the binding affinity of subsite 3. This result supports the finding that the BBA-7s K_m value for maltohexaose is about fivefold that of the original recombinant enzyme (Yoshigi *et al.*, 1995b).

In site 3, the mutated Ser376 OG makes two additional hydrogen bonds with the adjacent β -strand (between $\beta 7$ and $\beta 8$). In the structures of BBA-7s and SBA there is found only one tight hydrogen bond between $\beta 7$ and $\beta 8$, while other β -strands make three to five tight hydrogen bonds with adjacent strands (Mikami *et al.*, 1993). This packing is reinforced by the introduction of hydrogen bonds with Ser376 OG. The twisted β -strands in the β -barrel allow Ser376 OG to approach the main-chain atoms of Gly413. A similar type of hydrogen bond between the side-chain of Ser and the main chain of the adjacent strand in a β -bulge has been reported for a thermostable variant of subtilisin (Bryan *et al.*, 1986).

In contrast to sites 1 and 3, there seems to be no energetic advantage for the three tandem mutations of site 2. This site is the most effective for thermostability. The ΔT_{50} values were reported as 2.7 deg.C for Ser350Pro and 2.3 deg.C for Ser351Pro, respectively (Yoshigi *et al.*, 1995b), though they belong to different (ϕ, ψ) groups of proline residues (MacArthur & Thornton, 1991). It has been proved that the pyrrolidine ring of proline restricts the freedom of the unfolded backbone structure (Matthews *et al.*, 1987). In the case of the phage T4 lysozyme, the single mutation of Ala82-Pro increased the T_m value by 2.1 deg.C (Matthews *et al.*, 1987), which is compatible with the effects of the two Pro substitutions in BBA-7s. These substitutions seem to follow the so-called proline rule (Suzuki *et al.*, 1987; Watanabe *et al.*, 1997) and provide the new example of tandem mutations with main-chain torsion angles of an α -helix (Pro350)

and a β -strand (Pro351) in a loop region. We do not know the reason for the increased thermostability (by 1.6 deg.C) that accompanies mutation Gln352Asp, but this mutation may reduce side-chain freedom in the unfolded state by introduction of a shortened side-chain. These three successive mutations, however, suggest the importance of reduced chain movement of this loop region in the unfolded state. The facts that this loop region (residues 344-353) does not have any contact with other parts and does have a higher *B*-factor in the native state also support this view.

As to the mutation of site 4, Leu185 exists adjacent to the catalytic residue Glu184 in BBA-7s. The mutated Leu side-chain exists almost in the same position as Leu187 in SBA and faces the other side of the catalytic site. Though there is no experimental determination of the conformation of the original Met185 side-chain, the space occupied by the side-chain of Leu185 surrounded by helices α 4 and α 5, strands β 4 and β 5, and loop L5 is too narrow to accommodate the side-chain of Met185. The accessible surface area of Leu185 is found to be only 0.7 Å², suggesting that it is buried inside. The sequence comparison of plant and microorganism β -amylases reveals that they all have Leu residues at this position, except for BBA (Mikami *et al.*, 1993). This suggests possible unfavorable interactions of Met185 with the surrounding residues, or the favorable conformational entropy of the mutated Leu compared with the original Met side-chain in the unfolded state.

It should be noted that the thermal inactivation of BBA is an irreversible process, suggesting that the mutations do not necessarily influence the stability of the native state, but could act on a kinetic level. In this respect, the mutation Gln352Asp introducing a negative charge on the site 2 loop might slow the unfolding pathway. The mutation Met185Leu at site 4 may suggest the unfavorable effect of a possible oxidation of the Met side-chain in a partially unfolded state at high temperature.

C-terminal extended loop

BBA has a C-terminal sequence that is 45 residues longer than that of SBA. This extended C-terminal region has four repeats of the G(D)PTG(S)GMGGQ(E)A(V,L)E(K,P) sequence (Kreis *et al.*, 1987; Yoshigi *et al.*, 1994a). In the present analysis, we could follow the main-chain up to 504 residues, which contains the first repeat of the repeated C-terminal sequence. The remaining 28 residues were not visible. It has been reported that BBA in barley seeds and expressed in *E. coli* has multiple forms with different isoelectric points, which were ascribed to heterogeneity of the C-terminal amino acid residue owing to proteolysis by a contaminating pretease (Lundgard & Svensson, 1987; Yoshigi *et al.*, 1994b). We purified and crystallized a single component of BBA-7s from a mixture of the different isoelectric com-

Table 3. Interchain hydrogen bonds in the C-terminal region

Atom in C-terminal region	Pair atom	Secondary structure	Distance (Å)
N Gln481	O Pro398	α 7	3.0
NE2 Gln481	O His399	α 7	3.1
O Glu482	NH2 Arg345	L6	2.6
OD1 Asn485	N Ile401	L7	3.0
O Pro487	NE Arg438	α 8	3.0
N Val488	OD1 Asn390	α 7	2.9
O Thr491	ND2 Asn431	α 8	3.1

ponents. The fact that the C-terminal sequence is not visible even in this single component suggests a disordered structure of the 28 C-terminal residues, which appear to be susceptible to attacks by a contaminating protease. These structural features may support the view that this C-terminal repeat region functions as a linker to produce the latent form (Kreis *et al.*, 1987).

Yoshigi *et al.* (1995a) reported that deletion of 54 amino acid residues from the C terminus resulted in significant thermoinstability of the enzyme. This enzyme (Δ 54) has a molecular mass of 54,000 Da and Gln481 as its C-terminal residue. As SBA has a C-terminal residue corresponding to the position of Pro490 in BBA (Figure 3), the C-terminal sequence region 481-490 should be important for thermostability. There are eight hydrogen bonds between the C-terminal 481-492 residues and the other part of BBA-7s, including β 7, β 8, L6 and L7, as shown in Table 3. Of the eight hydrogen bonds, one is between main-chains (Gln481 N to Pro398 O), and three are in common with SBA (Gln481 N to Pro398 O, Asp485 OD1 to Ile401 N, and Val488 N to Asn390 OD1). No inter-chain hydrogen bond was found in the extended C-terminal region beyond residue 492. This suggests that the halted interaction of residues 481-491 with L6, α 7, L7 and α 8 seems to be responsible for the instability of the Δ 54 enzyme.

Experimental Methods

Purification and crystallization of BBA-7s

BBA-7s was expressed in *E. coli* and purified as described (Yoshigi *et al.*, 1995b). The purified enzyme gave a single band of protein on SDS-PAGE at an apparent molecular mass of 56,000 Da but showed heterogeneity on non-denaturing PAGE. The enzyme was further purified by an ion-exchange column as follows: the enzyme solution was first desalted by passing through a Sephadex G-25 column equilibrated with 0.05 M Tris-HCl (pH 8.0) containing 1 mM phenylmethanesulfonyl fluoride (PMSF). The eluted protein fraction was applied to a Mono-Q column equilibrated with the same buffer solution, and eluted with a linear gradient of 0.05 M to 0.3 M Tris-HCl (pH 8.0), 1 mM PMSF. The enzyme was eluted as several peaks. One major peak (20% of total) exhibiting a single band on both SDS-PAGE and PAGE, was collected and used for crystallization. The N-terminal sequence of this component was determined by Procise 492 protein N-terminal sequencer (Applied Bio-

system). The molecular mass was estimated to be 58,400 Da by matrix-assisted laser desorption ionization mass spectrometry using Voyager RP (PerSeptive Biosystem). A crystallization search using the hanging drop method with Crystal Screen (Hampton Research Co.) gave needle crystals at no. 18 drop (the bottom solution was 0.1 M sodium cacodylate, containing 0.2 M magnesium acetate and 20% PEG 8000, pH 6.5). The conditions were modified to yield large crystals. The drops of the final trial containing 3 mg/ml protein, 50 mM Pipes (pH 7.1), 0.1 M magnesium acetate, 7% PEG 6000, 1 mM PMSF, 0.01% (w/v) NaN_3 were equilibrated against bottom solution containing 0.1 M Pipes pH (7.1), 0.2 M magnesium acetate 14% PEG 6000 at 18 °C.

Data collection and structure determination

The crystal was soaked in bottom solution containing 100 mM maltose for one hour at 18 °C immediately before data collection. Diffraction data up to 2.5 Å were collected with CuK α radiation generated by a MAC Science M18XHF rotating anode generator on a Siemens HI-STAR multiwire area detector and were processed with the SAINT software package (Siemens).

The crystal structure of BBA-7s was solved by the molecular replacement method. XPLOR (Brünger, 1992) was used for calculation of the molecular replacement and refinement with the data $|F| > 2\sigma(F)$. The protein structure model of SBA complexed with cyclodextrin (1BTC of Brookhaven Protein Data Bank; Mikami *et al.*, 1993) was used as a search model. PC refinement after a cross-rotation search (10.0 to 5.0 Å resolution) gave a unique solution with a Patterson correlation value of 0.091 with the next peak of 0.051. The translation function search (10.0 to 5.0 Å resolution) using the PC-refined solution yielded an outstanding peak at 15.4 σ above the mean with the next peak at 8.6 σ . The appropriately rotated and translated model gave an *R*-factor of 0.44 for the data between 8.0 and 3.0 Å.

The rigid body refinement with the data between 10 and 3.0 Å resolution led to a decrease in the *R*-factor to 0.39. Refinement by simulated annealing with molecular dynamics using a slow-cooling protocol similar that described by Brünger (1992), from 3000 K to 300 K, yielded *R* = 0.240. In this step, the closed flexible loop, three glucose residues and an altered main-chain at residue 449 were clearly visible on an $|F_o| - |F_c|$ map. The SBA model was then assigned the correct BBA sequence according to Yoshigi *et al.* (1994a) by replacing residues using program TURBO-FRODO (Bio-Graphics), on an INDY computer (Silicon Graphics). $|F_o| - |F_c|$ and $2|F_o| - |F_c|$ maps were used to locate the correct model. Several rounds of positional refinement and *B*-factor refinement, followed by manual model building, were carried out to improve the model. Water molecules were incorporated where the difference density had values of more than 3 σ above the mean and the $2|F_o| - |F_c|$ map showed a density of more than 1 σ . The final *R*-factor was 0.189 for 18,732 data points with $|F| > 2\sigma(F)$ in the 10–2.5 Å resolution range (80.0% completeness). *R*_{free} calculated for the randomly separated 10% data was 0.262.

Brookhaven Protein Data Bank

Coordinates of the BBA-7s have been deposited with accession number 1BLY.

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