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Review

Promising α-Amylases for Modern Detergents

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Introduction

Amylose is a linear polysaccharide that is formed by α-1,4-linked α-D-glucose units. Amylopectin is also a polysaccharide of α-D-glucose monomers, but it contains α-1,6-branching points. α-Amylases (1,4-α-D-glucan glucanohydrolase; EC 3.2.1.1) catalyze the hydrolysis of the α-1,4-glucosidic linkages of starch. It randomly cleaves the internal bonds in amylose and amylopectin. Various starch-hydrolyzing enzymes catalyze a similar reaction (1). Cyclodextrin glucanotransferase (2), maltogenic α-amylases (3,4), and maltotetraose-forming amylases (5). Pullulanase (6,7) and isoamylase (8,9) hydrolyze the α-1,6-glucosidic linkages. Neopullualanase (10,11) and amylopullulanase (12) hydrolyze both the α-1,4-glucosidic linkages and the α-1,6-glucosidic linkages of starch. Such starch-hydrolyzing enzymes should be named the α-amylase family, because they are similar to α-amylases (13). According to the sequence and active site topology, α-amylase is grouped into family 13 (14). Well-known bacterial α-amylases are liquefying ones from *Bacillus* species, such as *Bacillus amyloliquefaciens* (BAA), *Geobacillus stearothermophilus* (BSA), and *Bacillus licheniformis* (BLA) (15-19). Their molecular masses and optimal pH for activity are around 55 kDa and. between 5 and 7.5, respectively. BLA is thermostable and its optimal temperature is around 80°C, depending upon the presence of Ca^{2+} ions (20).

The tertiary structures of α-amylases have been reported for TAKA-amylase A (21), porcine pancreas (22), *Aspergillus nigar* (23), bareley (24), and *Pseudomonas stutzeri* (25). Recently, Matius *et al*. (26,27) reported on the crystal structure of the industrially important BLA. Similar to the other α-amylases, BLA has a three-domain structure with the domains A, B, and C. The *N*-terminal domain A forms a so-called $(\beta/\alpha)_8$-barrel (TIM-barrel) domain. Domain B is located in a looped out part of the polypeptide chain between the third β-strand and the third helix of TIM-barrel. It forms a large part of the substrate-binding cleft and is presumed to be important for the substrate specificity differences observed between α-amylases. The *C*-terminal domain C forms an antiparallel β-sheet. Three Ca^{2+} and one Na^{+} ions were found in BLA, a linear triad CaI-Na-CaII

arrangement, is located at the interface between domain A and B. Another Ca^{2+} (CaIII) bridges between domains A and C.

α-Amylases are important, especially in the starch and food industries. The demand for liquefying α-amylase for use in laundry and automatic dishwashing detergents has been growing for several years (28). Detergent enzymes have to function in washing machines or dishwashers under conditions that are very unfavorable for the stability of the enzyme. The pH is high alkaline in washing conditions. The high temperature (55~60°C) in a dishwasher requires thermostable enzymes. Enzymes prefer to be resistant to various detergent ingredients, such as surfactants, chelators, and oxidants (bleach). However, most of bacterial liquefying α-amylases, such as BAA and BSA, including BLA, has optimal pH of between 5 and 7.5. Therefore, they are not practically suitable for use in laundry and dishwashing detergents with high alkalinity. We have found an alkaline, liquefying α-amylase, designated AmyK, from alkaliphilic *Bacillus* sp. strain KSM-1378 (29). Here, we describe the enzymatic properties, improvement in thermostability and oxidative stabilization of AmyK.

Enzymatic properties of AmyK

The homogeneous preparation of AmyK had the specific activity of 5,000 units (U)/mg when measured at 50°C and at pH 8.5 in 50 mM Tris-HCl buffer. The molecular mass of the purified enzyme was approximately 53 kDa by SDS-PAGE. The isoelectric point was around pH 9 (29). When AmyK was preincubated with 10 mM EDTA or EGTA, the activity decreased to 10% or 9% of the initial activity, respectively. AmyK was resistant to incubation at 40°C for 1 h with various surfactants, such as SDS, polyoxyethylene alkyl ether, sodium

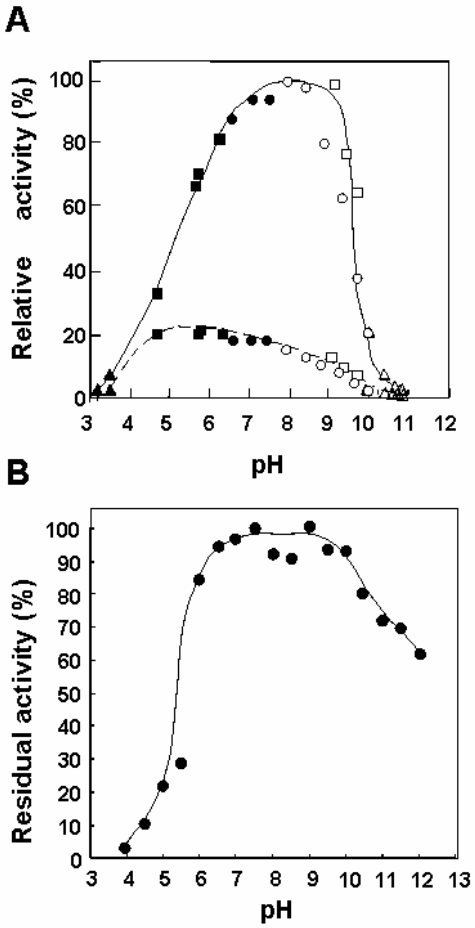

Fig. 1. Effect of pH on the activity and stability of AmyK. **A**. The pH-activity curves of purified AmyK and BLA (each at 0.2 U/ml) are shown by solid and dotted lines, respectively. The buffers used (50 mM each) were as follows: glycine-HCl, pH 3.0-3.5 (▲); acetate, pH 4.0-6.0 (■); Tris-HCl, pH 6.5-8.5 (●); glycine-NaOH, pH 9.0-10.5 (□); glycine-NaCl-NaOH, pH 8.0-10.5 (○); carbonate, pH 10.0-11.0 (△). The values are shown as percentages of the maximum specific activity of AmyK observed at pH 8.0-8.5, which is taken as 100%. **B**. To assess the pH stability of AmyK, the enzyme (2.0 U/ml) was preincubated at the indicated pH in 10 mM Britton-Robinson buffer and at 40°C for 30 min, and then samples (0.1 ml) were used for the measurements of the residual activity under the standard conditions of enzymatic assay. The values are shown as percentages of the original activity, which is taken as 100%.

α-sulfonated fatty acid ester, linear alkylbenzene sulfonate, and alkyl glucoside (each added at 0.1%, w/v). Moreover, the enzyme is unique in that at the early stage of hydrolysis of soluble starch, the enzyme produced G5 to G9 in quantities much larger than G2 and G3. G1 and G4 formed at this stage were trace. On further incubation, G3 and G5 increased while G6 to G8 decreased. These results indicate that AmyK is classified as liquefying α-amylase (20, 30).

As shown in Fig. 1A, the maximum activity was observed around pH 8.0-8.5 when measured in various buffers at 50 mM with soluble starch as substrate. Between pH 5 and 10, the specific activity was 2- to 5-fold greater than that of BLA. To assess the pH stability of AmyK, the enzyme was preincubated at 40°C for 30 min in 10 mM Britton-Robinson buffer and assayed at 50°C in 50 mM Tris-HCl buffer at pH 8.5. The enzyme was stable over a range between pH 6 and pH 10 (Fig. 1B).

The optimal temperature for activity was around at 55°C at pH 8.5 in 50 mM Tris-HCl buffer (Fig. 2A), while that of BLA was around at 80°C. At 80°C, the specific activities of BLA and AmyK were almost equal, and they were approximately 25% of the maximum activity of AmyK observed at 55°C. Time course of the thermal inactivation of AmyK was followed at various temperatures and at pH 8.5 in 20 mM Tris-HCl buffer (Fig. 2B). The enzyme retained its full activity after 60 min of incubation at 45°C, but only 32% and 3% of original activity remained after 60 min of incubation at 50°C and 60°C, respectively. However, in the presence of 0.1 mM $CaCl_2$, nearly 100% and 65% of the original activity remained at 50°C and 60°C, respectively. The enzymatic activity was abolished after heating at 70°C for 60 min even in the presence of 0.1 mM Ca^{2+} ions. In contrast, BLA was quite stable to incubation at 70°C at least up to 60 min under our test conditions.

Deduced amino acid sequence of AmyK

The entire gene for AmyK was cloned and sequenced (29). It contained an open reading frame of 1545-bp encoding 515 amino acids

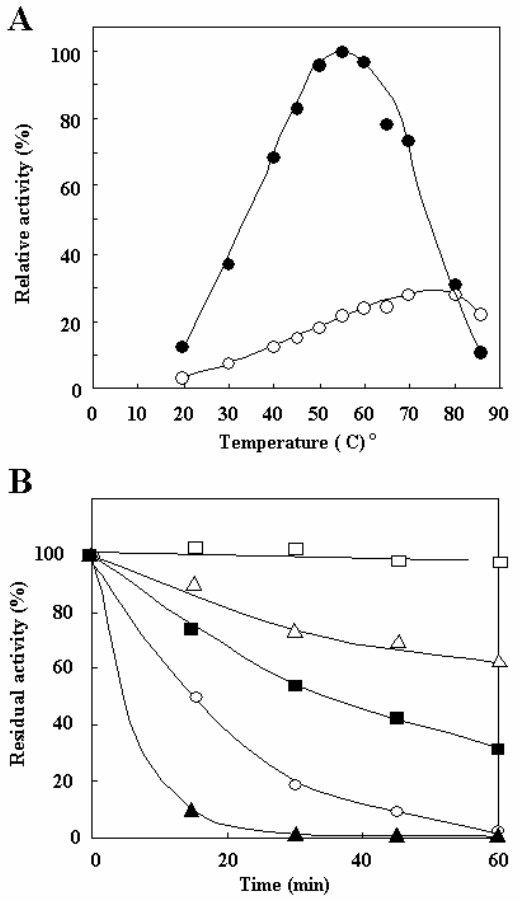

Fig. 2. Effect of temperature on the activity and stability of AmyK. **A**. The temperature-activity curves of purified AmyK (●) and BLA (○) (each at 0.18 U/ml) are shown. The reactions were done at the indicated temperatures for 10 min and at pH 8.5 in 50 mM Tris-HCl buffer. The values are shown as percentages of the maximum activity of AmyK observed at 55°C, which is taken as 100%. **B**. To asses the thermostability of AmyK, the enzyme (1.8 U/ml) was heated at the indicated temperatures in the presence at 50°C (□), 60°C (△), and 70°C (○)), or in the absence at 50°C (■) and 60°C (▲) of 0.1 mM Ca^{2+} ions in 50 mM Tris-HCl buffer (pH 8.5). Samples (0.1 ml) were used for the determination of the residual activity under the standard conditions of enzyme assay.

including a putative signal peptide of 31 amino acids. The molecular mass of the mature enzyme was 55,391 Da, a value close to the 53 kDa determined by SDS-PAGE of the purified AmyK from the culture broth of *Bacillus* sp. strain KSM-1378. When suitably aligned, the deduced amino sequence of AmyK exhibited only 66.7, 68.6, and 68.9% identity to those of BAA (31), BSA (32), and BLA (33), respectively, but almost no homology to those of saccharifying α-amylases reported to date. Essentially, AmyK has the $(\beta/\alpha)_8$ barrel structure. Four conserved regions, which are necessary for the catalytic activity of α-amylases (35) were conserved in AmyK as Asp102 to His107 (region I), Gly232 to His240 (region II), Glu266 to Lys269 (region III), and Phe328 to Asp333 (regions IV). They form the active center, the substrate binding sites, and the Ca^{2+} binding sites.

For the evaluation of properties of AmyK, we used an excretion vector pHSP64 to hype-rproduce it with *Bacillus subtilis* as the host (36,37). The gene for AmyK was amplified by PCR and cloned into *Sal*I-*Sma*I site of pHSP64. The transformed *B. subtilis* hyper-produced the AmyK activity, corresponding to approximately 1.0 g protein/ liter of an optimized liquid culture (38).

Improvement of thermostability of AmyK

Compared with BAA and BSA, BLA is more thermostable. Tomazic and Klibanov (39,40) and Declerck *et al*. (41) reported that temperature optima of native or engineered thermophilic enzymes are generally higher than those of mesophilic enzymes, possibly due to the stiff structures which stay folded at higher temperatures than mesophilic enzymes. Declerck *et al*. (41,42) and Joyet *et al.* (43) independently reported hyperthermostable mutants of BLA; two substitutions in the amino acid sequence, His133Ile (or His133Tyr) and Ala 209Val (or Ala 209Ile), can together increase in the half life of BLA at 90°C up to 10 folds. It is very interesting that the original amino acid sequence of AmyK conserves the corresponding amino acid residues at amino acids 135 (Tyr) and 214 (Ile), respectively. Van der Laan (44) and Aehle (45) showed that two substitutions, Val128Glu/His133Tyr or His133Tyr/Asn188Asp, improved the thermostability of BLA in the presence of excess $CaCl_2$ (1.5 mM). Again, we emphasize the unique amino acid sequence of AmyK in that the substituted residues in BLA are conserved as Tyr135 and Glu130 in the enzyme (although the substituted Asp188 corresponds to Ile193). Therefore, there may be another unidentified structural element(s) responsible for thermostabilization of AmyK, if its thermostability could be further improved as high as that of BLA or the engineered BAA.

Suzuki *et al*. (46) demonstrated that thermostability of BAA was drastically improved by deletion of Arg176-Gly177 and substitution of Lys269 for Ala, using site-directed mutagenesis. They proposed that an increase in hydrophobicity by changes in charged residues enhanced the thermostability of this enzyme. Machius *et al*. (27) also suggested that the loop containing the Arg-Gly residues 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. In fact, the amino acid sequences of AmyK, BAA and BSA, all conserved the corresponding Arg-Gly residues at the respective amino acid positions. However, substituted Ala269 in BAA corresponds to Ala274 for AmyK and Ala269 for BLA in their original amino acid sequences. In support of this scenario, we deleted Arg181-Gly182 residues from the AmyK molecule by site-directed mutagenesis (47).

AmyK and the dipeptide-deleted mutant protein (dRG) were very similar with respect to specific activity, pH-activity curve, temperature-active curve, susceptibility to inhibitors, and pattern of hydrolysis products from soluble starch and malto-oligosaccharides. As expected from the sequence alignment of AmyK and related amylolytic enzymes, dRG was found to acquire increased thermostability, regardless of whether $CaCl_2$ was present or not. It was stable to incubation up to 50°C and 60°C in the absence and presence of $CaCl_2$, respectively. In contrast, AmyK was stable up to 35-40°C and 45°C, respectively (Fig. 3).

To evaluate the possible role of calcium against thermoinactivation of AmyK and dRG, we examined their resistance to EDTA and EGTA by incubating for 30 min at varying concentrations of the individual chelating reagents at 40°C and pH 8.5. Wild-type AmyK lost its activity completely at very low concentrations of EDTA and EGTA (0.05 mM), whereas dRG remained approximately 60-70% active, independent of calcium concentrations up to 2 mM examined. The residual activities of both enzymes after incubation for various times at 45°C and at pH 8.5 in the presence of 0.25 mM EDTA or 0.05% SDS. Fifty % inactivation by 0.25 mM EDTA occurred after 3 min for AmyK and after 35 min for dRG, and that by 0.05% SDS occurred after 1.5 h for the former. However, the activity of dRG remained constant at 45°C for over a 20-h period even in the presence of SDS.

Proline residues in proteins are known to restrict backbone bond rotation because of their pyrrolidine rings. Suzuki *et. al*. (48) proposed a "proline rule" which states that many prolines at second position of β-turn make proteins more thermostable. Matthews *et al*. (49) improved the thermostability of T4 lysozyme by replacing

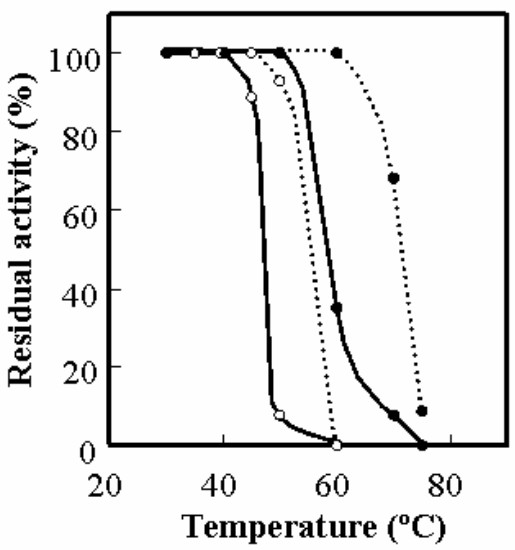

Fig. 3. Comparison of thermostability of AmyK and dRG. To assess the thermostability of AmyK and dRG, the enzymes (2.0 U/ml) were individually heated for 2 h at the indicated temperatures in the absence or presence of 2.0 mM $CaCl_2$ in 50 mM Tris-HCl buffer (pH 8.5). Samples were transferred after 2 h to wet ice (0°C) and the residual activity in them (0.1-ml aliquot) was assayed by measuring the rate of soluble starch hydrolysis under the standard conditions of the assay. The residual activities of AmyK (○) and dRG (●) in the absence (solid lines) or presence of $CaCl_2$ (dotted lines) are expressed as percentages of the respective original activities, which are each taken as 100%.

Ala87 at one of its β-turns with a proline residue, so as to decreased the backbone entropy of unfolding.

We showed that AmyK could also be thermostabilized by replacing proline for Arg124 (50). According to the homology alignment of AmyK with BLA, the amino acid residues of BAA, BSA, and BLA corresponding to Arg124 in AmyK were all proline. According to the structure of BLA, the position of Arg124 of AmyK is presumed to be located on a loop connecting between a two-stranded antiparallel β-sheet involving residues at amino acids from

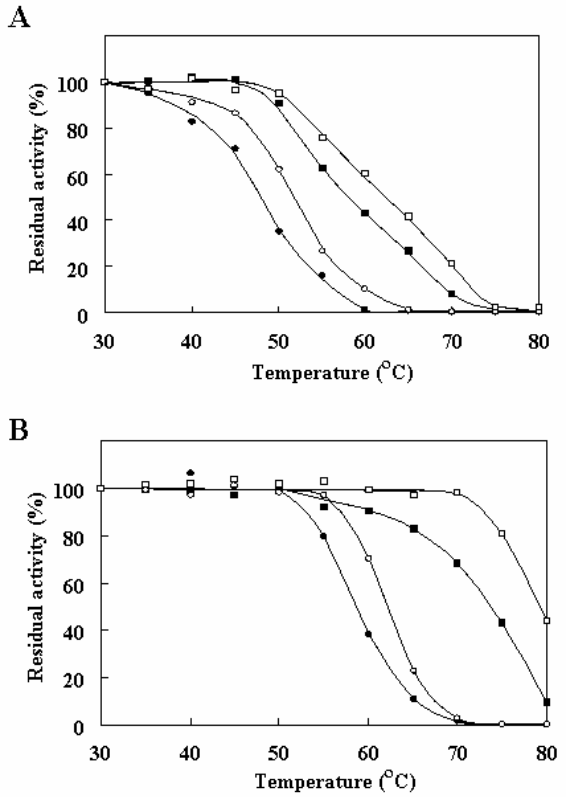

Fig. 4. Effects of temperature on the stability of wild-type AmyK (●), R124P (○), dRG (■), and dRG -R124P (□). Each enzyme (0.04 μg/ml) was heated for 10 min at different temperatures in 10 mM Tris-HCl buffer (pH 8.5) without (**A**) and with (**B**) 0.1 mM $CaCl_2$, and assayed for the activity remaining. The activity of each enzyme after treatment at 30°C is taken as 100%.

113 to 121 and from 135 to 142 in domain B. We chose the Arg124 residue in AmyK to examine the effects of proline substitution on thermostability and catalytic activity. Half lives at 50°C of the wild-type enzyme and an Arg124Pro mutant enzyme were 4.4 min and 11.3 min, respectively. Furthermore, the thermostabilization due to 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 enzyme and 62.5°C for the double mutant enzyme (Fig. 4). Although no information was given about the positions of β-turn in the BLA structure, the thermostabilization of the Arg124Pro mutant is caused as a result of the "proline rule".

Oxidative stabilization of AmyK

When AmyK was incubated with 1%, 2%, and 3% each of H_2O_2 in 10 mM Tris-HCl buffer (pH 8.5) plus 2 mM $CaCl_2$ at 30ºC for 30 min, the activity decreased to 27%, 4%, and 2% of the control, respectively. Such oxidative inactivation has also been reported for an alkaline serine protease (subtilisin) (51,52). Oxidative stabilities of detergent proteases have been improved by replacing a Met residue for non-oxidizable amino acids by site-directed mutagenesis.

In the AmyK molecule, there are 10 Met residues at positions 9, 10, 105, 116, 202, 208, 261, 309, 382, and 430, as deduced from its nucleotide sequence. When the amino acid

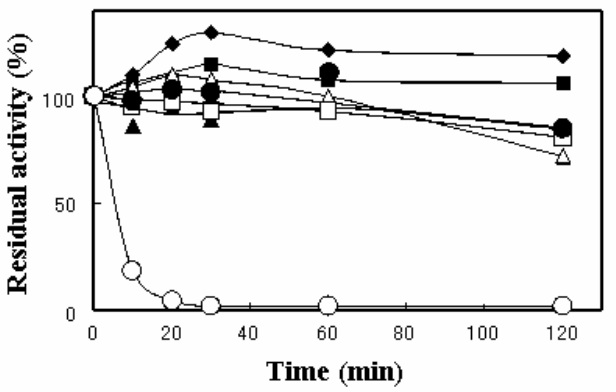

Fig. 5. Rates of oxidative inactivation by H_2O_2 of the wild-type and mutant enzymes.
Each enzyme was incubated in the presence of 0.5 M H_2O_2 at 30ºC and at pH 8.9 in 50 mM Tris-HCl buffer. Timed samples were withdrawn and mixed with 140 μg/ml catalase to quench excess H_2O_2, and the residual activities were measured under the standard conditions of enzyme assay. The original activity of each enzyme is taken as 100%. The wild-type and mutant enzymes retained full activities during the incubation without H_2O_2 up to 2 h. Symbols used are: ○, wild-type AmyK; ♦, Met202Ala; ▲, Met202Ser; ■, Met202Val, □, Met202Leu; ●, Met202Ile; △, Met202Thr.

sequence was suitably aligned with those of BSA, BLA, and BAA, Met10, 202 and 309 (in AmyK numbering) are conserved. This suggests that oxidation by H_2O_2 of some of the three residues would cause the reduced enzymatic activity. The oxidative stabilization of AmyK was achieved significantly by replacing Met202 with non-oxidizable amino acid residues**,** such as Thr, Leu, Ile, Ser, and Ala. These mutant enzymes showed strong oxidative stability when incubated with 2% H_2O_2 in 10 mM Tris-HCl buffer (pH 8.5) plus 2 mM $CaCl_2$ at 30°C for up to 60 min (Fig. 5). A computer-aided modeling of AmyK which was constructed using the 3D structure of BLA (27) as a template suggested that Met202 is located on the enzyme surface and may correspond to the substrate binding residue in AmyK. Substitutions with smaller amino acid residues such as Ser and Ala caused drastic increase in the K_m values toward a borohydride-reduced amylose, while substitutions with bulky Thr and Ile had essentially little effect. These results suggest that oxidation of Met202 to the sulfoxide derivative resulted in interference with substrate binding, thereby abolishing the enzyme activity (53).

Recently, we found an alkaline, Ca^{2+}-free α-amylase, designated AmyK38 (54,55), from an alkaliphilic *Bacillus* isolate that is highly resistant to chelating reagents and chemical oxidants and requires Na^+ for activity. We have already clarified why AmyK38 is resistant to EDTA and chemical oxidants by analyzing the crystal structure of the enzyme (56). Thus, detergent enzyme technologies will further develop with microbial screening for novel enzymes and protein engineering based on enzyme structures.

In this review, protein engineering of a detergent α-amylase AmyK was summarized. 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, and that the enzyme can also be thermostabilized by replacing proline for Arg124 on a loop region in domain B. The double mutant enzyme is suitable for practical use at high temperatures and high chelator concentrations under high alkalinity. α-Amylase is very promising for use not only in the starch and food industries but also in the detergent industry.

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