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The Error-prone PCR of α-amylase from *Bacillus amyloliquefaciens* Toward Enhanced Acid Tolerance and Higher Specific Activity

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In order to improve acid tolerance of *Bacillus amyloliquefaciens* α-amylase, error-prone PCR was used to randomize mutagenesis of the α-amylase gene from *Bacillus amyloliquefaciens*, and a mutant G300H was selected. The α-amylase activity of the mutant G300H (1,887 U/mg) was 9.6% higher than that of the wild type (WT) (1,722 U/mg). At pH 4.0, the α-amylase activity of the mutant G300H and the WT were 804 and 522 U/mg, respectively; this finding suggested that the acid tolerance of α-amylase G300H was 54.0% higher than that of the WT. The residual α-amylase activities of the mutant G300H and WT were 684 and 206 U/mg, respectively, after treatment at 70°C for 5 min, suggesting that the thermostability of α-amylase G300H was 2.3× higher than that of the WT. The *K*m of the α-amylase and the WT were 5.033 and 5.63 mg/mL, respectively, which indicated that the catalyzing efficiency of G300H was improved.

Key words: Acid tolerance; α-amylase; *Bacillus amyloliquefaciens*; Error-prone PCR.

The α-amylases (EC 3.2.1.1; α-[1,4]-D-glucan glucanohydrolase) are extracellular enzymes that randomly cleave the α-1,4 glycosidic bonds between adjacent glucose units in the linear amylose chain, generating dextrin and reducing sugar[1-3]. These α-amylases, which comprise approximately 25% of the enzyme market, are widely distributed among various plants, animals, and microorganisms[4, 5]. Although these enzymes can be derived from many plants and animals, those from microorganisms are used extensively for industrial demands[6]. *Bacillus* sp. in particular are widely used in industry.

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Mesophilic α-amylase, significant industrial enzymes, play major roles in the grain processing, food, brewing, fermentation, textile, medicine, and other industries. *Bacillus amyloliquefaciens* α-amylase (BAA) is one of the most ideal mesophilic α-amylases due to its high enzymatic activity and stability[7, 8]. However, the application of BAA is limited by its loss of hydrolysis ability in acidic environments. The pH value in several industrial processes is lower than the optimum pH of BAA, such as starch hydrolyzation, brewing and feed additives. As a result, significant raw material and process operating costs are needed for large-scale pH adjustments[9]. Therefore, it was extremely important and imperative to enhance the acid tolerance of BAA

Studies on starch-degrading strains examine the fermentation conditions, enzymatic

properties, cloning, and expression of thermostable α-amylase. Hence, screening and improving strains using new technology and/or methods to produce α-amylase could be useful and meet production demands. Directed evolution is a common genetic modification method, and particularly error-prone PCR[10] is a hot spot due to its wide applicability and powerful function. Rabbani *et al*.,[5] obtained three *Bacillus subtilis* mutants of α-amylase using error-prone PCR, which revealed that their specific activity significantly improved. Bessler *et al*.,[11] obtained a mutant of *B. amyloliquefaciens* α-amylase (BAA) using error-prone PCR and found that its specific activity was improved under high pH conditions. Therefore, error-prone PCR, the directed evolution skill that is most widely applied, can change enzyme stability, affinity, and characteristics.

Many studies on acid tolerance α-amylase have been conducted to date. Cai *et al*.,[12] established the *Bacillus licheniformis* α-amylase (BLA) mutant L134R/S320A via site-directed mutagenesis for acid-resistant capability. After modifying α-amylase from *Bacillus* sp. TS-25 using error-prone PCR, Jones *et al*.,[13] selected a mutant whose thermostability was improved under low pH conditions. Though error-prone PCR, Liu *et al*.,[14] obtained a BLA mutant, Thr353Ile/His400Arg, that exhibited stronger acid tolerance. In this study, the α-amylase gene from *B. amyloliquefaciens* was modified by error-prone PCR in vitro to explore an accurate, simple, and effective screening method for obtaining strains with enhanced acid tolerance.

MATERIALS AND METHODS

Material

The plasmid vector pET-32a, *Escherichia coli* strains BL21 (*DE3*), and engineering bacteria *E. coli* BL21 (*DE3*)-pET32a-amylase were all stored in the Sichuan Centre of Typical Cultures Collection. In our earlier work, a strain with high α-amylase activity was screened and identified as *B. amyloliquefaciens* by 16S rRNA molecular identification methods. The α-amylase gene was cloned and inserted into the multicloning site of the pET32a vector. *BamH* I, *Xho* I, *Taq* DNA polymerase, T4 ligase, dATP, dGTP, dCTP, dTTP, and Premixed Protein Marker were obtained from TaKaRa (Dalian, China). The plasmid extraction reagent and a glue recycling kit were purchased from OMEGABIO-TEK (USA). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was obtained from Merck (Germany). The PCR primers were designed by Primer Premier 5.0 and synthesized by Shanghai Invitrogen Biotechnology Co. Ltd (Shanghai). The chemicals used in this study were of analytical grade (Chengdu Changzheng Glass Co. Ltd, China).

METHODS

Construction of a mutant library using error-prone PCR. Plasmid pET-32a-amylase was extracted from the engineering bacteria *E. coli* BL21 (*DE3*)-pET32a-amylase by alkaline lysis as the template of the error-prone PCR. Two primers were used: 5' -CG*GGATCC*GTAAATGGCACGCT GATGCAGTA-3' and 5' -GGC*GAGCTC*TTATTT CTGAACATAAATGGAGACG-3' . The reaction system consisted of 12 μL of 25 mmol/L $MgCl_2$; 2, 4, 6, 8, or 10 μL of 5 mmol/L $MnCl_2$; 2 μL each of 10 mmol/L dATP and dGTP; 10 μL of 10 mmol/L dCTP and dTTP; 1 μL of TaKaRa *Taq* DNA polymerase; 2 μL of each primer (20 μmol/L); 2 μL of plasmid pET32a-amylase; and 10 μL of 10× *Taq* buffer; ddH_2O was added to create a final volume of 100 μL. PCR was carried out at 94°C for 45 s, 56°C for 30 s, and 72°C for 120 s for a total of 30 cycles, with further extension at 72°C at the end for 10 min. The amplified fragments were digested by *BamH* I and *Xho* I after DNA extraction and then cloned into the multi-cloning site of the pET32a vector. The plasmids were transformed into competent *E. coli* BL21 (*DE3*), then brushed on the Luria-Bertani (LB) medium (1% peptone, 0.5% yeast extract, 1% NaCl, 1% agar, 50 μg/mL ampicillin) and cultured at 37°C for 16 h. The clones were then transferred into 96-well plates.

High-throughput screening to enhance acid tolerance (96-well plate method)

The clones were cultivated in a 96-well plate with 200 μL of LB medium (1% peptone, 0.5% yeast extract, 1% NaCl, 50 μg/mL ampicillin) at 37°C until the value at OD_{600} reached 0.6–0.8. Then IPTG (final concentration 0.1 mmol/L) was added to each well and cultured the samples at 16°C overnight to produce recombinant protein. 8 μL of lysozyme (20 mg/mL) was added to each well and incubated the plates at 37°C for 1 h for lysis. The cell lysate was used to measure the enzyme activity. 250 μL

of soluble starch (0.5%, pH 4.0) was into each well of the second 96-well plate, maintained it for 10 min at incubation temperature, and then added 25 µL of the cell lysate to each well. After incubation at 40°C for 5 min, then 250 µL of the stopping reagent (0.1 mol/L H_2SO_4) was added to each well. After being mixed, 25 µL of this mixture was added to 250 µL of iodine solution in the third 96-well plate. Finally, the absorbance of the mixture was detected by a Microplate Spectrofluorometer (Molecular Devices Spectra Max Gemini XPS) at 620 nm. The WT α-amylase was used as a control. The mutant strains with higher activity compared with the WT strain were sequenced by Shanghai Invitrogen Biotechnology Co. Ltd, and all of the mutants were sequenced three times to confirm the mutation.

Protein purification and enzymatic activity detection

The mutant strain was cultured in 50 mL of LB medium (1% peptone, 0.5% yeast extract, 1% NaCl, and 50 µg/mL ampicillin) at 37°C until the value at OD_{600} reached 0.6–0.8. Then IPTG (final concentration, 0.1 mmol/L) was added to induce expression at 16°C overnight. The cells in 10 mL NaH_2PO_4-$C_6H_8O_7$ buffer (pH 6.0) were suspended after centrifugation at 15,000 × *g* for 10 min, crushed ultrasonically, and centrifuged again at 15,000 × *g* for 10 min. The supernatant was collected for protein purification, which was carried out using a BioLogic Duo Flow (BIO-RAD, USA). Enzyme purity was verified by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and its concentration was evaluated according to the Bradford method using Coomassie brilliant blue (CBB G-250) using bovine serum albumin as a standard protein[15]. The enzyme activity was measured based on the procedure described by Yoo YJ[16]. One unit of the enzyme activity was defined as the amount of enzyme that degraded l mg of starch under the assay conditions.

Analysis of Enzymatic Characterization

pH performance

The pH performance of α-amylase was determined at 40°C using three different buffers: 0.2 mol/L NaH_2PO_4-$C_6H_8O_7$ buffer for pH 3.0–6.0; 0.2 mol/L Na_2HPO_4-NaH_2PO_4 buffer for pH 7.0 and 8.0; and 0.1 mol/L Na_2CO_3-$NaHCO_3$ buffer for pH 9.0–11.0. The purified enzymes were diluted with each buffer at the different pH values and the enzyme was assayed using an improved version of Yoo YJ' s method[16].

Optimum temperature

The enzymatic activity was determined at various temperatures: 30°C, 40°C, 50°C, 60°C, 70°C, and 80°C based on the optimal pH.

Thermostability

The residual activities were eventually determined as described previously (40°C, pH 6.0) after heat treatment in an intelligent temperature metal bath (CHB-100, Hangzhou Bioer Technology Co. Ltd. China) at 70°C/min (0–5 min).

Kinetic characterization

The assay reactions were initiated by the addition of 500 µL of enzyme into 5 mL of various starch concentrations dissolved in a solution containing NaH_2PO_4-$C_6H_8O_7$ (pH 6.0). After incubation at 40°C for 5 min, the reaction was stopped by the addition of 5 mL of 0.1 mol/L H_2SO_4. According to the Lineweaver–Burk method[17], the *K*m and *V*max values were then calculated by fitting the initial rates as a function of substrate concentration to the Michaelis -Menten equation[18].

Salt bridges and hydrogen bond analyses

Salt bridge analyses of mutant and WT α-amylase have been performed by Visual Molecular Dynamics 1.8.7 with the cutoff distance of 4 Å. Hydrogen bond analyses were carried out by the DeepView-The Swiss-PdbViewer.

RESULTS

Error-prone PCR condition determination and mutation library screening

The PCR product yield was remarkably decreased when the Mn^{2+} concentration was >0.3 mmol/L. Five randomized mutation libraries were conducted with different Mn^{2+} concentrations (final concentrations, 0.1, 0.2, 0.3, 0.4, and 0.5 mmol/L), and then four clones from each library were randomly chosen for sequence analysis. It was found that the mutation frequency increased as the Mn^{2+} concentration increased; 2–4 bp mutations could be found, while there were one–three amino acid mutations at 0.3 mmol/L Mn^{2+}. Therefore, it was appropriate when Mn^{2+} concentrations were 0.3 mmol/L. Error-prone PCR was performed at 0.3 mmol/L Mn^{2+} and the mutation library was constructed. An acid-tolerant mutant was selected from the library using the 96-well plate

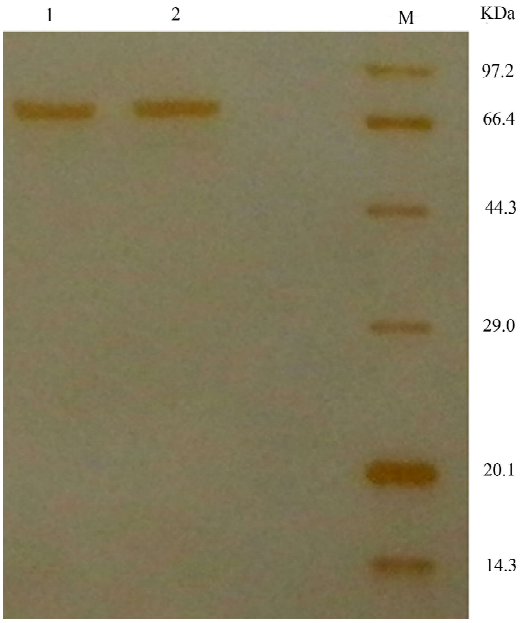

Fig. 1. SDS-PAGE and silver staining analysis of the purified protein

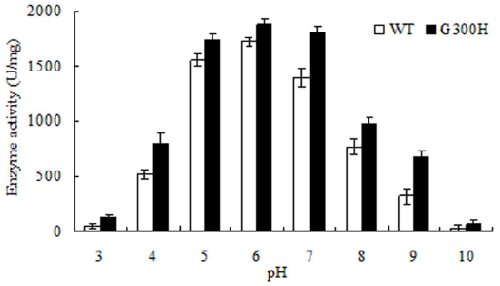

Fig. 2. The pH performance of wild type (WT) and mutant α-amylase

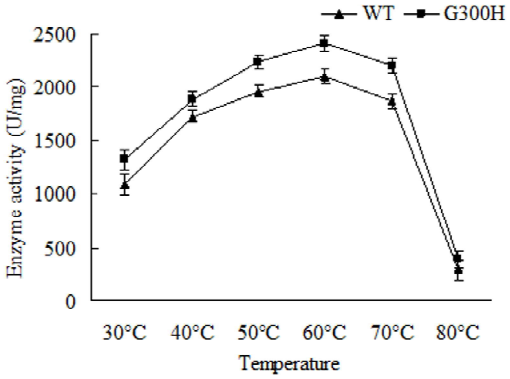

Fig. 3. The optimum temperature of wild type (WT) and mutant α-amylase

method. Sequence analysis was performed of one mutant that was named G300H in which glycine was changed to histidine in the 300th.

Purification of the protein and detection of enzymatic activity

The α-amylase was purified by BioLogic Duo Flow (Fig. 1). The WT and mutation α-amylase were approximately 72.5 kDa as expected. Figure 1 shows that the proteins were pure enough for the subsequent assay. The enzyme activities were measured based on the procedure described by Yoo YJ, the WT and G300H α-amylase activity were 1,722 and 1,887 U/mg, respectively. G300H showed 9.6% enzyme activity, higher than that of the WT.

Analysis of Enzymatic Characterization

pH performance

Both mutant and WT α-amylase were active at pH 3–10; in fact, the optimal pH of both was 6.0 (Fig. 2), and α-amylase activity of the mutant G300H was higher than that of the WT. At pH 4.0, the α-amylase activity of mutant G300H and WT were 804 and 522 U/mg, respectively; this finding suggested the acid tolerance of α-amylase G300H was 54.0% higher than that of the WT. Moreover, the α-amylase activities of the WT were 1,560, 1,772, and 1,394 U/mg at pH 5.0, 6.0, and 7.0, respectively. The activities of mutant G300H were 1,743, 1,887, and 1,812, U/mg at pH 5.0, 6.0, and 7.0, respectively. This finding suggested that the acid tolerances of α-amylase G300H were 11.8%, 9.6%, and 29.5% higher than those of the WT at pH 5.0, 6.0, and 7.0, respectively.

Optimum temperature

The optimum temperature was determined at the optimal pH of 6.0. The temperature curve of

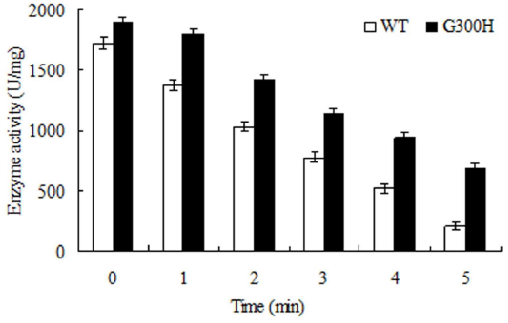

Fig. 4. The thermostability of wild type (WT) and mutant α-amylase

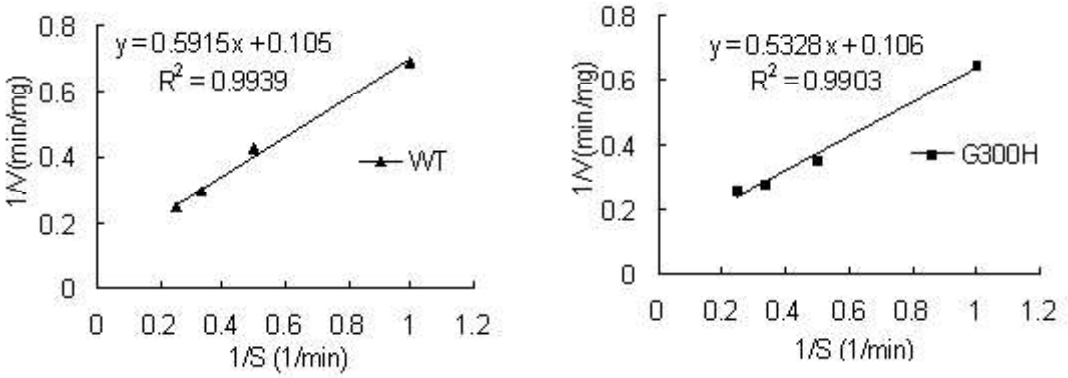

Fig. 5. The Lineweaver-Burk of the wild type (WT) plot and mutant G300H

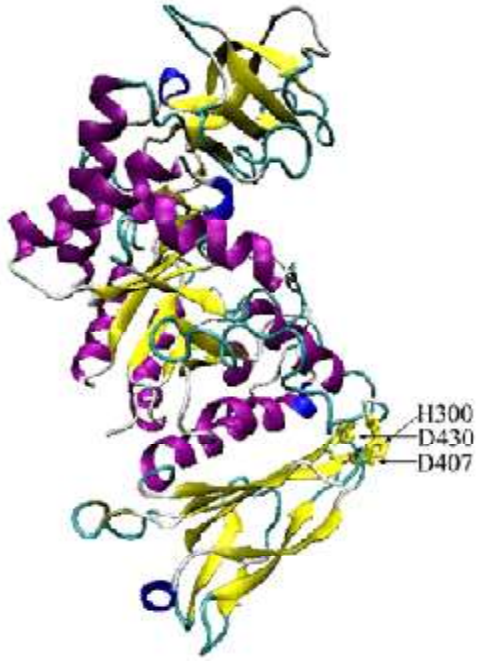

Fig. 6. Salt bridges analyses of the mutant.

the mutant was the same as that of the WT (Fig. 3). The optimal reaction temperature of the α-amylase was 60°C for both the mutant G300H and the WT, and >80% of the relative activity was retained at temperatures of 40–70°C. The activity decreased rapidly when the temperature was increased to 80°C.

Thermostability

At 70°C, the α-amylase activity gradually decreased over time. After intelligent temperature metal bath treatment for 5 min (Fig. 4), the residual α-amylase activities of mutant G300H and the WT were 684 and 206 U/mg, respectively. The enzymatic activity of the mutant G300H was 3.3× higher than that of the WT. This finding suggests that the thermostability of α-amylase G300H was 2.3× greater than that of the WT.

Kinetic characterization

At pH 6.0 and 40°C, using starch as the substrate and based on the Lineweaver–Burk method, the enzymatic activity and other parameters of the mutant G300H and WT enzymes

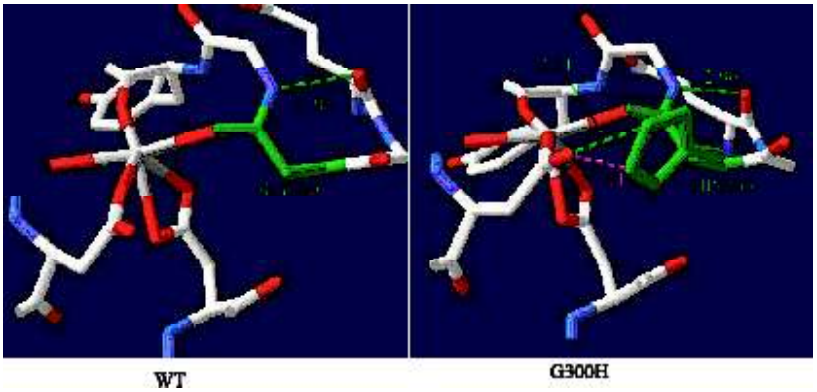

Fig. 7. The hydrogen bonds of Gly300 and His300

were calculated (Fig. 5). The kinetic parameters showed that the *K*m of the G300H α-amylase (5.033 mg/mL) was lower than that of WT (5.63 mg/mL), while the *V*max of the G300H and the WT α-amylase were 9.43 mg/min and 9.52 mg/min, respectively. This finding indicated increased G300H α-amylase affinity and enhanced catalytic efficiency.

Salt bridges and hydrogen bond analyses

Salt bridges analyses of the mutant and WT α-amylase revealed that the mutant α-amylase had two more salt bridges than the WT, Asp407-His300 (2.55 Å) and Asp430-His300 (4.63 Å) (Fig. 6), respectively. Hydrogen bond analyses of the mutant and WT α-amylase showed that the mutant α-amylase had two more hydrogen bonds than the WT α-amylase (Fig. 7).

DISCUSSION

The acid tolerant α-amylase was extensively used in starch hydrolyzation, brewing process and the feed. The mutant G300H obtained via error-prone PCR raised the acid tolerances by 54.0% and 11.8% at pH 4.0 and pH 5.0, respectively. It indicates that the mutant had significantly improved acid tolerance, and was very conducive for these fields.

It is report that the three-dimensional structure of BAA consists of three domains. Domain A is the central region folding into an $(\alpha/\beta)_8$ barrel and contains three active site residues Asp231, Glu261 and Asp328. Domain B, which is located between Aα3 and Aβ3, is created by β-strands. The C-terminal portion of the protein, which folds into a Greek-key motif, is named Domain C[19]. The previous research showed that Asp231 was the nucleophile, and Glu261 was hydrogen donor to glycosidic oxygen in the reaction mechanism[20,21]. The Asp328 was suggested to play an important role in the catalytic reaction by hydrogen bonding to the substrate and by elevating the pK_a of Glu261 [22]. The chemistry of the catalytic mechanism demands on that both the catalytic nucleophile (Asp231) and the proton donor (Glu261) should be in a particular protonation state. It was generally assumed that the pH-activity profile of the alpha amylases was determined by the titration of the catalytic nucleophile during pH 4.0 to pH 7.0, and affected by the titration of the catalytic proton donor during pH 7.0 to pH 10.0 (23-25). It was suggested that alpha amylase catalysis was limited at low pH conditions by the protonation of the nucleophile (Asp231). Moreover, the relationship between the pH and enzyme activity was determined by the pK_a values. Altering the electrostatic field, such as charged groups, was an important way of shifting the pK_a value of a residue. In the mutant of G300H, the residue of His with the imidazole group could be expected to bear positive charges than the residue of Gly which was a neutral amino acid. Although the positive charges were far from nucleophiles (Asp231), the interaction energy between a unit charge and titratable group was less dependent on distance. Hence, it could still stabilize the negative charge on Asp231 residue to stabilize its deprotonated form and thereby reduced its pK_a. Therefore, the mutant G300H would produce a shift of the acidic limb to more acidic values. Meanwhile, it could also stabilize the negative charge on the Glu261 residue and thereby decreased its pK_a, resulting in an acidic shift of the pH-activity profile.

Moreover, salt bridges and hydrogen bonds were related to protein stability. As the numbers of salt bridges and hydrogen bonds increased, the proteins became more stable[26-28]. Once a protein became more stable, it developed an improved ability to adapt to the external environment (acidic environment, thermal environment, etc.) In our study, the acid tolerance of G300H α-amylase was improved 54% compared to the WT, and the mutant had two more salt bridges and two more hydrogen bonds compared to the WT. It indicated that the improved acid tolerance may be due to the additional salt bridges and hydrogen bonds. 300th amino acid near the Domain C was located at the edge of Domain A; yet 407th and 430th amino acid near the Domain A were located at the edge of Domain C (Fig. 6). Compared with the WT, the substitution of Gly300 to His added two salt bridges (Asp407-His300 [2.55 Å], Asp430-His300 [4.63 Å]) and two hydrogen bonds (His300↔Asp407 [1.91 Å, 2.31 Å]) between domain A and C. Hence, the conformational rigidity between the Domain A and C was increased, and the protein stability was improved after the mutation occurred. Then the adaption of the acidic environment was also improved. Furthermore, according to the hydropathy index (Gly [-0.4], His

[-3.2]) [29], the surface hydrophilicity of mutant was increased, causing the improvement of the protein stability[14]. It was also conducive for improving acid tolerance.

Interestingly, the mutant of G300H improved thermostability. The amino acid sequences of BLA and BAA are approximately 80% identical. Although their amino acid sequences and protein structures are similar, their thermostabilities were widely different since that of BLA was much higher than that of BAA. Sequence alignment analysis showed that BLA contained 10 more histidine residues than BAA and that these additional histidine residues in the structure of BLA may be associated with protein thermostability[30]. It indicated that histidine played an important role in BLA thermostability. In this study, after the amino acid changed from glycine to histidine in the 300th, the thermostability was enhanced 2.3× after 5-min treatment at 70°C, which suggests that histidine plays an important role in BAA thermostability.

It provides a theoretical basis and the needed materials to improve acid tolerance in BAA by protein engineering. In a future study, we could try to screen new mutants whose acid tolerance is higher based on the mutant G300H using directed evolution or site-directed mutagenesis. We could also try to change the 300th amino acid to other amino acids to obtain a protein with higher stability and/or acid tolerance using site-directed mutagenesis. According to other reports on acid tolerance sites, we could also try to integrate multiple mutants to establish strains that are more suitable for food production.

ACKNOWLEDGEMENTS

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