Thermostabilization by Proline Substitution in an Alkaline, Liquefying α-Amylase from Bacillus sp. Strain KSM-1378

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α-Amylase (LAMY) from alkalophilic Bacillus sp. strain KSM-1378 is a novel semi-alkaline enzyme which has 5-fold higher specific activity than that of a Bacillus licheniformis enzyme. The Arg124 in LAMY was replaced with proline by site-directed mutagenesis to increase thermostability of the enzyme. The wild-type and engineered LAMYs were very similar with respect to specific activity, kinetic values, pH-activity curve, and degree of inhibition by chelating reagents. Thermostability and structure stiffness of LAMYs as measured by fluorescence were increased by the proline substitution. The change of Arg124 to proline is assumed to stabilize the loop region involving amino acid residues from 122 to 134. This is the first report that thermostability of an α-amylase is improved by proline substitution.

Key words: alkaliphile; Bacillus; α-amylase; thermostability; site-directed mutagenesis

Amylolytic enzymes, such as α-amylase (1,4-α-D-glucan glucohydrolase; EC 3.2.1.1) and pullulanase (α-dextrin 6-glucanohydrolase; EC 3.2.1.41), are industrially very important, particularly in the food and detergent industries. We have found and characterized some unique alkaline debranching enzymes and a biheaded alkaline amylopullulanase from alkalophilic strains of Bacillus, which can be used as effective additives in dishwashing and laundry detergents, as also reported by us for alkaline cellulases and a protease from Bacillus strains.

Recently, we reported the isolation and molecular cloning of the gene for a novel liquefying α-amylase (LAMY) from alkalophilic Bacillus sp. strain KSM-1378, and also succeeded in hyperexpressing the LAMY gene in Bacillus subtilis cells. This enzyme is highly active at alkaline pH, compared with other liquefying α-amylases, such as enzymes from Bacillus licheniformis (BLA), Bacillus amylooliquifaciens (BAA), and Bacillus steaetheromophilus (BSA). LAMY is essentially homologous with BLA, the complete tertiary structure of which was solved by Machius et al., although the two enzymes show diversity in specific activity and thermostability, as well as in pH-activity profile. LAMY is less thermostable than BLA, and therefore it is desirable that the thermostability of LAMY is improved for use at high temperatures under alkaline conditions, especially in automatic dishwashing machines.

Some factors that decide the thermostability of α-amylase have been proposed. Declerck et al. and Joyet et al. have independently reported hyperthermostable mutants of BLA with two substitutions in the amino acid sequence, His133Tyr (or His133Ile) and Ala209Val (or Ala209lle). It is very interesting that the original amino acid sequence of LAMY conserves the corresponding amino acid residues at 135 (Tyr) and 214 (Ile), respectively. Suzuki et al. demonstrated that the thermostability of BAA was drastically improved by deletion of Arg176-Gly177 and substitution of Lys269 with Ala and suggested that an increase in hydrophobicity by decrease of positive residues increased the thermostability of this enzyme. In a previous paper, we reported that thermostability of LAMY could also be improved by deletion of the Arg181-Gly182 residues which correspond to Arg176-Gly177 of BAA, which was caused by stronger calcium binding to the enzyme molecule. We report here that further stabilization of LAMY can be done by proline substitution, which is known to restrict the conformational freedom of the backbone of the polypeptide chain.

Materials and Methods

The organisms and culture conditions. Bacillus sp. strain KSM-1378 was used for the source of genomic DNA and grown at 30°C for 2 days, with shaking, in an alkaline medium, as described previously. B. subtilis ISW1214 (lueA8 metB5 hsrM1) harboring plasmids was propagated at 30°C for 72 h, with shaking, in 50-ml portions of an optimized medium placed in 500-ml flasks. The medium was composed of (w/v) 5% maltose, 0.05% yeast extract (Difco), 8% corn steep liquor, 1% meat extract, 0.02% KH₂PO₄, 5 mM CaCl₂, and 15 μg/ml tetracycline (pH 7.4). Transformed Escherichia

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Abbreviations: BAA, α-amylase from Bacillus amylooliquifaciens; BSA, α-amylase from Bacillus stearothermophilus; BLA, α-amylase from Bacillus licheniformis; LAMY, α-amylase from Bacillus sp. strain KSM-1378; dRG-LAMY, engineered LAMY with deletion of Arg181-Gly182; R124P-LAMY and ΔR124P-LAMY, engineered LAMY and ΔRG-LAMY with substitution of Arg124 to proline; Tspan, temperature at which enzyme was 50% inactivated at pH 8.5 in 10 min; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PCR, polymerase chain reaction(s)
coli HB101 (hsdS20 (r5 m5) recA13 ara14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44 leuB6 thi-1) and E. coli MV1184 (Δ(lac-proAB) ara rpsL thi(680 lacZ ΔM15) Δ(srl-recA)306::Tn10(ter+)/F′) [rad36 proAB+ lacZΔM15]) cells were grown in LB broth containing kanamycin.

**Purification of recombinant enzymes.** Wild-type and mutant recombinant enzymes were individually purified at temperatures below 4°C. The centrifugal supernatant of the culture broth was treated with ammonium sulfate and the fraction precipitated at 60% saturation was collected. Precipitates formed were dissolved in a small volume of 10 mM Tris-HCl (pH 7.5) containing 2 mM CaCl₂ and the solution was dialyzed several hours against 50 volumes of the same buffer. The retentate was then passed through a column of DEAE-Toyopearl 650M (10 × 20 cm; Tosho) that had been equilibrated with 10 mM Tris-HCl containing 2 mM CaCl₂ (pH 7.5). The unadsorbed eluate was concentrated by ultrafiltration (PM-10, 10,000-M, cutoff; Amicon), and put directly on a Poros CM-M column (4.6 × 10 mm; Perseptive Biosystems), and purified by HPLC (Bio-Cad; Perseptive Biosystems). The column was initially washed with 3 column volumes of 10 mM Tris-HCl containing 2 mM CaCl₂ (pH 7.0), and proteins were eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer at a flow rate of 15 ml/min. The active fractions were combined and concentrated by ultrafiltration on a PM-10 membrane. The concentrate was dialyzed overnight against 10 mM Tris-HCl buffer (pH 7.5). The resultant retentate was used exclusively throughout experiments.

**Enzyme assay.** α-Amylase activity was routinely measured at 50°C in a 1-mL reaction mixture that contained 0.5 ml of a 1.0% (w/v) solution of soluble starch (from potato; Sigma), 0.2 ml of 250 mM Tris-HCl buffer (pH 8.5), 0.2 ml of distilled water, and 0.1 ml of a suitably diluted solution of enzyme. The reducing sugar formed was measured by the dinitrosalicylic acid procedure.

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. Inactivation of the enzyme in the presence of substrate was not observed during the assay. Kinetic parameters were calculated by Lineweaver-Burk plots using amylase (degrees of polymerization, 17 (DP-17); Hayashibara Biochem.) as substrate which had been reduced by NaBH₄ to lower background for the assay. Preparation of reduced amylase was as follows. Ten grams of amylase DP-17 were dissolved in 125 ml of water on a boiling water bath. After the solution was cooled, 1 g of NaBH₄ was added to it and the reducing reaction was done for 24 h at room temperature. The reaction was stopped by adjusting the pH to 5.0 with acetic acid. The reduced amylase was precipitated by 250 ml of acetone, washed with 65% acetone 3 times on a glass filter, and then dried in a desiccator (yield, 73.6%). Protein was measured, using a protein assay kit (Bio-Rad) with bovine serum albumin as the standard protein.

**Fluorescence assay.** Purified wild-type LAMY and mutant LAMYs were each diluted appropriately to 8 µg/ml in 10 mM Tris-HCl (pH 8.5). Intrinsic fluorescence changes were followed on an F2500 Hitachi spectrofluorometer, using excitation and emission wavelengths of 278 nm and 338 nm, respectively. Fluorescence of wild-type LAMY in the same buffer at 30°C was taken as 100%.

**Electrophoretic analysis.** Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) was done essentially as described by Laemmli. Molecular masses were estimated by SDS-PAGE on slab gels [10% (w/v) acrylamide] with low range molecular mass standards (Bio-Rad) as molecular mass markers.

**Isolation of DNA and transformation.** Genomic DNA from *Bacillus* sp. strain KSM-1378 and plasmid DNA were prepared, as described by Saito and Miura and Birnboim and Doly, respectively. Transformations of *E. coli* and *B. subtilis* with plasmids were done by the methods of Hanahan and Chang and Cohen, respectively.

**Construction and expression of LAMY gene.** Primer DNAs were designed for the amplification of the LAMY gene in the genomic DNA. Since hSP64 (5.5 kbp) contained part of the signal peptide sequence, MMLRKKTQKQLR, of a *Bacillus* endoglucanase, we inserted PAQA between the incomplete signal peptide and the LAMY structural amino acid sequences. The incomplete signal sequence has been shown to be very important for the hyperexpression of foreign genes in our host-vector system. The primer sequences used were 5′-GAGTCGACCCAGCAAGCCATCATATGG-3′, which contained a SalI site and 5′-AAGCTT- CCAATTATATTGGTGTTAT-3′. They were prepared with a DNA synthesizer (model 392A; Applied Biosystems) and were purified by a DNA refinement system (model Dnastec-1000; Astec). The polymerase chain reaction (PCR) was done in a DNA thermal cycler (model 480; Perkin-Elmer), using each primer (30 pmol) plus genomic DNA (1.0 µg) from *Bacillus* sp. strain KSM-1378. Cycling parameters were 5 min at 94°C followed by 20 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, and a final 15 min at 72°C. The reaction mixture contained 200 µM dNTPs, 25 mM KCl, 5 mM (NH₄)₂SO₄, 2 mM MgSO₄, 2.5 units *Pwo* DNA polymerase, and 10 mM Tris-HCl buffer (pH 8.85) (Boehringer Mannheim) in a reaction volume of 100 µl. Products of PCR were purified with a PCR product purification kit (Boehringer Mannheim) and they were used for sequencing or for subcloning. The 1.5-kbp PCR product was digested with *Sal*I and the digest was inserted into the *Sal*I-Smal site of pHSP64. The resultant plasmid, designated hPSPLAMY (7.1 kbp), was introduced into *B. subtilis* ISW1214 cells and one of the transformants obtained was grown at 30°C for 70 h, with shaking, in an optimized liquid medium.
Replacement of Arg124 by proline by site-directed oligonucleotide mutagenesis. A 2.1-kbp DNA fragment that contained a 0.6-kbp promoter region from Bacillus sp. strain KSM-6420 and the structural gene for the 1.5-kbp LAMY (or ΔRG-LAMY) gene27 was amplified by PCR, using 30 pmoles each of appropriate primers, 5′-ACTTACATTATAGTCATAAAG-3′ and 5′-AAGCTTCAATTTATATGGTGTTAT-3′, and 1 ng of pHSPLAMY (or PHSP ΔRG-LAMY) as template. The PCR conditions were as follows: 5 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, and a final 15 min at 72°C. The reaction mixture was the same as that used for amplification of the LAMY gene. Both PCR products (2.1 kbp) generated were purified and subcloned into the Smal site of the plasmid pKF19k (Takara Shuzo) using E. coli HB101 as a host strain, and the constructed plasmids were designated pKF LAMY and pKΔRG-LAMY (4.3 kb), respectively.

The Arg124-substituted mutant proteins of LAMY and ΔRG-LAMY were prepared by site-directed mutagenesis, using a Mutan super express Km kit (Takara Shuzo) with pKF LAMY as template and a 27-bp synthetic primer, 5′-GTGGAGTGAAACCCAGACCGAAAC-3′. The PCR conditions for the mutagenesis were as follows: 1 min at 94°C, 1 min at 50°C, and 4 min at 72°C for 30 cycles. The PCR product was collected by ethanol precipitation and the precipitates were used for transformation of E. coli MV1184. The substitution mutation was confirmed by sequencing the recombinant plasmid extracted from the transformed E. coli cells. To hyperproduce the engineered LAMy in B. subtilis cells, each mutated gene was PCRamplified, using appropriate primers, 5′-GAGTCGACACGACGACAGCCATCATATGG-3′ and 5′-AAGCTTCAATTTATATGGTGTTAT-3′, as described above. The amplified products (1.5 kbp) were purified and digested with Sal I. Each digest was subcloned into the SalI-Smal site of pHSP64, designated pHSPR124p and pHSΔRG-R124p, which were then used to transform B. subtilis ISW1214 cells.

Sequencing was done by the dideoxy chain termination method, using fluorescent terminators and an automated DNA sequencer (model 377A; Perkin-Elmer).27

Nucleotide sequence accession number. The original nucleotide sequence data reported in this paper have been submitted to the DDBJ/EMBL/GenBank with the accession number AB008763.

Results and Discussion

Prediction of targeting site of thermostability

Figure 1 shows the comparison of a partial alignment of amino acid sequences of α-amylases from different sources. The amino acid residues of BAA, BSA, and BLA corresponding to Arg124 in LAMY were all proline. A refined three-dimensional structure of BLA26 has been solved by X-ray crystallographic analysis, and overall folding patterns of BAA, BSA, and BLA are thought to closely resemble each other. According to the structure of BLA, the position of Arg124 of LAMY appears to be on a loop in domain B. We chose the Arg124 residue in LAMY to examine the effects of proline substitution on thermostability and catalytic activity.

Purification and physicochemical properties of LAMY and mutant enzymes

The Arg124 residues of wild-type LAMY and ΔRG-LAMY were each replaced by proline by site-directed mutagenesis, and the resultant mutant enzymes were designated R124P-LAMY and ΔRG-R124P-LAMY, respectively. ΔRG-LAMY is a thermostabilized mutant of LAMY that has acquired strong capture of calcium ion by deletion of Arg181-Gly182.27 The mutated genes were PCR-amplified, digested with Sal I, and subcloned into the SalI-Smal site of pHS64, which was then introduced into B. subtilis ISW1214 cells. The transformed B. subtilis cells were grown in an optimized medium6 to hyperproduce the engineered proteins, R124P-LAMY and ΔRG-R124P-LAMY, both at a level corresponding to approximately 2×10^6 U/liter (approximately 0.4 g/liter) of the medium after incubation at 30°C for 3 days. Highly purified preparations of LAMY and its mutant enzymes were obtained by the simple purification procedure (yields approximately 70% for all) by HPLC (Bio-Cad; Perseptive Biosystems). Molecular masses of the wild-type and mutant enzymes were electrophoretically identical (53 kDa for all on SDS-PAGE). The N-terminal amino acid sequence of R124P-LAMY was HHNGTNQTMQYFREW, which was identical to the sequence of the wild-type enzyme.9 When amyloytic activities toward soluble starch and amylase (DP-17) were compared between the wild-type and mutant enzymes, no significant differences appeared in specific activities, catalytic parameters (Table1), pH-activity curves, or degree of inhibition by chelating reagents (data not shown). These results suggested that no extensive conformational changes of the LAMY structure, especially around the active site, were induced by the proline substitution. However, there remains a possibility that the mutant enzymes endure the decreased flexibility caused by the substitution of the side chain of Arg124 for a pyrrolidine ring of proline.

Increase of thermostability by proline substitution

To examine whether replacement of Arg124 with proline increases the stability of LAMY, the thermal stability of the mutant enzymes was assessed by incubating

| LAMY | 113 | GTENWAVEVESARNQNQRIESGETTIEAWTK |
| BAA | 109 | ATEVTVAVEVSPHRQRQSTQVEQIVQWTD |
| BSA | 112 | GTENWAVEVESARNQNNRQIESGETTIEQMTK |
| BLA | 111 | ATEVTVAVEVSPHRQRQSTQVEQIVQWTD |

Fig. 1. Partial Alignment of the Amino Acid Sequences of α-Amylases from Different Sources.

Amino acid sequences of liquefying α-amylases from Bacillus amylogeiquefaciens, Bacillus steatorrhophilus, Bacilluslicheniformis, and Bacillus sp. strain KSM-1378 are labeled by BAA, BSA, BLA, and LAMY, respectively. Each numbering starts from the N-terminal amino acid of the respective mature enzyme. β-Sheet structures are shaded. Mutated position is boxed.
the enzymes at various temperature in 50 mM Tris-HCl buffer pH 8.5 for 10 min with and without 0.1 mM calcium ion. The thermal stability curves of proline-substituted mutants, R124P-LAMY and ΔRG-R124P-LAMY, were compared with those of wild-type LAMY and ΔRG-LAMY. As shown in Fig. 2, the proline substitution caused an increase in thermal stability. The temperature at which the enzyme was 50% inactivated in 10 min (t_m) in the absence of calcium ion was estimated to be 47.4°C, 51.6°C, 57.9°C, and 62.5°C for wild-type LAMY, R124P-LAMY, ΔRG-LAMY and ΔRG-R124P-LAMY, respectively. The stability effect of the double mutation of R124P and ΔRG was cumulative, since the difference in t_m value between ΔRG-LAMY and ΔRG-R124P-LAMY was similar to that between wild-type LAMY and R124P-LAMY. In the presence of 0.1 mM calcium ion, t_m values of wild-type LAMY, R124P-LAMY, ΔRG-LAMY, and ΔRG-R124P-LAMY were 58.9°C, 62.5°C, 74.3°C, and 79.4°C, respectively.

The thermostability of amylase involves two distinct steps, a reversible unfolding step and subsequent irreversible changes of the enzyme molecule, as proposed by Tomazic and Klabinov. They also reported that temperature optima of native and engineered thermophilic enzymes are generally higher than mesophilic enzymes, possibly due to the stiff structures which keep folded at high temperature compared with mesophilic enzymes. In this study, the optimum temperatures were found to be around 52°C for wild-type LAMY and 54°C for the proline-replaced mutant, as calculated by the second approximation from 45°C to 60°C (Fig. 3). The slight increase in optimum temperature observed here by the proline substitution could be the consequence of the mutant enzyme keeping folded at higher temperatures than wild-type LAMY. By contrast, as described in our previous paper, wild-type LAMY and ΔRG-LAMY had the same temperature optimum. This would be the result if the mutation did not protect the protein against reversible unfolding, as has been reported for BAA and its thermostabilized enzymes.

The denaturation processes of wild-type and mutant

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**Table 1.** Catalytic Properties of Wild-type and Mutant LAMYS.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-type</th>
<th>ΔRG-LAMY</th>
<th>R124P-LAMY</th>
<th>ΔRG-R124P-LAMY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity (U/mg protein)</td>
<td>5009</td>
<td>4848</td>
<td>5280</td>
<td>3952</td>
</tr>
<tr>
<td>K_m (mg/ml)</td>
<td>4.35</td>
<td>3.21</td>
<td>4.06</td>
<td>4.12</td>
</tr>
<tr>
<td>V_max (μmol/min/mg protein)</td>
<td>4.41</td>
<td>4.05</td>
<td>4.21</td>
<td>3.64</td>
</tr>
</tbody>
</table>

*a* Specific activities were measured at 50°C with soluble starch as substrate.

*b* Kinetic parameters were calculated by Lineweaver-Burk plots using amylose DP-17, as described in Material and Methods.

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**Fig. 2.** Effects of Temperature on the Stability of Wild-type LAMY (●), R124P-LAMY (○), ΔRG-LAMY (●), and ΔRG-R124P-LAMY (○).

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 CaCl₂, and assayed for the activity remaining. The activity of each enzyme after treatment at 30°C was taken as 100%.

**Fig. 3.** Effects of Temperature on the Activity of Wild-type LAMY (●) and R124P-LAMY (○).

The reactions were done at the indicated temperatures for 5 min and at pH 8.5 in 50 mM Tris-HCl buffer. The values are shown as percentages of the maximum activity of each enzyme, which is taken as 100%.
Amylase Thermostabilized by Proline Substitution

![Graph showing time courses of fluorescence changes observed for wild-type LAMY (○) and R124P-LAMY (●).](image)

Each enzyme (10 μg/ml) was incubated at 50°C in 50 mM Tris-HCl (pH 8.5) buffer. Fluorescence strength of wild-type LAMY at 30°C in the same buffer was taken as 100%.

LAMY were followed fluorometrically. As shown in Fig. 4, fluorescence of R124P-LAMY decreased more slowly than that of wild-type LAMY. Half lives at 50°C of the wild-type enzyme and the mutant enzyme were 4.4 min and 11.3 min, respectively. This indicates that the steric stability of R124P-LAMY was greatly improved. The fluorescence intensity of both enzymes did not return to the original level after cooling, even if calcium ion was added. This suggests that the denaturation by heating observed here was not reversible. In addition, the denaturation slope of wild-type LAMY was biphasic, suggesting that several modification processes might occur in the enzyme molecules. The proline substitution improved denaturation rate only in the first phase (from 0 min to 6 min in Fig. 4), in which rapid conformational changes by protein unfolding would occur. In the second phase (from subsequent 7 min to 15 min), denaturation rates of wild-type LAMY were almost the same as that of R124P-LAMY, in which irreversible processes might occur. Therefore, denaturation of R124P-LAMY at 50°C seems to occur in a monophasic fashion. Similar phenomena were also reported by Declerck et al.\(^{29}\) in their fluorescence study on thermoinactivation of BLA and its thermostabilized mutants. Thermostabilizing mutation at His133 and Ala209 of BLA was shown to decrease the denaturation rate only in the first phase.

Proline residues are known to restrict backbone bond rotation because of their pyrrolidine rings. Suzuki et al.\(^{30}\) proposed a “proline rule” which states that many prolines at second position of β-turn make proteins more thermostable. Matthews et al.\(^{31}\) improved the thermostability of T4 lysozyme by replacing Ala87 at one of its β-turn with a proline residue, so as to decrease the backbone entropy of unfolding. Similar effects of single-site proline substitution have been found for human lysozyme,\(^{32}\) Bacillus stearothermophilus neutral protease,\(^{33}\) Bacillus sp. alkaline serine protease,\(^{34}\) barley β-amylase,\(^{35}\) and Aspergillus awamori glucoamylase.\(^{36}\) Watanabe et al.\(^{37}\) found that the thermostability of a Bacillus cereus oligo-1,6-glucosidase can be cumulatively increased by substituting proline residues for nine critical residues of this enzyme; Lys121, Glu208, and Glu290 on the second site of β-turn, Asn109, Glu175, and Thr 261 on the α-helix, and Glu216, Glu270, and Glu378 on the coils within loops.

According to the homology alignment of LAMY with BLA,\(^{38}\) Arg124 is presumed to be located on a loop connecting between a two-stranded antiparallel β-sheet involving residues at amino acids from 113 to 121 and from 135 to 142. Although it was reported that the thermal stability of a B. cereus oligo-1,6-glucosidase was effectively improved by proline substitution at the second position of β-turn and the first turn of α-helix,\(^{39}\) no information was given about the positions of β-turn in the BLA structure.\(^{40}\) The change of Arg124 to proline is assumed to increase structural stiffness of the LAMY molecule by decreasing flexibility of the possible β-turn in the loop.

We have constructed a thermostable LAMY by combination of two different mechanisms; one is loop stabilization by proline substitution and another is an increase of calcium binding by specific amino acid deletion. We are now acquiring the analyzed thermostability of mutant proteins by computer-aided structure modeling. The results will shortly be published elsewhere.

References
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