Investigation on the Effects of Three X→Histidine Replacements on Thermostability of α-Amylase from *Bacillus amyloliquefaciens*

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*Bacillus licheniformis* α-amylase (BLA), a thermophilic counterpart of *Bacillus amyloliquefaciens* α-amylase (BAA), is an appropriate model for the design of stabilizing mutations in BAA. BLA has 10 more histidines than BAA. Considering this prominent difference, in the present study, three out of these positions (I34, Q67, and P407; located in the thermostability determinant 1 region and Ca-III binding site of BAA) were replaced with histidine in BAA, using the site-directed mutagenesis technique. The results showed that the thermostability of P407H and Q67H mutants had increased, but no significant changes were observed in their kinetic parameters compared to that of the wild type. I34H replacement resulted in complete loss of enzyme activity. Moreover, fluorescence and circular dichroism data indicated a more rigid structure for the P407H variant compared with that of the wild-type BAA. However, the flexibility of Q67H and I34H mutants increased in comparison with that of wild-type enzyme.

**Keywords:** *Bacillus amyloliquefaciens* α-amylase, histidine, site-directed mutagenesis, thermal stability

α-Amylase (α-1,4-α-glucan glucanohydrolase; E.C. 3.2.1.1) cleaves α-1,4-glycosidic linkages in starch, glycogen, and the related polysaccharides and produces oligosaccharides of a relatively low molecular weight [43]. α-Amylases are produced by animals, plants, fungi, and bacteria. However, the industrial interest for *Bacillus* α-amylases, owing to their high thermostability, is more than other α-amylases. In particular, α-amylases from species such as *Bacillus amyloliquefaciens* α-amylase (BAA) and *Bacillus licheniformis* α-amylase (BLA) have been widely studied in both academic and industrial laboratories [3, 11, 13, 26, 36, 39]. These enzymes are known to have common properties. For example, BAA and BLA structures are formed by the three distinct A, B, and C domains. Domain A is composed of an (α/β)₈ barrel and the N-terminus as well as the active site. Domain B is almost exclusively created by β-strands, indicating the highest structural variability [24, 25]. Domain C folds into a Greek key motif. Calcium ion is needed for preserving the structural integrity of α-amylases. In addition, the amino acid sequence of BLA is approximately 80% identical to that of BAA [44]. Despite the similarity of their amino acid sequences and structures, these enzymes exhibit different stabilities toward heat; the half-life of BLA is more than 100-fold longer than that of BAA at 90°C [42]. Several mutational approaches have been fulfilled to determine the amino acid residues responsible for the thermostability of *Bacillus* α-amylases [8, 12, 19, 29, 38, 40]. Moreover, the comparisons of homologous enzymes having different thermostabilities provide a special opportunity for determining the thermal adaptation strategies [17]. BLA, a thermophilic counterpart of BAA, is a suitable model to design the stabilizing mutations in BAA. BLA uses a combination of mechanisms to reach its superior thermostability. After resolving the crystal structure of BAA, two other hypotheses were suggested to explain the causes of unusual thermostability of BLA compared with BAA [1]. One of these hypotheses proposed that BLA contains 10 more histidine residues than BAA and these additional histidine residues in the structure of BLA may be associated with the thermostability of the protein. Accordingly, in the present study, three residues located in important regions were separately replaced with histidine in BAA; then, the effects of these mutations were investigated. Conrad *et al.* [10] proposed four regions, containing thermostability determinants (TSDs).
BLA and BAA differ from each other regarding several amino acid residues, five of which are histidines in the BLA, in the TSD regions. We investigated two out of them located in the TSD1. In addition to TSD regions, Ca\(^{2+}\) binding sites also play an important role in the thermostability of \(\alpha\)-amylases [24]. BAA and BLA contain three common Ca\(^{2+}\) binding sites [5, 33, 41], but BAA has one more Ca\(^{2+}\) binding site than BLA [1]. Interestingly, there is only one amino acid difference for BAA compared with the BLA in the common Ca\(^{2+}\) binding sites. This amino acid is Pro407 in BAA, which is replaced by histidine in BLA. We, therefore, replaced these three amino acids in BAA with corresponding histidine in BLA. Two of these substitutions (I34H and Q67H) are located in the TSD1 region and the other (P407H) is located at the third common Ca\(^{2+}\) binding site in BAA and BLA. The effects of these mutations on the structural and kinetic parameters of the enzyme were then assessed.

**Materials and Methods**

**Materials**

The growth media and reagents were purchased from Liofichem (Rosteto degli Abruzzi, Italy). The genomic DNA extraction kit was obtained from R-real Biotech Corporation (Banqiao, Taiwan). The oligonucleotide sequences were synthesized by MWG Biotech-AG (Roseto degli Abruzzi, Italy). The restriction enzymes, T4 ligase, DpnI, Isopropyl-\(β\)-D-thiogalactopyranoside (IPTG), and PCR reagents were purchased from Fermentas Life Sciences (Vilnius, Lithuania). The genomic DNA extraction kit was obtained from Amersham Biosciences (Piscataway, USA). All the other chemicals were obtained from Merck (Darmstadt, Germany).

**Bacterial Strains, Plasmid, and Growth Conditions**

* Bacillus amyloliquefaciens* (ATCC 23350) was purchased from the Industrial Fungus and Bacteria Center of Iran and used as the source of the \(\alpha\)-amylase gene. *Escherichia coli* XL1 Blue and plasmid pET21a (Novagen, USA) were used as the hosts and expression vectors for cloning the \(\alpha\)-amylase gene, respectively. *Escherichia coli* BL21 (DE3; Stragatene) was used for the expression of wild type and engineered \(\alpha\)-amylases. All the strains were grown in Luria–Bertani (LB) medium at 37°C.

**Cloning of \(\alpha\)-Amylase Gene from B. amyloliquefaciens**

The genomic DNA was isolated from BAA. To introduce vectors for cloning the pET21a (Novagen, USA) were used as the hosts and expression vectors. The \(\alpha\)-amylase gene, the BAA peptide-encoding sequence, the BAA NotI restriction sites (shown underlined) and remove the signal binding site than BLA [1]. Interestingly, there is only one amino acid difference for BAA compared with the BLA in the common Ca\(^{2+}\) binding sites. This amino acid is Pro407 in BAA, which is replaced by histidine in BLA. We, therefore, replaced these three amino acids in BAA with corresponding histidine in BLA. Two of these substitutions (I34H and Q67H) are located in the TSD1 region and the other (P407H) is located at the third common Ca\(^{2+}\) binding site in BAA and BLA. The effects of these mutations on the structural and kinetic parameters of the enzyme were then assessed.

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**Expression and Purification of Wild-Type and Mutant Enzymes**

The plasmids harboring wild-type and mutant \(\alpha\)-amylase genes were transformed into BL21 competent cells, grown in LB medium containing ampicillin (100 \(\mu\)g/ml) at 37°C, and then induced by IPTG [21, 22]. The pellet was resuspended in the lysis buffer (100 mM NaCl, 50 mM Tris-HCl, 2 mM imidazole, and 1 mM PMSF; pH 7.5). After sonication and centrifugation, the inclusion bodies pellet was resuspended in denaturing buffer (300 mM NaCl, 50 mM Tris-HCl, 2 mM imidazole, and 6 M urea; pH 8) for 1–2 h at 25°C. The extract was centrifuged and the supernatant was subjected to dialysis in a buffer containing 50 mM NaCl, 50 mM Tris-HCl, 2 mM imidazole, and 5 mM CaCl\(_2\); (pH 7.5). The refolded proteins were then loaded onto a Ni-NTA agarose chromatography column. The purity of proteins was confirmed by SDS-PAGE according to the method of Laemmli [27]. Protein concentrations were estimated by the Bradford method [4].

**Enzyme Assay and Kinetic Characterization**

The enzyme activity was measured based on the procedure described by Miller [32]. One unit of the enzyme activity was defined as the amount of enzyme that released 1 \(\mu\)mol of reducing sugars per minute under the assay conditions. Assay reactions were initiated by adding 20 \(\mu\)l of enzyme into 480 \(\mu\)l of various starch concentrations dissolved in a solution containing 5 mM CaCl\(_2\); (pH 7.5). After incubation at 37°C for 10 min, the reaction was stopped by adding 500 \(\mu\)l of DNS reagent. The \(k_\text{m}\) and \(k_\text{cat}\) values were then calculated through fitting the initial rates as a function of substrate concentration to the Michaelis–Menten equation.

**Thermal Inactivation of BAA and Mutants**

The effect of temperature on enzyme activity was evaluated in 50 mM Tris-HCl and 5 mM CaCl\(_2\); (pH 7.5) at three temperatures of 70°C, 75°C, and 80°C. Then, at the time intervals of 0, 10, 20, 45, 60, 80, 100, 120, 150, 180, 210 min, the samples were removed and cooled on ice. The residual activities were eventually determined as described previously. In assay solution, the final concentrations of the enzyme were 10 \(\mu\)g ml\(^{-1}\). The activity of the enzyme at the interval zero was considered the control (100% activity).
Chemical Stability Measurements
The unfolding profiles of wild-type BAA and its mutants (I34H, Q67H, and P407H) were determined by intrinsic fluorescence measurements. The wild-type and mutant enzymes were unfolded using different concentrations of guanidine hydrochloride (Gdn-HCl) in 50 mM Tris–HCl and 5 mM CaCl$_2$ buffer (pH 7.5) at room temperature for 6 h. The unfolding of the variants of BAA in the presence of Gdn-HCl was independent of the protein concentration.

Fluorescence Analysis
Intrinsic protein fluorescence spectra of the samples were measured at 25°C using a Perkin Elmer LS50B spectrophotometer. The excitation wavelength was set at 280 nm and the emission spectra were then recorded between 300 and 400 nm in a 10-mm path-length quartz cuvette. All the experiments were carried out at 25°C in 50 mM Tris buffer (pH 7). The protein concentration was the same in all the samples (20 µg/ml).

Dynamic Quenching
Fluorescence quenching was carried out by adding different concentrations of acrylamide to protein solutions (final concentration = 0.02 mg/ml) at an excitation wavelength of 280 nm and an emission wavelength of 344 nm in a Perkin Elmer LS50B spectrophotometer. The quenching data were analyzed regarding the emission wavelength of 344 nm in a Perkin Elmer LS50B spectrofluorometer. The quenching data were analyzed according to the Stern–Volmer constant, $K_{SV}$, which was calculated from the ratio of the fluorescence intensities in the absence and presence of the quencher, $F_0/F$, using the equation $F_0/F = 1 + K_{SV}[Q]$. $Q$ is the molar concentration of the quencher [25]. The intrinsic protein fluorescence $F$ was corrected for the acrylamide inner filter effect $f$, defined as shown in the following equation,

$$f = 10^{-0.05}$$

The extinction coefficient $ε$ for acrylamide at 280 nm was 4.3 M$^{-1}$ cm$^{-1}$.

Circular Dichroism Measurements
Circular dichroism (CD) experiments were performed on an Avivo model of 215 CD spectropolarimeter (USA) in 50 mM borate and 5 mM CaCl$_2$ buffer (pH 7.5). Far-UV CD (200–250 nm) was monitored using a cell with 2-mm path-length with an enzyme concentration of 0.2 mg/ml. The results are expressed as molar ellipticity $[θ]$ (deg cm$^2$ dmol$^{-1}$) based on a mean amino acid residue weight (MWR) assuming average weights of 110. The molar ellipticity $[θ]$ was calculated from the formula $[θ] = (ε100MWR)/(cθl)$, where $c$ is the protein concentration in mg/ml, $l$ the light path-length in centimeters, and $θ$ the measured ellipticity in degrees at wavelength $λ$.

RESULTS
Evaluation of the Kinetic Parameters for Wild-Type and Mutant Enzymes
A 1.45 kb α-amylase gene fragment from *Bacillus amyloliquefaciens* was amplified and cloned into the expression vector pET-21a. The resulting plasmid was used as the template for mutagenesis. For IPTG-induced gene expression, the plasmids containing wild-type or any other mutated α-amylase genes were transformed into *E. coli* BL21 cells. SDS-PAGE result revealed a predominant band corresponding to the molecular mass of approximately 58 kDa in the crude extracts of the recombinant cells. In the next step, refolding of insoluble inclusion bodies into bioactive forms was performed. We used specific activity to compare the refolding efficiency of enzyme variants [14]. Since the specific activity of all variants is almost the same, it is assumed that the refolding yield of all enzyme variants is similar (Table 1). The one-step purification procedure using a Ni-NTA agarose chromatography column resulted in a protein yield of 95% for wild-type and mutant enzymes. Only the SDS-PAGE profile of wild type is shown in supplementary data, Fig. S1. The kinetic constants of the wild-type and mutant α-amylases were obtained using Michaelis–Menten and Lineweaver–Burk curves of these variants (supplementary data, Fig. S2). As shown in Table 1, no significant changes in the kinetic constants between wild type and mutants were observed, except I34H, of which its activity was completely missed.

Thermal Stability of BAA and Its Mutants
As shown in Fig. 1, the thermal inactivation pattern of Q67H mutant was almost unchanged compared with the wild-type enzyme after incubation at 80°C. After 35 min, the initial activity of the Q67H mutant and wild-type enzymes was reduced by almost 50%, meaning that their half-life could be 30 min. The half-life of P407H mutant was 56 min under the same conditions. Fifty percent of the initial activity of the wild type enzyme was retained after 125 min of incubation at 75°C. Furthermore, the half-life of Q67H and P407H mutants at 75°C was 145 and 185 min, respectively. Seventy-five percent of the initial activity of P407H, Q67H, and the wild type enzyme was

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity (U/mg)</th>
<th>$k_{on}$ (s$^{-1}$)</th>
<th>$K_m$ (mg/ml)</th>
<th>$k_{on}/K_m$ (ml mg$^{-1}$ s$^{-1}$)</th>
<th>$K_m$ (M$^{-1}$)</th>
<th>$t_{1/2}$ at 75°C (min)</th>
<th>$t_{90}$ at 80°C (min)</th>
<th>$C_m$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>183±7</td>
<td>162.3±5</td>
<td>3.6±0.3</td>
<td>45.1</td>
<td>3.3±0.1</td>
<td>125±3</td>
<td>34±1</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>P407H</td>
<td>196±4</td>
<td>168.3±3</td>
<td>3.8±0.2</td>
<td>44.3</td>
<td>3.1±0.1</td>
<td>185±6</td>
<td>57±3</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>Q67H</td>
<td>175±3</td>
<td>154.4±2</td>
<td>3.9±0.5</td>
<td>39.6</td>
<td>3.4±0.1</td>
<td>145±5</td>
<td>36±2</td>
<td>1.6±0.2</td>
</tr>
<tr>
<td>I34H</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>3.9±0.2</td>
<td>-</td>
<td>-</td>
<td>1.1±0.3</td>
</tr>
</tbody>
</table>
retained after 185, 125, and 80 min of incubation at 70°C, respectively. These data showed that the thermal stability of P407H and Q67H mutants had partly enhanced in relation to the wild type.

Gdn-HCl-Induced Denaturation of BAA and Its Mutants

The unfolding of the wild-type BAA and its mutants, as a function of Gdn-HCl concentration, was followed by monitoring fluorescence emission at 344 nm, which decreased as the denaturant concentration increased (Fig. 2). Apparent midpoint concentrations of unfolding ($C_{m}$; the denaturant concentration at which the fractions of folded and unfolded protein are equal) for all variants are given in Table 1. Our results demonstrated that P407H mutant was more resistant to the chemical denaturant compared with the wild-type enzyme. In contrast, the stability of Q67H and I34H mutants decreased against the chemical denaturant.

Fluorescence Intensity

As shown in Fig. 3, an increase in fluorescence intensity can be observed upon replacement of Pro 407 by His (curves 1 and 2). This result indicates that less solvent is exposed to Trp and Tyr residues in the P407H enzyme variant. However, the intrinsic fluorescence of the enzyme decreased in I34H and Q67H variants (curves 3 and 4). These results can suggest that upon replacement of Ile 34 and Gln 67 to His, some changes in the structure of the enzyme variants containing I34H and Q67H result in exposure of the buried aromatic residues to the solvent.
These findings may reflect the reduction of compactness in the structure as the consequential effect of such mutations.

**Dynamic Quenching by Acrylamide**

The results of quenching experiments allowed us to assess the relative solvent exposure of different types of fluorophores [15]. Fig. 4 shows the Stern–Volmer plots for acrylamide quenching of the wild-type and mutant forms of BAA. The quenching constants ($K_{SV}$ values) for all variants were calculated and the results are given in Table 1. It is evident that the I34H mutant can be effectively quenched by acrylamide, which indicates a higher accessibility of aromatic residues to the quencher, meaning that the structure of I34H mutant is more flexible. $K_{SV}$ values for Q67H mutant increased slightly compared with the wild type enzyme, whereas this value for P407H mutant decreased. These results also indicated that the structural flexibility of P407H mutant decreased and the flexibility of Q67H mutant increased slightly in relation to the wild-type $\alpha$-amylase.

**Circular Dichroism**

The far-UV CD spectra of $\alpha$-amylases showed a considerable increase in negative ellipticity upon replacement of Pro 407 by His (Fig. 5). In addition, the intensity of the negative ellipticity of the wild-type enzyme was clearly greater than those of Q67H and I34H. We believe that this finding supports the notion of enhanced flexibility of the structure of Q67H and I34H enzyme variants, which was also obvious in the intrinsic fluorescence experiment. In addition, the CD data indicate a more rigid structure for the P407H mutant compared with the wild-type $\alpha$-amylase.

**DISCUSSION**

Our previous structural studies emphasized the 10 extra histidines in the structure of BLA in comparison with BAA and demonstrated that this property may be related more or less directly to the stability of the protein [1].
the present study, we separately replaced three points in BAA with corresponding histidine in BLA and assessed the effects of these replacements on the protein stability. On the other hand, it has been demonstrated that the deamidation of glutamine and asparagine residues is mainly responsible for \(\alpha\)-amylases inactivation at high temperatures [42]. Therefore, we supposed that the substitution of histidine with glutamine at position 67 in the TSD1 region might improve the thermostability of BAA. In addition, we studied the effects of histidine replacement for isoleucine at residue 34 in the TSD1 region. The mutagenic studies of other amylases have also indicated that the residues involved in metal binding are very critical for the thermostability [31, 37]. There is only one amino acid difference between BLA and BAA in the regions involved in the common calcium-binding sites. This amino acid at the Ca-III binding site of BAA is Pro407, which corresponds to histidine in BLA. Accordingly, we decided to replace the Pro407 residue in BAA with histidine.

**I34H variant.** Previous studies have demonstrated that TSD1 correlates with the alpha-amylase’s substrate specificity [10]. The total loss of activity observed for the I34H mutant may be due to the conformational changes of substrate binding-site residues. As shown in Fig. 6A, Ile34 is located within a hydrophobic environment. It is possible that substitution of histidine for isoleucine in position 34 leads to a bulky imidazol group protruding into the substrate-binding cavity. Furthermore, Ile34 can play an important role in formation of this site. These changes are not readily explainable, but an unsuitable conformational change is possible.

**Q67H variant.** Structural analysis of Q67H mutant showed that the flexibility of this variant increased compared with the wild type. However, the thermostability of the mentioned mutant was significantly increased as compared with the wild type. The extreme thermal stability of proteins is widely believed to arise from an increased conformational rigidity in the native state, but this is not always true. For example, some mutagenic studies have shown that despite the substantially increased thermal stability of mutant proteins, their rigidity is equal to or lesser than that of wild type [2, 6, 9, 18, 28, 30, 34]. Lazaridis et al. [28] point out that flexibility cannot, in itself, be offered as a reason for decreased stability since increased flexibility implies increased conformational entropy in the native state and should therefore be stabilizing. As the entropy difference between the native and the denatured states decreases, the folded protein becomes more stable [2, 34]. On the other hand, there have been several reports that thermophilic proteins have a lower frequency of deaminating residues (Asn and Gln) compared with mesophilic proteins, especially in the exposed state [7, 35]. As shown in Fig. 6B, Gln67 lies in a surface turn. In Q67H mutant, glutamine was substituted by histidine; therefore, a possible further stabilization of Q67H variant against thermal inactivation may be attainable through the glutamine replacement, of which its potential deamidation likely leads to the noticeable disruption of the enzyme structure.

**P407H variant.** A significant change in thermostability of the BAA was observed upon conversion of proline to histidine at position 407. The surrounding environment of Pro407 showed that the distance between this residue and His406 is almost 4.3 Å (Fig. 6C). We suggest that the imidazole ring of the His407 in the P407H variant may be stacked against the ring of His406 [23], and this compactness may reduce the His406 solvent accessibility. This change in the surface of hydrophobicity content and the placement of the hydrophobic constriction rings into the nonpolar core may affect the rigidity of P407H variant.
Furthermore, it is possible that these conformational changes positively affect the Ca-III binding site and, therefore, improve the stability of the enzyme.

In summary, mutagenesis data partially supported our hypothesis that extra histidines may play a role in improving BLA thermal stability. This hypothesis was corroborated by the P407H and Q67H mutations, which significantly improve the thermal stability of BAA. These results really encourage us to study other histidine replacements and combinational mutations in more details. In the present study, we only investigated one feature hypothesized to contribute toward BAA thermostability. This mechanism is somewhat responsible for the decreased difference between the thermal stability of BLA and BAA (P407H mutant). The simultaneous study of this mechanism with other known mechanisms might better explain the causes of the difference in thermostability between BAA and BLA.

REFERENCES


