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Critical Factors to High Thermostability of an α-Amylase from Hyperthermophilic Archaeon *Thermococcus onnurineus* NA1

LIM, JAE KYU, HYUN SOOK LEE, YUN JAE KIM, SEUNG SEOB BAE, JEONG HO JEON, SUNG GYUN KANG, AND JUNG-HYUN LEE*

Korean Ocean Research & Development Institute, Ansan P.O. Box 29, Seoul 425-600, Korea

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Abstract Genomic analysis of a hyperthermophilic archaeon, *Thermococcus onnurineus* NA1 [1], revealed the presence of an open reading frame consisting of 1,377 bp similar to α-amylases from *Thermococcales*, encoding a 458-residue polypeptide containing a putative 25-residue signal peptide. The mature form of the α-amylase was cloned and the recombinant enzyme was characterized. The optimum activity of the enzyme occurred at 80°C and pH 5.5. The enzyme showed a liquefying activity, hydrolyzing maltooligosaccharides, amylopectin, and starch to produce mainly maltose (G2) to maltoheptaose (G7), but not pullulan and cyclodextrin. Surprisingly, the enzyme was not highly thermostable, with half-life ($t_{1/2}$) values of 10 min at 90°C, despite the high similarity to α-amylases from *Pyrococcus*. Factors affecting the thermostability were considered to enhance the thermostability. The presence of Ca^{2+} seemed to be critical, significantly changing $t_{1/2}$ at 90°C to 153 min by the addition of 0.5 mM Ca^{2+}. On the other hand, the thermostability was not enhanced by the addition of Zn^{2+} or other divalent metals, irrespective of the concentration. The mutagenetic study showed that the recovery of zinc-binding residues (His175 and Cys189) enhanced the thermostability, indicating that the residues involved in metal binding is very critical for the thermostability.

Keywords: Genomic analysis, hyperthermophile, cloning and expression, α-amylase

Starch-hydrolyzing enzymes are widely distributed in a variety of microbes, and are currently used in a broad array of industrial applications such as in the clinical, medical, and analytical chemistries, as well as in starch saccharification and in the textile, food, fermentation, paper, brewing, and distilling industries [3, 5, 10, 16, 31]. There is a huge demand for enzymes suitable for specific applications to economize processes with respect to temperature, utilization of high concentrations of starch, substrate specificity, and pH. In starch liquefaction and saccharification processes occurring at 80–90°C, it is desirable that enzymes should be active at the high temperature, and therefore, there has been a need for thermophilic and thermostable enzymes. Hyperthermophilic enzymes could be advantageous because of their resistance to denaturing agents, solvents, and proteolytic enzymes, in addition to their extreme thermostability. Thermostable amylolytic enzymes including α-amylases have been reported from mesophilic *Bacillus* sp., and thermophilic archaea and eubacteria [4, 6–9, 12–14, 17, 18, 21, 24, 27, 28]. Most of them belong to the glycosyl hydrolase family 13 [11], based on amino acid sequence homology. The family shares four conserved catalytic regions and adopts a common $(\alpha/\beta)_8$-barrel structure, although the overall sequence similarity is low [30].

The screening of useful enzymes and engineering of the available enzymes made it possible to improve the stability of enzymes to meet the important criteria for commercialization [30].

With a generally applicable combination of conventional genetic engineering and genomic research techniques, the genome sequences of some hyperthermophilic microorganisms are of considerable biotechnological interest because of their heat-stable enzymes, and many extremely thermostable enzymes are being developed for biotechnological purposes. We isolated a new species of a thermophilie, *T. onnurineus* NA1, and the whole genome sequence was determined to search for many extremely thermostable enzymes. The analysis of the genome information showed that the strain possesses starch-hydrolyzing enzymes, such as α-amylase, α-glucosidase, pullulanase, and cyclodextrinase. In this study, the gene encoding an α-amlyase from *T. onnurineus* NA1 was cloned and expressed in *E. coli*. The recombinant enzyme was purified and characterized. In addition, factors affecting the thermostability were considered.

*Corresponding author
Phone: 82-31-400-6243; Fax: 82-31-406-2495;
E-mail: jlee@kordi.re.kr

MATERIALS AND METHODS

Strains and Growth Conditions

E. coli DH5A was used for plasmid propagation and nucleotide sequencing. *E. coli* BL21-CodonPlus(DE3)-RIL, *E. coli* Rosetta(DE3)pLysS (Stratagene, LaJolla, CA, U.S.A.) and plasmid pET-24a(+) (Novagen, Madison, WI, U.S.A.) were used for gene expression. *E. coli* strains were cultivated in Luria-Bertani medium at 37°C, and kanamycin was added to the medium at a final concentration of 50 μg/ml.

DNA Manipulation and Sequencing

DNA manipulations were performed by standard procedures, as described by Sambrook and Russell [25]. Restriction enzymes and other modifying enzymes were purchased from Promega (Madison, WI, U.S.A.). Small-scale preparation of plasmid DNA from *E. coli* cells was performed with a plasmid mini kit (Qiagen, Hilden, Germany). DNA sequencing was performed with the automated sequencer (ABI3100) using BigDye terminator kit (PE Applied Biosystems, Foster City, CA, U.S.A.).

Cloning and Expression of the α-Amylase-encoding Gene

The full-length *T. onnurineus* NA1 α-amylase gene flanked by the NdeI and XhoI sites was amplified by PCR with genomic DNA and two primers for the full length of α-amylase (sense [5'-CG ACC CGG CAT ATG GCC AGA AAA GCA GCC GTT GCA GTT TTG-3'] and antisense [5'-CT CCA CAT CTC GAG GCC AAC ACC ACA GTA GCT CCA GAC-3']), and the mature form of α-amylase (sense [5'-CG ACC CGG CAT ATG GCG GAA ACA CTG GAA AAC GGC GGA GTC-3'] and antisense [5'-CT CCA CAT CTC GAG GCC AAC ACC ACA GTA GCT CCA GAC-3']); the underlined sequences indicate the NdeI sites in the sense primers and the XhoI sites in the antisense primers. The amplified DNA fragments were digested with NdeI and XhoI, and were ligated with the corresponding sites of plasmid pET-24a(+). The resultant plasmids, pET-amyl*f* (full-length) and pET-amyl*m* (mature form), were used to transform *E. coli* DH5α, and the recombinant plasmids were introduced into *E. coli* BL21-CodonPlus(DE3)-RIL and *E. coli* Rosetta(DE3)pLysS, respectively, for the expression after the sequence confirmation. Overexpression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at the mid-exponential growth phase and incubated for 3 h at 37°C. To purify the mature form of enzyme, the cells were harvested by centrifugation (6,000 ×*g* for 20 min at 4°C) and resuspended in 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 M KCl and 10% glycerol. The cells were disrupted by sonication and centrifuged (20,000 ×*g* for 1 h at 4°C). The resulting supernatant was applied to a column of TALON metal affinity resin (BD Biosciences Clontech, Palo Alto, CA, U.S.A.) and washed with 10 mM imidazole (Sigma, St. Louis, MO, U.S.A.) in 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 M KCl and 10% glycerol, and the α-amylase was eluted with 300 mM imidazole in the buffer. The pooled fractions were then buffer exchanged to 50 mM Tris-HCl buffer (pH 8.0) containing 10% glycerol using Centricon YM-10 (Millipore, Bedford, MA, U.S.A.).

The protein concentration was determined by the colorimetric assay of Bradford [3]. The protein purity was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis performed according to the standard procedure [15].

Site-directed Mutagenesis and Purification of the Mutant Proteins

The combinatorial mutations at the residues involved in metal binding (S175H, Y176C, A177C, and D189C) were introduced by site-directed mutagenesis using the QuickChange kit (Stratagene) and following primers: SCCD (sense [5'-AAT GAC GTC AGC TGT TGC GAT GAA GGC ACC TTC GGA GGT TTC-3'] and antisense [5'-GGT GCC TTC ATC GCA ACA GCT GAC GTC ATT GGG ATG GAA GTC-3']); SYAC (sense [5'-TTC CCC GAC ATA TGC CAC GAC AAA GAG TGG AAC CAG TAC-3'] and antisense [5'-CTC TTT GTC GTG GCA TAT GTC GGG GAA ACC TCC GAA GGT-3']); HYAD (sense [5'-AAT GAC GTC CAC TAC GCC GAT GAA GGC ACC TTC GGA GGT TTC-3'] and antisense [5'-GGT GCC TTC ATC GGC GTA GTG GAC GTC ATT GGG ATG GAA GTC-3']); HYAC (sense [5'-AAT GAC GTC CAC TAC GCC GAT GAA GGC ACC TTC GGA GGT TTC-3'] and antisense [5'-GGT GCC TTC ATC GGC GTA GTG GAC GTC ATT GGG ATG GAA GTC-3']); and HCCC (sense [5'-CCA ATG ACG TCC ACT GTT GCG ATG AAG GCA CCT TCG GAG GTT-3'] and antisense [5'-GCC TTC ATC GCA ACA GTG GAC GTC ATT GGG ATG GAA GTC GAG-3']). The recombinant plamids were introduced into *E. coli* BL21-CodonPlus(DE3)-RIL for the expression after the sequence confirmation. The mutant proteins were purified similarly to the wild-type protein.

Enzyme Assays

T. onnurineus NA1 α-amylase (TO_amyl) activity was determined by measuring the amount of reducing sugar released during enzymatic hydrolysis of 1% soluble starch in 50 mM sodium acetate buffer (pH 6.0) at 80°C for 15 min. The amount of reducing sugar was measured by a modified dinitrosalicylic acid method [2].

Nucleotide Sequence Accession Number

The reported nucleotide sequences have been submitted to the GenBank/EMBL Data Bank with Accession No. DQ144136.

RESULTS

Primary Structure of the TO_amyl-encoding Gene

In the genome sequence data of *T. onnurineus* NA1, an open reading frame (1,377 bp) encoding a protein consisting of 458 amino acids was found and showed high similarity to α-amylases from *T. hydrothermalis* (85% identity; [18]) and *T. kodakaraensis* KOD1 (82% identity; [25]), and *P. furiosus* or *P. woesei* (82% identity; [7]) with a predicted molecular mass of 51,948 Da. The deduced amino acid sequence of the α-amylase coding region includes the putative signal peptide of 25 amino acids and mature enzyme of 433 amino acids (predicted molecular mass of 50,531 Da) (Fig. 1). Sequence analysis displayed that four active-site consensus regions characteristic of the glycosyl hydrolase family 13 [22] were present in TO_amyl. TO_amyl displayed a lower number of Lys and Arg residues (32, similar to 35 in *P. furiosus* α-amylase) comparable to the 54 in *Bacillus licheniformis* amylase

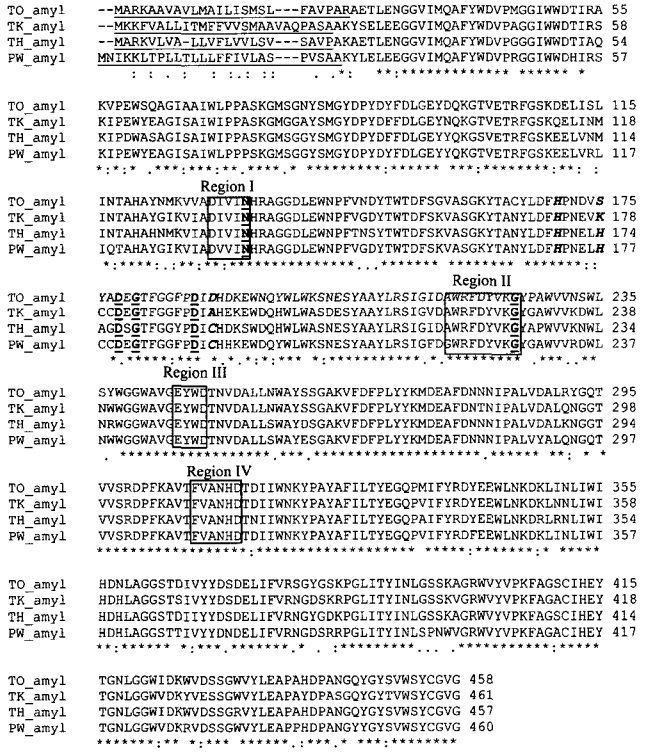

Fig. 1. Sequence comparison of the α-amylase from *T. onnurineus* NA1 (TO_amyl), *T. kodakaraensis* KOD1 (TK_amyl, gi:57641390), and *T. hydrothermalis* (TH_amyl, gi:14521082), and *P. woesei* (PW_amyl, gi:14590819).

Dashes indicate gaps, and numbers on the right represent the position of the last residue in the original sequence. Identical residues among the four enzymes are marked as asterisks (*), and residues with conserved substitutions and semiconserved substitutions are marked as colons (:) and periods (.), respectively. The four conserved active-site residues are boxed. The residues participating in Ca^{2+} and Zn^{2+} binding, critical for thermostability, are bold underlined and bold italic, respectively. The identified or putative signal peptides are indicated by underlines.

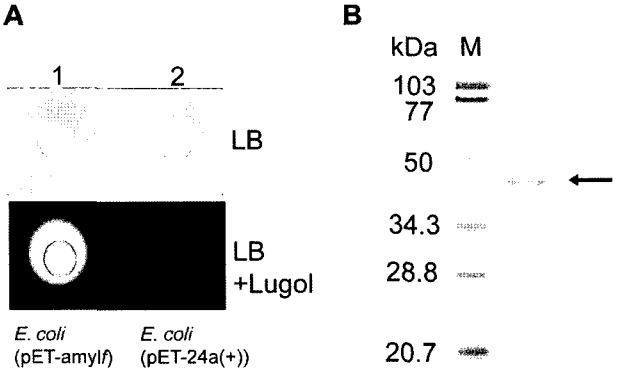

Fig. 2. Expression of the α-amylase gene in *E. coli* (**A**) and SDS-PAGE of the purified enzyme (**B**).

A. Plate assay of α-amylase activity of *E. coli* transformants. **B**. Coomassie blue staining of the purified TO_amyl*m* enzyme. The molecular mass standards (lane M) were phosphorylase *b* (103 kDa), bovine serum albumin (77 kDa), ovalbumin (50 kDa), carbonic anhydrase (34.3 kDa), soybean trypsin inhibitor (28.8 kDa), and lysozyme (20.7 kDa). The band corresponding to the enzyme is indicated by the arrow.

[21], and is probably responsible for the low isoelectric point of the enzyme (pI of 4.61).

Cloning and Expression of the TO_amyl-encoding Gene in *E. coli*

The full length of the TO_amyl gene was cloned into a pET-24a(+) vector by PCR (pET-amyl*f*) and was transformed into *E. coli* BL21-CodonPlus(DE3)-RIL to express the gene. When cultured on a starch medium plate, *E. coli* transformed with the empty vector was unable to degrade this substrate after incubation at 60°C, but *E. coli* with pET-amyl*f* formed a distinct halo indicating effective starch degradation of the TO_amyl gene (Fig. 2). The α-amylase activity was detected in the extracellular fraction of recombinant *E. coli* harboring pET-amyl*f*. Since very little α-amylase was produced from *E. coli* (pET-amyl*f*), we constructed a plasmid pET-amyl*m* without the sequence of the signal peptide. The mature form of α-amylase was purified from a soluble fraction of induced *E. coli* (pET-amyl*m*), and the purified TO_amyl remained soluble after the repeated thaw and freezing steps. In SDS-PAGE, the band of purified enzyme was observed at a size smaller than 51 kDa, which was estimated from the amino acid sequence of the mature form including the His_6-tag (Fig. 2).

Biochemical Characterization of TO_amyl

The specific activity of the purified TO_amyl was 7,238 U/mg. The enzyme exhibited a temperature optimum at 80°C, with less than 20% of maximal activity observed at 40 to 50°C, and the optimum pH for the enzyme was 5.5 to 6.0 (data not shown). The thermostability of TO_amyl was evaluated by incubating the enzyme in 50 mM sodium acetate buffer (pH 6) at 80 and 90°C. TO_amyl lost enzymatic

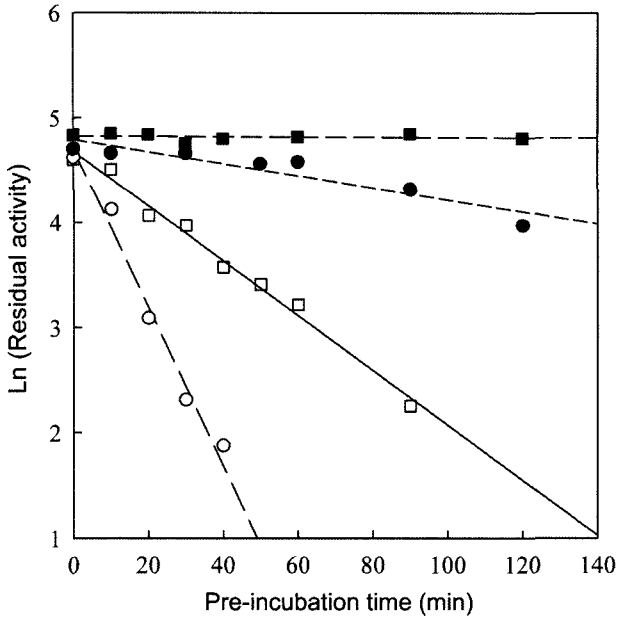

Fig. 3. Thermal inactivation of α-amylase.
Semilog plots of the remaining activity versus incubation time are shown. α-Amylase (2 nM) was incubated at 80°C (squares) or 90°C (circles) in 50 mM sodium acetate buffer (pH 6.0) in the absence (open symbols) or presence (filled symbols) of 0.5 mM Ca^{2+}. At the times indicated, aliquots were taken out, and the activities were measured in the same buffer at 80°C using soluble starch as substrate. The lines were obtained by linear regression of the data.

activity with half-life ($t_{1/2}$) values of 30 min at 80°C and 10 min at 90°C, respectively (Fig. 3).

TO_amyl could hydrolyze a wide variety of substrates such as amylose, oligomaltosaccharide, amylopectin, and soluble starch to form maltose (G2) to maltoheptaose (G7) as the main products. The activity to hydrolyze pullulan and cyclodextrin could not be detected (data not shown). Conclusively, TO_amyl can be classified as liquefying enzyme like *P. furiosus* α-amylase. Kinetic analysis was carried out using soluble starch, and K_m (4.57 mg/ml) and V_{max} (10,000 μmol/min/mg) values were calculated from the measured activity using a Lineweaver-Burk plot (data not shown), and compared with α-amylases from other *Thermococcus* strains (Table 1).

Table 2. The residues involved in the zinc binding of PWA and the corresponding residues in the wild-type and various mutants of TO_amyl.

Name	Residues in various mutant constructs				
PWA	His172	His177	Cys178	Cys179	Cys191
TO_amyl	His170	Ser175	Tyr176	Ala177	Asp189
SYAC	His170	•	•	•	Cys189
SCCC	His170	•	Cys176	Cys177	Cys189
HYAD	His170	His175	•	•	•
HYAC	His170	His175	•	•	Cys189
HCCC	His170	His175	Cys176	Cys177	Cys189

Factors Affecting the Thermostability of TO_amyl

As mentioned above, TO_amyl showed high similarity to α-amylases from *P. furiosus* or *P. woesei* (82% identity, [7]). However, TO_amyl was not highly thermostable, losing enzymatic activity within 30 min at 90°C. What could be a critical change of TO_amyl lowering the thermostability, and is it possible to enhance the thermostability by considering the factors? To address the issue, the effects of divalent metals were evaluated, and changes of residues involved in metal binding were carried out to favor the metal binding (Table 2).

It seemed that Ca^{2+} binding was critical for the thermostability of TO_amyl. As shown in Fig. 3, the thermostability of TO_amyl was significantly increased in the presence of 0.5 mM Ca^{2+} with $t_{1/2}$ of 7,581 min at 80°C and 153 min at 90°C although the activity was not changed by addition of 0.5 mM Ca^{2+}. It seemed obvious that TO_amyl requires calcium ion for the activity as well, since the activity was decreased up to 4% when the enzyme (2 ng) was treated with 1 mM ethylenediaminetetraacetic acid (EDTA), and the activity with EDTA treatment was recovered by the addition of Ca^{2+}.

On the other hand, other divalent cations including Zn^{2+} could not replace Ca^{2+} for the enhanced thermostability. In our research, Zn^{2+} significantly inhibited TO_amyl activity in a relatively high concentration (data not shown) and the thermostability was not enhanced at low concentrations, inconsistent with the result of the amylase of *P. woesei* (PWA) [20].

Table 1. Comparison of general biochemical properties of TO_amyl and α-amylases from *Thermococcales*.

	TNA1[a]	TK	TH	TP	PF
[c]K_m (mg/ml)	4.6	7	ND[b]	2.3	3.6–6.8
[c]V_{max} (mmol/min/mg)	10,000	5,672	ND	1,807	ND
Optimum temp (°C)	80	90	75-85	80	100
Optimum pH	5.5–6	6.5	5-5.5	5.5	6.5–7.5
Ion requirement	Ca^{2+}	None	Ca^{2+}	Ca^{2+}, Cs^{2+}, Mg^{2+}	None

[a]TNA1, this study; TK, α-amylase from *T. kodakaraensis* KOD1 [28]; TH, α-amylase from *T. hydrothermalis* [18]; TP, α-amylase from *T. profundus* [17]; PF, α-amylase from *P. furiosus* [7].
[b]ND, not determined.
[c]K_m and V_{max} toward soluble starch.

Mutagenesis of Residues Involved in Metal Binding

The PWA structure showed a two-metal center (calcium or zinc binding) in close proximity to the active-site cleft. The comparison of the amino acid sequence between PWA and TO_amyl showed that the amino acid residues involved in calcium binding (Asn133, Asp178, Gly180, Asp187, and Gly225 corresponding to Asn135, Asp180, Gly182, Asp189, and Gly227 of PWA) were found in TO_amyl, supporting that calcium addition could enhance the thermostability as well as the activity of TO_amyl. In contrast, only one residue for zinc binding (His170 corresponding to His172 of PWA) could be found, whereas the other two residues were changed to Ser175 (His177 of PWA) and Asp189 (Cys191 of PWA) (Table 2). It is possible that the changes in TO_amyl might unfavor metal binding, resulting in decreased thermostability. It has been also reported that cysteine residues affected the thermostability of amylase. To address the issue, the combinatorial mutant proteins at Ser175, Tyr176 (Cys178 of PWA), Ala177 (Cys179 of PWA), and Asp189 were constructed as described in Materials and Methods, and the thermostability of purified proteins was evaluated in comparison with that of wild-type protein. As shown in Fig. 4, the thermostability of HYAC or HCCC among the mutants was significantly increased ($t_{1/2}$: HYAC (114.1 min), HCCC (181.1 min)) compared with wild-type protein ($t_{1/2}$: 39 min), even in the absence of supplemented divalent ions. It indicates that the presence of a zinc-binding site consisting of His170, His175, and Cys189 was critical for the enhanced thermostability of TO_amyl. Unlike the result of PWA, a single mutation at Cys189 did not affect the thermostability. It seemed likely that tight binding of metal ions is essential for the high thermostability of TO_amyl.

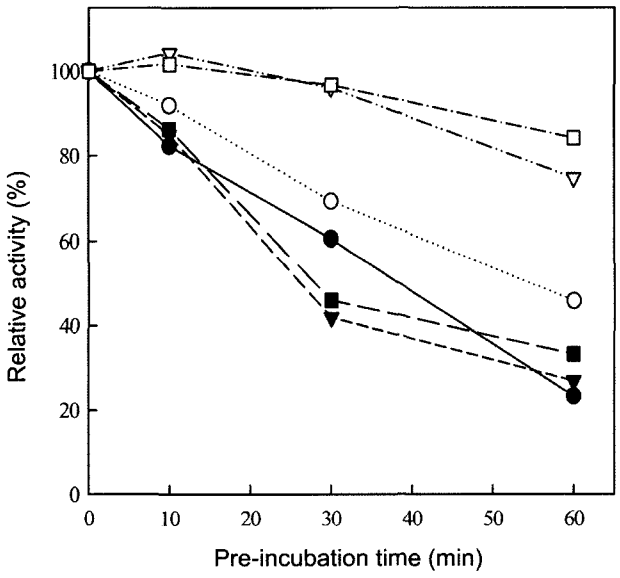

Fig. 4. The thermostability of various TO_amyl mutant proteins.
The thermostability of wild-type (SYAD, ●) and mutants (HYAD, ○; SYAC, ▼; HYAC, ▽; SCCC, ■; HCCC, □) were measured under standard conditions.

DISCUSSION

In this work, we could characterize an α-amylase from a hyperthermophilic archaea, *T. onnurineus* NA1, by combining conventional genetic engineering and genomic research techniques. The α-amylase gene showed high similarity to a highly thermostable amylase from *P. furiosus*. It has been reported that Ca^{2+} enhanced the thermostability and amylolytic activity of *P. furiosus* α-amylase even if the enzyme was also active in the absence of Ca^{2+}. In *P. furiosus* α-amylase, calcium ion seemed to be bound so tightly to the enzyme that EDTA cannot remove it, showing that 95% activity of α-amylase (11.5 ng) remained by 1 mM EDTA and even 86% activity by 5 mM EDTA [20]. However, recent reports on the presence of calcium-binding residues in *P. furiosus* and the data of EDTA treatment at high temperature after extensive dialysis against metal-free buffer suggest that Ca^{2+} binding was essential for the amylolytic activity of *P. furiosus* α-amylase [26]. Compared with *P. furiosus* α-amylase, TO_amyl was not very thermostable, losing enzyme activity within 30 min at 90°C. The inhibition of TO_amyl with EDTA treatment was quite significant, and reversible by the presence of Ca^{2+}. It suggests that the calcium binding was not tight enough to TO_amyl, compared with *P. furiosus* α-amylase. Nonetheless, Ca^{2+} was a critical factor for the activity and thermostability of TO_amyl.

The residues of TO_amyl essential for zinc binding previously reported in *P. furiosus* α-amylase were not conserved. As discussed earlier, one residue (His175) could be found, but other residues were changed to serine or aspartate, which may affect zinc binding. Consistent with the changes, zinc ion did not enhance the thermostability. It was thought that the changes in the zinc-binding motif were one of the reasons for the decreased thermostability of TO_amyl. The possibility was proved by mutagenic study, where the recovery of the zinc-binding motif at HCCC or HYAC mutant proteins could improve the thermostability significantly, confirming that the low thermostability of TO_ amyl was due to zinc-binding change.

It seems likely that extracellular α-amylases originated from *Thermococcus* are less thermophilic, with optimum temperature close to 80°C, than those from *Pyrococcus* [21]. The difference may reflect the living environment of the members belonging to the two genus, although exceptions to this correlation between optimal growth temperature of a strain and optimal temperature of activity of its α-amylase(s) also exist. *T. onnurineus* NA1 grows under a slightly milder temperature than *Pyrococcus*, and it makes

sense that the extracellular amylolytic enzyme may be needed to support the growth of the own organism or the own relatives, from an ecological point of view.

The analysis of the genome information of *T. onnurineus* NA1 showed that the strain possesses various starch-hydrolyzing enzymes, such as α-amylase, α-glucosidase, pullulanase, and cyclodextrinase including several putative glycosyltransferases. The development of other starch-hydrolyzing enzyme is under way.

Acknowledgments

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