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A Highly Active Alpha Amylase from *Bacillus licheniformis*: Directed Evolution, Enzyme Characterization and Structural Analysis

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The stability of *Bacillus licheniformis* alpha-amylase (BLA) under acid condition was enhanced through direct evolution using the error-prone polymerase chain reaction. One beneficial mutation site, H281I, was obtained in BLA. The specific activity of H281I was 161/352 U/mg, which was 62.6/27.5% higher than that of the wild-type (WT) (99/276 U/mg) at pH 4.5/6.5 and 95°C. The pH optimum for H281I was decreased about 1 unit, whereas no significant changes of optimum temperature and thermostability were observed compared with the wild type (WT). The k_{cat}/K_m value of H281I was 1.7-/1.4-fold higher at pH 4.5/6.5, respectively, than that of WT. The structure model analysis indicated that the H281I mutation altered the predicted interaction between the amino acid residues at 281 and 273, thus creating a conducive local environment for substrate binding, as reflected by its decreased K_m, and consequently increased the specific activity.

Keywords: Direct evolution, *Bacillus licheniformis*, alpha-amylase, characterization, specific activity, structure model

Introduction

Alpha-amylase (E.C. 3.2.1.1. 1.4-α-D-glucan glucanohydrolases), representing 30% of the world's enzyme market, is a starch-hydrolyzing enzyme with important industrial applications [6]. Thermostable *Bacillus licheniformis* alpha-amylase (BLA) is widely used in industrial processes such as starch processing, brewing, sugar production, baking, and detergents because of its good thermal stability [3]. Under industrial starch liquefaction conditions, industrially used BLA currently functions at pH 6.0 and above (optimum pH 6.0–7.0) and it becomes unstable below pH 6.0 [1]. However, the natural pH of starch slurry is 4.5, and thus adjustment of the pH is required for liquefaction. After liquefaction, the pH has to adjust back to pH 4.2–4.5 for the saccharification process [11]. Thus, it is desirable to develop a BLA variant capable of accommodating the acidic conditions in the process of converting starch to sugars. It is expected that the use of engineered acid-stable BLA in various starch processing industrial sectors should cut production costs and benefit the environment.

In recent years, protein engineering techniques have been successfully applied to improve the properties of alpha-amylases by introducing mutations to the coding gene. For instance, directed evolution and site-directed mutagenesis have been applied to improve alpha-amylase acid stability [7, 15], the product specificity [13], and to increase specific enzyme activity [12, 20].

In this study, a novel BLA mutant, H281I, was obtained by screening the BLA error-prone PCR mutant library. The

catalytic efficiency, enzymatic properties, and kinetic parameters of H281I were investigated. The potential mechanism underlying the improved enzymatic activities of H281I at both pH 4.5 and 6.5 was studied by three-dimensional structure analysis.

Materials and Methods

Bacterial Strains and Plasmids

Escherichia coli BL21 (DE3) and plasmid pET-22b (+) were preserved in our laboratory. *E. coli* BL21 (DE3) was used as the host for BLA expression. All bacteria were cultivated at 37°C in Luria–Bertani (LB) medium (Bactotryptone 10 g/l, yeast extract 5 g/l, and NaCl 10 g/l). Ampicillin (100 μg/ml) was added to the growth medium when necessary.

Mutant Library Construction by Error-Prone PCR

The amylase gene of *B. licheniformis* CICC 10181 was used as the template for the construction of a mutant library. A set of primers, N-terminal primer (5′-cgcggatccggcaaatcttaatgggacgct-3′) and C-terminal primer (5′-cccaagctttctttgaacataaattgaaacc-3′), was used to incorporate *Bam*HI and *Hin*dIII restriction sites (underlined), respectively. The mutagenic buffer (100 μl) was prepared, which contained 0.2 mM dATP and dGTP, 1 mM dCTP and dTTP, 3 mM $MgCl_2$, 0.1 mM $MnCl_2$, 10 ng of template, 10× buffer (Tiangen, Hai Dian District, Beijing, China) and 5 units (U) of Taq polymerase (Tiangen). The PCR profile was 30 sec at 95°C, 30 sec at 55°C, and 90 sec at 72°C for 30 cycles, and a final 72°C extension step for 10 min. The PCR products were purified and digested with *Bam*HI and *Hin*dIII and then subcloned into the *Bam*HI-*Hin*dIII site of pET-22b(+) with the 6-His tag at the C terminus for purification. The plasmids were then transformed into competent *E. coli* BL21 through electroporation. The transformed *E. coli* BL21 was spread on LB medium containing ampicillin (100 μg/ml) and grown overnight at 37°C. An amount of 15% sterile glycerol was then added. The culture was then mixed and stored frozen at −80°C to serve as the BLA random mutagenesis library.

Screening of Mutant Library

Colonies from LB-agar plates with ampicillin were picked into 96-well plates containing 200 μl of LB supplemented with 100 μg/ml ampicillin and were grown at 37°C until the OD_{600} value reached 0.6. This was followed by the addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mmol/l to induce the expression of recombinant enzymes. The BLA was directed into the periplasmic space by using *pelB* in *E. coil* BL21(DE3). After incubation at 16°C for 16 h, one freeze-thaw cycle (−80°C, 15 min; 37°C, 15 min; 0°C, 15 min) followed by centrifugation (1,000 ×*g*, 10 min, 4°C) yielded the enzyme-containing supernatant. The supernatant was inoculated into replica 96-well plates, followed by dilution in sodium acetate buffer solution (100 mM, pH 4.5) containing 0.5% (w/v) soluble starch. These plates were used for the initial activity assay.

Protein Expression and Purification of the Wild Type (WT) and Mutant

Recombinant *E. coli* BL21 (DE3) carrying the plasmid pET-*amy* or pET-*amyM* was cultivated at 37°C in LB medium supplemented with 100 μg ampicillin/ml overnight, and then 1 ml of the culture was re-inoculated into 100 ml of LB media. When the culture reached an OD_{600} of 0.6–0.8, IPTG was added at a final concentration of 0.1 mM, and the cells were cultivated at 16°C with shaking at 80 rpm for 24 h. The culture supernatant was harvested by centrifugation at 4°C and 2,500 ×*g* for 10 min. After disrupting the cells by ultrasonication, the lysates were centrifuged at 2,500 ×*g* at 4°C for 20 min. The samples were loaded onto a Ni-NTA agarose gel column equilibrated with PBS buffer (50 mM sodium phosphate, 0.5 mM NaCl, pH 7.4). The enzymes were eluted by a linear imidazole gradient (10–400 mM) in the same PBS buffer. The purity and molecular mass of the proteins were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% polyacrylamide).

Enzyme Activity Assay

One unit of amylase was defined as the amount of amylase needed to complete the liquefaction of 1 mg of starch into dextrin per minute at 70°C and pH 6.0. Amylase activity was measured by the Chinese Industrial Standard (QB/T 2306-97). Twenty milliliters of substrate solution (20 mg/ml starch in H_2O) and 5 ml sodium phosphate buffer (pH 6.0) were added to a test tube and maintained for 5 min at 70°C. Subsequently, 1 ml of appropriately diluted enzyme solution was added to the substrate solution, incubating for 5 min at 70°C. Then 1 ml of this mixture was added to a test tube with 0.5 ml of stopping reagent (0.1 M HCl) and 5 ml of iodine-iodide solution, which was prepared fresh by adding 20 g of potassium iodide and 2.0 ml of stock solution (11 g iodine and 22 g potassium iodide/500 ml water) to 500 ml of water. The intensity of the blue color (absorbance at 660 nm), which is characteristic for the substrate-iodine complex, was measured using the 10 mm colorimetric utensil in a spectrometer. The enzyme activity was calculated by the following formula: Activity (unit/ml) = c × n × 16.67, where c is the concentration of the control enzyme (unit/ml) corresponding to the absorbance, which can be obtained from the appendix of the Standard (QB/T 2306-97), and n is the dilution factor of the enzyme. The protein concentration was measured by the Bradford method (Bio-Rad Laboratories, USA), using bovine serum albumin as a standard.

Determination of Enzymatic Properties and Kinetic Parameters of the WT and Mutant

For the thermostability of WT and H281I, purified enzymes were tested by incubating the enzymes, without substrate, for different times (20, 40, 60, 80, 100, 120 min) in 100 mM sodium

phosphate buffer (pH 6.5) at 95°C. After cooling the treated enzyme samples on ice for 10 min, the residual activity was determined based on the Chinese Industrial Standard (QB/T 2306-97). The original activities of WT and H281I without preincubation were defined as 100%, respectively.

For the pH stability of WT and H281I, purified enzymes were incubated for different times (10, 20, 30, 40, 50, 60 min) in 100 mM sodium acetate buffer (pH 4.5) without the substrate at 70°C. After cooling the treated enzyme samples on ice for 10 min, the residual activity was determined based on the Chinese Industrial Standard (QB/T 2306-97). The original activities of WT and H281I without preincubation were defined as 100%, respectively.

To determine the pH profile of the purified WT and H281I, the enzymatic activity was measured at 70°C using the 100 mM sodium acetate buffer (pH 4–6), 100 mM sodium phosphate buffer (pH 6–8), of 100 mM sodium carbonate buffer (pH 9–10), with the substrate. The activity of alpha-amylase was determined as described by Chinese Industrial Standard (QB/T 2306-97).

The kinetic parameters of WT and mutant BLA were measured by a modified dinitrosalicylic acid method [2]. Amylase assays were performed using soluble starch as substrates of different concentrations, from 1 to 20 mg/ml in different pH buffers (pH 4.5 and 6.5) at 70°C. Data were fitted to the Michaelis–Menten equation using GraphPad Prism ver. 5.0 (GraphPad Software, San Diego, CA, USA) to generate estimates of K_m and k_{cat} values. The k_{cat}/K_m was calculated for both WT and mutant, and the results were compared.

Three-Dimensional Structural Analysis

The three-dimensional models of the WT and mutant were generated using a public website, Swiss-Model server (http://swissmodel.expasy.org/), using the BLA (1VJS pdb entry) as a template [4]. All structural figures were generated with PyMOL (http://www.pymol.org).

Results and Discussion

Screening and Selection from a Mutant Library

An error-prone PCR mutagenesis library containing 5,500 clones was subjected to screening to identify mutants with increased acid tolerance. A mutant exhibiting the highest amylase activity at pH 4.5 was selected and the target gene sequenced. Analysis of the selected mutant BLA gene sequence revealed that one mutation resulted in substitution of Ile for His at position 281.

Expression and Purification of the WT and Mutant BLAs in *E. coli* BL21 (DE3)

Expression of the WT and H281I BLAs in *E. coli* BL21 (DE3) was induced with 1 mM IPTG at 16°C for 24 h, and the respective amylase activity of WT and H281I was detected in the supernatant of the culture broth. The WT and H281I were purified by Ni-NTA agarose gel column equilibrated with the binding buffer, and their concentrations and purity were determined by the Bradford method and SDS-PAGE, respectively. SDS-PAGE analysis showed that the target protein has a molecular mass of about 53 kDa, in agreement with the expected BLA size (data not shown).

Characterization of Enzymatic Properties of WT and H281I

The optimal temperature for the H281I mutant was found to be around 95°C, quite similar to that for the WT (data not shown). The specific activities of WT and H281I at various pHs were measured using soluble starch as the substrate, respectively. As shown in Fig. 1A, the WT had an optimum activity at pH 6.5 and was active over the pH range 5.5–8.0, whereas the optimum pH for H281I was pH 5.5 and it was active over the pH range 5.0–8.5.

The heat stability of the enzymes at pH 6.5 was assayed by measurement of the residual activities at 95°C for different time points. The purified WT and H281I retained approximately 61% of their initial activity after incubation for 60 min at pH 6.5 and 95°C (Fig. 1B). Thermal denaturation assay showed that there was no significant change in temperature stability of H281I compared with WT at 95°C and pH 6.5.

The acid stability of the enzyme was examined at 70°C and pH 4.5 for various times in 100 mM sodium acetate buffer. After incubation at pH 4.5 and 70°C for 30 min, the activity of WT declined rapidly, and only 19.5% of enzyme activity was retained. Compared with WT, 30.7% of the amylase activity could be detected for H281I. The results demonstrated that H281I exhibited improved acid stability (Fig. 1C).

Specific Activity of the WT and H281I

The specific activities of WT and H281I were determined at 95°C and pH 4.5/6.5. The specific activity of H281I was 161/352 U/mg at pH 4.5/6.5 and 95°C, which was 62.6%/27.5% higher than that of the WT (99/276 U/mg), suggesting that the substitution of Ile for His significantly improved the specific activity of alpha amylase at pH 4.5/6.5.

Kinetic Characterization of the WT and H281I

The WT and H281I were purified to homogeneity and used to measure their kinetic properties. Table 1 shows the kinetic parameters of WT and H281I. Kinetic property analyses showed that the k_{cat} of WT and H281I at pH 4.5/6.5 was approximately equal, respectively. Compared with WT, the H281I showed a lower K_m, and therefore, increased

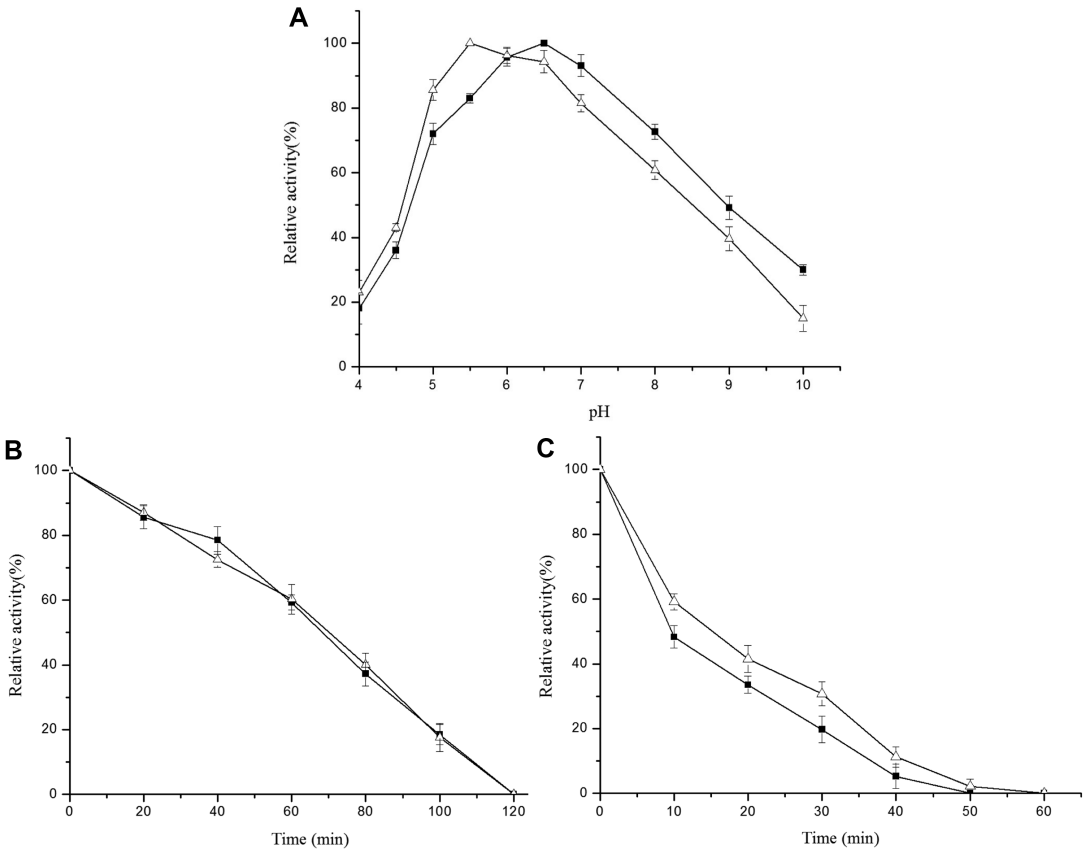

Fig. 1. Characterization of purified BLA.

(**A**) Effects of pH on the specific activity of the purified enzymes. To determine the pH profile of the purified WT and H281I, the enzymatic activity was measured at 70°C using the 100 mM sodium acetate buffer (pH 4–6), 100 mM sodium phosphate buffer (pH 6–8), or 100 mM sodium carbonate buffer (pH 9–10) with the substrate. The activity of alpha-amylase was determined as described by Chinese Industrial Standard (QB/T 2306-97). (■) WT and (△) H281I in different pHs (p <0.05). (**B**) Thermostability of enzymes. For the thermostability of WT and H281I, purified enzymes were tested by incubating the enzymes, without substrate, for different times (20, 40, 60, 80, 100, 120 min) in 100 mM sodium phosphate buffer (pH 6.5) at 95°C. After cooling the treated enzyme samples on ice for 10 min, the residual activity was determined based on the Chinese Industrial Standard (QB/T 2306-97). The original activities of WT and H281I without preincubation were defined as 100%, respectively. Recombinant BLAs: (■) WT and (△) H281I (p <0.05). (**C**) Acid-stability of enzymes. For the pH stability of WT and H281I, purified enzymes were incubated for different times (10, 20, 30, 40, 50, 60 min) in 100 mM sodium acetate buffer without the substrate (70°C) at pH 4.5. After cooling the treated enzyme samples on ice for 10 min, the residual activity was determined based on the Chinese Industrial Standard (QB/T 2306-97). The original activities of WT and H281I without preincubation were defined as 100%, respectively. Recombinant BLAs: (■) WT and (△) H281I (p <0.05).

Table 1. Comparison of the kinetics of WT and H281I BLAs.

Enzyme	4.5			6.5		
	k_{cat} (s^{-1})	K_m (mg/ml)	k_{cat}/K_m (ml mg^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mg/ml)	k_{cat}/K_m (ml mg^{-1} s^{-1})
WT	67 ± 0.4	14.6 ± 0.6	4.6	195 ± 5.1	11.8 ± 0.7	16.5
H281I	73 ± 0.6	9.5 ± 0.3	7.7	204 ± 4.2	8.6 ± 0.8	23.7

Enzyme reactions (n = 3) were conducted at 70°C, pH 4.5 and 6.5, using soluble starch as the substrate (p < 0.05).

affinity for starch. At pH 4.5/6.5 and 70°C, the k_{cat}/K_m of H281 was about 1.7-/1.4-fold higher than that of the WT, due to the decreased K_m value of H281I for soluble starch, demonstrating that H281I has improved catalytic efficiency.

In this study, we discovered that the k_{cat}/K_m value of H281I was increased at both pH 4.5 and 6.5. Meanwhile, compared with WT, H281I exhibited increased specific activity at pH 4.5 and pH 6.5. Therefore, we reasoned that the mechanism for the improved tolerance to acidity of H281I and our previously reported BLA mutants is likely to be different.

Comparative Structural Analysis of the WT and H281I

The three-dimensional models for the WT and H281I were generated using the public website Swiss-Model. The overall topology of BLA is typical of alpha-amylases, with three domains called A, B, C. The central A domain contains a $(\alpha/\beta)_8$-barrel (TIM barrel), which forms the core of the enzyme – the active sites, and the N-terminus [4,8]. BLA interacts with polymeric substrates (starch) *via* multiple binding sites on the enzyme surface, such as His105, Asp231, His235, Glu261, His289, and Asp328.

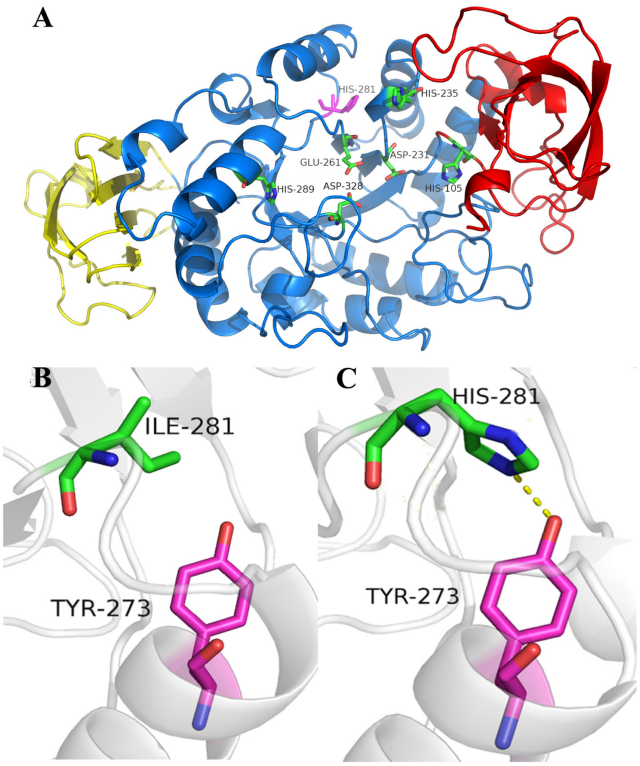

Fig. 2. Model structure of BLA and the expanded view of 281 and its surroundings.

(**A**) The three-dimensional structural model of BLA: domain A (red), domain B (blue), and domain C (yellow). (**B**) The models of the amino acid of 281 and its surroundings. The amino acid of 281 is Ile. (**C**) The models of the amino acid of 281 and its surroundings. The amino acid of 281 is His. The dotted lines indicate a hydrogen-bonding network around the residue at the position 281.

These sites, which are believed to play important roles in adsorption and hydrolysis of starch, not only contains three catalytically active residues (Asp231, Glu261, and Asp328), but also includes two substrate binding sites (His105 and His235) [8] (Fig. 2A). The His281 site is located between the fifth α-helix and the sixth β-strand of domain A, and is predicted to form a hydrogen bond with Try at position 273. The stability of BLA and the ionization state of its catalytic residues, which depend on the pKa values, have a fundamental role in maintaining enzymatic activity in acidic conditions [14]. Several previous studies have shown that intramolecular weak interactions, such as hydrophobic interactions, electrostatic effects, and hydrogen binding, play a critical role in stabilizing protein structures and altering the pKa values of the catalytic residues [17]. H281I mutation resulted in the loss of its hydrogen bonding with Try273. Although substitution of Ile for His at position 281 resulted in the loss of one hydrogen bond (Figs. 2B and 2C), the introduction of a hydrophobic side chain at this interior site to replace a hydrophilic one allows the formation of new hydrophobic interactions with the neighboring amino acids, namely Leu230 and Val260. As residue Val260 is close to the catalytic residue Glu261, the substitution reinforces the stabilization of the catalytic residue of H281I, resulting in increased stability of BLA activity in low pH. It has been established that decreasing hydrophobic effects can lead to a reduction of the pKa of Asp [5]. In the proposed catalytic mechanism [9, 16], the catalytic nucleophile, Asp231, must remain deprotonated in the first step of catalysis, and activity in acidic conditions is determined by the catalytic nucleophile. Thus, mutant H281I exhibits a shift of the acidic limb to more acidic values.

It was previously found that the mutations near the catalytic residues most likely influenced the catalytic mechanism [10, 19]. The catalytic efficiency of H281I, as indicated by its k_{cat}/K_m, was 1.7-/1.4-fold higher than that of WT at pH 4.5/6.5. The substitution led to a decrease in K_m, indicating a possible increase in relative affinity of H281I for the substrate, presumably owing to the changing spatial position, the size of the amino acid, and electrostatic effects. The refined structures of the WT and H281I were analyzed by comparing the distance of the residues targeted for site-directed mutagenesis to the catalytic triad of the active site residues (Asp231, Glu261, Asp328), the two substrate binding site residues (His105, His235) and His289 before and after mutation (Table 2). The variations in the refined structures of BLA could be characterized based on the changed distance between these residues. The H281I showed an increased distance of 0.13 Å and 0.68 Å

Table 2. Structural analysis data of the WT and H281I.

Asp231 (Å)		Glu261 (Å)		Asp328 (Å)	
WT[a]	H281I[b]	WT	H281I	WT	H281I
12.29	12.29	11.65	11.88	16.17	16.30
His105 (Å)		His235 (Å)		His289 (Å)	
WT	H281I	WT	H281I	WT	H281I
16.22	16.35	14.04	14.72	20.20	20.48

Asp231, Glu261, and Asp328 are the three active site residues of BLA.
His105 and His235 are the two substrate binding site residues of BLA.

[a]Distance between the WT and active site/substrate binding residues (*e.g.*, distance from His281 to Asp231 = 12.29).

[b]Distance between the H281I and active site/substrate binding residues (*e.g.*, distance from His281 to Asp231 = 12.29).

between Ile281 and substrate binding site residues (His105, His235). Moreover, basic amino acids with a positive charge might reduce the substrate affinity [18]; therefore, the replacement of histidine with a non-polar amino acid (Ile) resulted in increased substrate affinity. These results indicate that substitution of Ile for His might increase the distance between the target residue and the substrate binding site residues and alter the electrostatic effects, creating a potentially favorable environment in substrate binding site regions to interact with the substrate.

In conclusion, these results indicate that the substitution of Ile for His at position 281 has a beneficial effect on BLA in terms of substrate affinity and specific activity. It is expected that the introduction of additional mutations targeting the other amino acid residues near the active site regions could further enhance the BLA stability and activity under lower pH conditions.

Acknowledgments

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