Protein-engineered Bacillus α-Amylases That Have Acquired Both Enhanced Thermostability and Chelator Resistance

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Thermostability and chelator resistance of the liquefying alkaline α-amylase (AmyK) from alkaliophilic Bacillus sp. strain KSM-1378 were examined by deletion of either Arg181-Gly182 or Thr183-Gly184 on a loop in domain B. In the tertiary structure of Bacillus stearothermophilus α-amylase (BSA), Ile181-Gly182 (Thr183-Gly184 in AmyK) pushes away a spatially contacting region containing Ca²⁺-coordinating Asp207 (Asp209 in AmyK). Therefore, the deletion of Ile181-Gly182 rather than Arg179-Gly180 was predicted to result in a higher thermostability of BSA. However, our results with AmyK were clearly contrary to this prediction. The resistance to EDTA of both mutant enzymes from AmyK was essentially equal, and the Arg181-Gly182-deleted mutant was more thermostable than the Thr183-Gly184-deleted one. It strongly implies that the microenvironmental topology around the loop containing these dipeptides in AmyK is different from that in BSA.

α-Amylases (1,4-α-D-glucan glucanohydrolases; EC 3.2.1.1) are industrially important, particularly in the food and detergent industries.1-3) We have found an alkaline liquefying α-amylase, AmyK (formerly designated LAMY), from alkaliophilic Bacillus sp. strain KSM-1378.4) The liquefying α-amylases that have been used for detergents so far are all acid or neutral enzymes like the enzymes from Bacillus amyloliquefaciens (BAA),5) Bacillus stearothermophilus (BSA)6) and Bacillus licheniformis (BLA).7) AmyK is highly active at alkaline pH, compared with BAA, BSA and BLA, and resistant to surfactants.4) Thus, this enzyme can be used as an effective additive in dishwashing and laundry detergents.

Based on the amino acid alignment of typical α-amylases (Fig. 1A), Suzuki et al.8) showed that the thermostability of BAA was drastically improved by deletion of Arg176-Gly177 and substitution of Lys269 for Ala, using site-directed mutagenesis. They proposed that the enhanced thermostability due to the deletion and substitution mutations is caused by increased hydrophobicity. Machius et al.9) also suggested that the loop containing the Arg-Gly residue in BAA has two more amino acid residues than BLA and that this could cause increased mobility of this region and decreased thermostability of the whole protein. We reported that AmyK acquired thermostability and, at the same time, resistance to EDTA, EGTA and SDS by the Arg-Gly residue in BAA has two more amino acid residues than BLA and that this could cause increased mobility of this region and decreased thermostability of the whole protein. We reported that AmyK acquired thermostability and, at the same time, resistance to EDTA, EGTA and SDS by the Arg181-Gly182 deletion mutation.10) Therefore, the above hypotheses cannot explain why and how the mutant enzyme (dRG) acquires resistance to chelating reagents. Recently, Suvd et al.11) analyzed the 3D structure of BSA and showed that the insertion of Ile181-Gly182, instead of Arg179-Gly180 (see Fig. 1B), pushes away a spatially contacting region containing Asp204, which may correspond to Ca²⁺-coordinating Asp207 in BLA and Asp209 in AmyK. According to them, this may be the reason why BSA, BAA and AmyK are less thermostable than BLA. To examine whether this...
Fig. 1. Amino acid sequence alignment of the region including Arg 181-Gly 182 and Thr183-Gly 184 in AmyK and the corresponding regions in BLA, BAA and BSA.

(A) Alignment computed using a GENETYX program.
(B) Alignment based on the 3D structure of BSA. The two dipeptide residues shown in bold face are presumably located on a loop in domain B of AmyK, BAA and BSA (see Fig. 2). The identical amino acid residues among the four enzymes are boxed.

MATERIALS AND METHODS

Organisms, transformation and culture conditions. The AmyK producer Bacillus sp. strain KSM-1378 was used as the source of genomic DNA and propagated, with shaking, as described previously. Preparation of the genomic Bacillus sp. strain KSM-1378 DNA, isolation of plasmids, and transformation of Escherichia coli HB101 and B. subtilis ISW1214 were done by conventional methods, as described. Transformed E. coli cells were grown in Luria-Bertani broth containing 50 μg/mL kanamycin or 15 μg/mL tetracycline. B. subtilis harboring plasmids was grown, with shaking, at 30°C for appropriate lengths of time in an optimized liquid medium placed in 500 mL flasks. The medium was composed of (w/v) 5% maltose, 0.05% yeast extract (Difco), 1% Polypepton S (Nippon Pharmaceutical), 1% meat extract, 0.02% KH₂PO₄, 5 mM CaCl₂, and 15 μg/mL tetracycline (pH 7.4).

Construction and purification of recombinant enzymes. Recombinant wild-type AmyK and dRG were prepared as described previously. dTG was obtained by site-directed mutagenesis, using a Mutan Super Express Km kit (TaKaRa) and a plasmid gene containing the AmyK gene (pKFLAMY) as template for PCR. The mutagenic primer for dTG was 5’-TATAAATTCAGAGTAAAGCATTGGAGCTGG-3’ and 5’-phosphorylated by T4 polynucleotide kinase (TaKaRa). PCR cycling parameters used were as described previously. Plasmid pHSP64, constructed with pHY300PLK and the promoter region of an endo- glucanase from Bacillus sp. strain KSM-64, was used for high-level expression of AmyK and engineered enzymes in B. subtilis ISW1214.

The wild-type AmyK, dRG and dTG expressed in B. subtilis cells were purified to homogeneity by column chromatography on DEAE-Toyopearl 650M and CM-Toyopearl 650S (Tosoh) as described. The purified enzymes were concentrated and dialyzed against a large volume of 10 mM Tris-HCl buffer (pH 7.0) overnight.

Enzyme assay. α-Amylase activity was measured routinely at 50°C for 15 min in a 1-mL reaction mixture composed of 0.5 mL of a 1.0% solution of soluble starch from potato (Sigma), 0.2 mL of 250 mM Tris-HCl buffer (pH 8.5), 0.1 mL of a suitably diluted solution of enzyme (usually 1:1200 dilution with distilled water), and 0.2 mL of distilled water. The reducing sugar formed was quantified as glucose by the dinitrosalicylic acid procedure of Miller et al. One unit (U) of enzymatic activity was defined as the amount of protein that produced 1 μmol of reducing sugar as glucose per min under the conditions of the assay. Protein was measured, using a protein assay kit (Bio-Rad) with bovine serum albumin as standard.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done using slab gels containing 10% acrylamide, and samples were stained for protein with Coomassie Brilliant Blue R-250. The gels were calibrated with a low molecular mass standard protein.
kit (Bio-Rad), which included bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and trypsin inhibitor (20.1 kDa). Electrofocusing PAGE was done with a Multiphore II gel electrophoresing system with a PAG-Plate and a broad pH calibration kit (Pharmacia Fine Chemical), as described previously.4)

**Homology modeling of tertiary structures of α-amylases.** The structural models of AmyK and its engineered proteins were constructed by the homology modeling method, based on the 3D structure of BSA (the PDB code lhvx)11) and the deduced amino acid sequence of AmyK.4) All data sets were processed on a Silicon Graphics O2 workstation using an Insight II/Discover software package (Molecular Simulation, Inc.).

**Nucleotide sequence accession number.** The original nucleotide sequence data of AmyK reported in this paper has been deposited with the accession number AB008763 (DDBJ/EMBL/GenBank).

**RESULTS**

**Homology modeling of AmyK.**

First, we constructed a model of AmyK with BSA as template. The validity of the modeled structure was examined by 3D-ID, XPLORE and PROCHECK programs. The 3D structures of several α-amylases from different origins appear very similar and contain three domains, A, B and C. Essentially, AmyK has the $(\beta/\alpha)_8$ barrel core structure, as shown in Fig. 2A. Domain B includes the loop that contains Arg181-Gly184 and Thr183-
Gly184. If the 3D structure of BSA were essentially the same as that of AmyK, the presence of Thr183-Gly184 (Ile181-Gly182 in BSA) would push away the spatially contacting region containing Asp209 (Asp207 in BSA), which corresponds to the Ca$^{2+}$-coordinating Asp204 in BLA. As a result, a water molecule would coordinate to the Ca$^{2+}$ in place of the side chain of Asp209 in AmyK (Fig. 2B), thereby making BSA, BAA and AmyK less thermostable than BLA.11)

Production and purification of recombinant enzymes.
To examine whether dTG is really more thermostable than dRG, we constructed the respective mutant genes and expressed them in B. subtilis cells. B. subtilis cells harboring a plasmid containing the gene for wild-type AmyK, dRG or dTG were grown at 30°C for 72 h, with shaking, in an optimized liquid medium plus tetracycline (15 µg/mL). AmyK, dRG and dTG were produced extracellularly at levels of 26.2×10^4, 48.3×10^4 and 44.8×10^4 U/L, respectively. The difference in the productivity among the wild-type and engineered enzymes was reproducible.

AmyK, dRG and dTG were separately purified to homogeneity as judged by SDS-PAGE, as shown in Fig. 3. Their molecular masses were approximately 53 kDa for all, values very close to that of native enzyme produced by Bacillus sp. strain KSM-1378.4 Apparent pI values of AmyK, dRG and dTG were around pH 7.9, 7.7 and 8.3, respectively, as judged by electofocusing PAGE. When measured at 50°C and at pH 8.5, specific activities of AmyK, dRG and dTG were 4900, 4500 and 3700 U/mg protein, respectively.

Effect of temperature on activity.
The activities of AmyK, dRG and dTG were measured at various temperatures for 5 min at pH 8.5 in 50 mM Tris-HCl buffer. The temperature optima of the three enzymes were around 55°C for all. In the temperature range examined, the specific activity of dTG is 70–80% of that of AmyK and dRG, as shown in Fig. 4.

Effect of pH on activity.
The activities of the three recombinant enzymes were measured at various pHs and at 50°C for 15 min. The pH-activity curves of the enzymes coincided well, and their pH optima were around 8.5–9.0. In the pH range examined, the specific activity of dTG is lower than 80% of that of AmyK and dRG, as shown in Fig. 5.
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Fig. 5. Effect of pH on the activities of AmyK, dRG and dTG.

The pH-activity curves of wild-type AmyK (open circles), dRG (solid circles) and dTG (solid squares) are shown. The reactions were done at 50°C and at the indicated pHs. The buffers used (50 mM each) were as follows: acetate, pH 4.0–6.0; potassium phosphate, pH 7.0–8.0; glycine-NaOH, pH 9.0–10.0. The values are shown as percentages of the specific activity of wild-type AmyK observed at pH 8.0, which is taken as 100%.

Fig. 6. Thermostabilization of wild-type AmyK by Na+ and Ca2+.

The time courses of inactivation of the wild type enzyme at various temperatures without additives (A) and with both 10 mM Na+ and 0.1 mM Ca2+ (B) are shown. The thermal stability was examined at 30°C (open circles), 40°C (open squares), 50°C (open triangles), 60°C (solid circles), 65°C (solid squares), and 70°C (solid triangles) and at pH 8.5 in 50 mM Tris-HCl buffer. Samples (0.1 mL) were withdrawn, and their residual activities were measured under the standard conditions of the assay. The original activity before heating is taken as 100%.

Thermal stability of wild-type AmyK in the presence of Na+ and Ca2+.

When wild-type AmyK was heated at 60°C for 15 min without exogenous additives in 50 mM Tris-HCl buffer (pH 8.5), the enzyme activity was reduced to 10% of the original activity. In contrast, the activity was protected from thermal inactivation by the addition of both CaCl2 and NaCl at optima of 0.1 and 10 mM, respectively (data not shown). The thermostabilization was not observed in the presence of any of the following ions: Ag2+, Be2+, Mg2+, Mn2+, Fe2+, Co2+, Cd2+, Sr2+, Pb2+, Ba2+, Al3+, Fe3+ (1 mM each), or K+ ions (50 mM). Next, we followed the time courses of inactivation of AmyK at various temperatures either without additives or with both 10 mM Na+ and 0.1 mM Ca2+ and found that AmyK retained approximately 60% of its original activity even after incubation with both cations at 60°C for 60 min (Fig. 6B), while it lost the activity almost completely after heating without additives (Fig. 6A).

Thermostabilization of mutant enzymes by Na+ and Ca2+.

The effects of Na+ and Ca2+ on thermal stability of wild-type AmyK were examined for dRG and dTG with wild-type AmyK as control. As shown in Fig. 7, the deletion mutation made both mutants more stable than AmyK, even after being heated at 70°C for 60 min in the presence of both cations. Further, dTG was clearly more thermostable than dRG, regardless of whether the cations were present or not. The half life (t1/2, min) at 70°C without cations was 1 for AmyK, 22 for dRG, and 7 for dTG; t1/2 in the presence of 0.1 mM CaCl2 was 4 for AmyK, 68 for dRG, and 15 for dTG; and t1/2 in the presence of 10 mM NaCl was 9 for AmyK, at 70°C and at pH 8.5 were examined for dRG and dTG with wild-type AmyK as control. As shown in Fig. 7, the deletion mutation made both mutants more stable than AmyK, even after being heated at 70°C for 60 min in the presence of both cations. Further, dTG was clearly more thermostable than dRG, regardless of whether the cations were present or not. The half life (t1/2, min) at 70°C without cations was 1 for AmyK, 22 for dRG, and 7 for dTG; t1/2 in the presence of 0.1 mM CaCl2 was 4 for AmyK, 68 for dRG, and 15 for dTG; and t1/2 in the presence of 10 mM NaCl was 9 for AmyK,
Fig. 7. Thermostabilization of AmyK by deletion mutations.

The thermal stability of wild-type AmyK (A), dRG (B) and dTG (C) was assessed by incubation at 70°C and at pH 8.5 in 50 mM Tris-HCl buffer. The enzymes were heated without cations (open circles), in the presence of 10 mM Na⁺ (solid squares), in the presence of 0.1 mM Ca²⁺ (solid triangles), and in the presence of both 10 mM Na⁺ and 0.1 mM Ca²⁺ (solid circles). Samples (0.1 mL) were withdrawn at timed intervals, and the residual activities were measured. The respective original activities before heating are taken as 100%.

Fig. 8. Resistance to EDTA of AmyK, dRG and dTG.

The stability of wild-type AmyK (open circles), dRG (solid circles) and dTG (solid squares) was examined by incubation with 10 mM EDTA at 40°C and at pH 8.5 in 50 mM Tris-HCl buffer. Samples (0.1 mL) were withdrawn and used for measurement of the residual activity. The respective original activities before the EDTA treatment are taken as 100%.

270 for dRG, and 260 for dTG.

Resistance to EDTA.

Next, we examined the resistance of AmyK, dRG and dTG to EDTA. Their residual activities were measured after incubation for various times at 40°C and at pH 8.5 in the presence of 10 mM EDTA. As shown in Fig. 8, wild-type AmyK lost its activity because of EDTA within 5 min, whereas dRG and dTG remained very active even after a 90-min incubation. The resistance to EDTA of the RG deletion was completely equal to that of the TG deletion.

DISCUSSION

In this study, we showed that the deletion of Arg181-Gly182 or Thr183-Gly184 on a loop of domain B in AmyK caused enhanced thermostability and chelator resistance. Our present results reconfirm our previous conclusion that these mutational effects, regardless of the deletion of Arg181-Gly182 or even Thr183-Gly184, arise primarily from enhanced Ca²⁺ binding. Furthermore, this suggests that the thermostability mechanism proposed by Suvd et al. for BSA cannot explain why dRG and dTG (dIG in the case of BSA) of AmyK acquire the same degree of EDTA resistance and dRG is more thermostabilized than dTG. Suvd et al. predicted that the deletion of Ile181-Gly182 rather than Arg179-Gly180 would result in a higher thermostability of BSA. However, our present results with AmyK are clearly contrary to their prediction. It is suggested strongly that the microenvironmental topology around the dipeptide-containing loop in AmyK (possibly also the case for BAA) may be different from that in BSA. The contribution of loop stabilization by shortening it.
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Two amino acid residues to the thermostabilization of the enzyme may be marginal, because only two more hydrogen bondings are gained on the loop (data not shown). At present, we cannot explain how the deletion mutations acquire chelator resistance (or thermostabilization). By deletion of either Arg181-Gly182 or Thr183-Gly184, the coordination of Ca²⁺ with Asp209 could be restored by an unidentified mechanism, or a new Ca²⁺ coordination geometry could be generated in the AmyK structure. We have already succeeded in the crystallization of AmyK, and its tertiary structure will be published shortly.

It has recently been shown that the Ca²⁺-binding site conserved in both BLA and BSA forms part of an unprecedented linear triadic array, with two Ca²⁺ flanking a central Na⁺ (Cal-Na-CalII) (see Fig. 2). The metal triad and an additional Ca²⁺ (CalIII) located between domains A and C are believed to contribute to increased thermostability in all bacterial α-amylases. Exogenous Na⁺ and Ca²⁺ effectively thermostabilize AmyK and its engineered enzymes. Na⁺ stabilizes the enzyme very much, but the positive effect of Ca²⁺ is marginal. This suggests that the three Ca²⁺ in AmyK are tightly bound to the enzyme, but Na⁺ is easily removable from the enzyme, for instance, by dilution or dialysis. Since natural environments do not always contain excess Na⁺, the tertiary structures of α-amylases with the metal triad may be a transitional architecture that occurs only when excess Na⁺ is present. In most cases, the enzymes may partially or not fully contain Na⁺. Recently, we found an alkaline, Ca²⁺-free α-amylase, designated AmyK38, from an alkaliphilic Bacillus isolate, which is highly resistant to EDTA and chemical oxidation and requires Na⁺ for activity. However, Na⁺ does not stabilize this novel enzyme.

AmyK has very high specific activity compared with BLA, BSA and BAA. The high activity is not destroyed by the deletion mutation. However, the specific activity of dTG is lower than the 80% of the wild-type enzyme, while that of dRG is more than 90%. Since the Arg181-Gly182 and contiguous Thr183-Gly184 residues in AmyK are spatially positioned far away from the catalytic residues Asp236 and Asp333, we have no idea what in the dTG mutation causes such low activity, as well as its low thermostability, compared with that of the dRG mutation. Anyway, taking into account the commercial production of AmyK, the dTG mutation is clearly more disadvantageous than the dRG mutation. We have shown that AmyK can also be thermostabilized by replacing proline for Arg124 on a loop region in domain B, as a result of the “proline rule” proposed by Suzuki. The thermostabilization due to the double mutation with Arg124Pro and dRG is cumulative and remarkable even in the absence of any exogenous additives. The temperature at which the enzyme is inactivated in 10 min is 47.4°C for the wild type and 62.5°C for the double mutant enzyme. The double mutant enzyme is now suitable for practical use at high temperatures and high chelator concentrations under highly alkaline conditions.

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