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Improvement of the activity of L-asparaginase I improvement of the catalytic activity of L-asparaginase I from *Bacillus megaterium* H-1 by *in vitro* directed evolution

Xiaohong Lu, Juhua Chen, Linshu Jiao, Lei Zhong, Zhaoxin Lu, Chong Zhang, and Fengxia Lu*

College of Food Science and Technology, Nanjing Agriculture University, Xuanwu District, Weigang No. 1, Nanjing 210095, China

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Directed evolution methodologies have been used as promising strategies for improving the catalytic properties of many existing enzymes. In the presented work, this approach was applied to improve the enzyme activity of L-asparaginase I obtained from *Bacillus megaterium* H-1. After two rounds of error-prone polymerase chain reaction (epPCR) and two generations of sequential DNA shuffling, all of 5 different mutants showed a significant increase in the enzyme activity of L-asparaginase I, ranging from 6.27 to 22.78 IU/mL. Among these mutants, D-9B and DD-12G displayed the relatively high catalytic activity, which were 20.22-fold and 21.33-fold higher than the wild-type enzyme (WT), respectively. Furthermore, the catalytic efficiency (k_{cat}/K_m) of D-9B and DD-12G were also improved, which were 132.73 $min^{-1}mM^{-1}$ and 146.39 $min^{-1}mM^{-1}$, respectively, in comparison to that of WT (3.39 $min^{-1}mM^{-1}$). In addition, mutant DD-12G showed tolerance toward wider range of pH values and higher temperatures than its WT counterpart. Homology modeling of above two mutants reflected a reduction of hydrogen bonds and an introduction of flexible residues in the loops near the active catalytic site Thr15. These changes contributed to the flexibility of loops, which may lead to further enhancement in catalytic efficiency. Results also showed that approximately 88.5% (0.978 mg/kg) acrylamide could be removed from mutant DD-12G pre-treated fried potato chips. This study clearly shows that directional evolution methods can indeed be utilized to improve the activity of L-asparaginase, which could also provide research basis for future application in food industry.

[**Key words:** *Bacillus megaterium* H-1; L-Asparaginase; Directed evolution; Catalytic activity; Amino acid substitutions]

L-Asparaginase (L-asparagine amidohydrolase; EC 3.5.1.1) catalyzes L-asparagine conversion to ammonia and L-aspartic acid (1). L-Asparaginases from bacterial resources have been categorized type I and II isoenzymes according to the distribution and structure (2). Type II L-asparaginases have shown promising effects in therapy of childhood acute lymphoblastic leukemia and in inhibiting the proliferation occurred in breast cancer cells (3,4). Recently, it has been widely reported L-asparaginases (type I and II) could control the content of acrylamide of numerous deepfried or baked foodstuffs (5–8). Acrylamide, a carcinogenic compound to humans (1,9,10), is mainly generated via the Maillard reaction taking place between L-asparagine and the reducing sugar at high temperatures (11–13). An effective measure to minimize acrylamide in high-temperature processed foods is reducing L-asparagine levels from the raw materials through the addition of L-asparaginase (14,15). Other than effectively controlling the levels of acrylamide, the organoleptic properties and nutritional values of final products could also be retained by L-asparaginase treatment (14,16). Therefore, L-asparaginase has a great potential in controlling acrylamide levels of fried and baked foods within regulations. However, the poor activity and instability of reported L-asparaginases limit the large-scale usage of these enzymes (17,18). Hence, L-asparaginases with efficient catalytic activity and superior stability in high temperature are urgently requested in meeting the conditions of industrial food manufacturing.

Multiple protein engineering approaches are presently available to overcome the defects of natural enzymes and enhance their industrial performance (19,20). Among the molecular modifications of L-asparaginase, rational design was the most adopted strategy to improve the enzymatic properties (19,21–25). In essence, researchers use site-directed mutagenesis to probe molecular interactions and obtain mutant strains with increased catalytic activity and/or thermal stability (24–27). Commonly noticed and shared by the aforementioned studies, the site mutation that improves the enzyme's thermostability is often accompanied by a decrease in the overall enzyme activity (28,29). This flaw in performance enhancement of the engineered enzymes can be addressed by directed evolution (28,30). The *in vitro* directed evolution of enzymes is a promising strategy of protein engineering, and the modified proteins could obtain one or more desirable properties under little or no previous knowledge of crystal structure or the related catalytic mechanism (31,32). As far as we know, there is only one report on the directional evolution of L-asparaginase which aimed at improving the thermal stability (33). No earlier studies were undertaken to obtain mutants of L-asparaginase with enhanced activity through directed evolution.

* Corresponding author. Tel./fax: +86 25 84395963.
E-mail address: lufengxia@163.com (F. Lu).

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In our previous work, *Bacillus megaterium* H-1 as a novel L-asparaginase-producing bacteria was isolated from the soil (2) and L-asparaginase (type I and II) genes were cloned and expressed in *Escherichia coli* BL21(DE3). However, the enzymatic activity of wild-type L-asparaginase (type I) was only 1.02 IU/mL, restricting its effect in removing acrylamide from food materials. In this present work, the approach that combined error-prone PCR with DNA shuffling was pursued to obtain mutants with superior enzyme activity and catalytic efficiency. The obtained results could attest the effectiveness of directed evolution in improving the activity of L-asparaginase and more importantly, they help elucidating the influence and role of certain amino acid residues on the enzyme catalytic activity.

MATERIALS AND METHODS

Materials Plasmid pET-30a(+)-BmansA that harbored L-asparaginase I gene (*ansA*) from *B. megaterium* H-1 was preserved in our laboratory. The vector pET-30a(+) was obtained from Novagen (Darmstadt, Germany). *E. coli* BL21(DE3), FastPure Plasmid Mini Kit, FastPure Gel DNA Extraction Mini Kit, 2 × Taq Master Mix (Dye Plus), ClonExpress II One Step Cloning Kit were obtained from Vazyme (Nanjing, China). Modified Bradford Protein Assay Kit was purchased from Sangon Biotech (Shanghai, China). Restriction enzymes and Super Pfu DNA polymerase were obtained from Takara Biotechnology (Dalian, China). Kanamycin, isopropyl-β-D-thiogalactopyranoside (IPTG) and lysozyme were obtained from Solarbio (Beijing, China). L-Asparagine was purchased from Aladdin (Shanghai, China). All other chemicals were analytical grade.

Random mutagenesis and the recombinant library construction Plasmid pET-30a(+)-BmansA encoding 993 bp native *ansA* gene was served as the template for epPCR. Forward (F) and reverse (R) primers containing *EcoR* I and *Xho* I restriction sites (underlined) were designed for this amplification, respectively: F (5′-gctgatatcggatccgaattcATGTCGGTTTTGAAAAAACTATTATTG-3′); R (5′-gtggtggtgg tggtgctcgagTTAATTAATATCTTCTGCAATTGACGTT-3′).

The mutagenesis frequency could be adjusted to requested levels (three to five amino acid mutations per kb) through controlling the addition of Mg^{2+} and Mn^{2+} as reported previously (34). The error-prone PCR system (50 μL) contained 100 ng template, 1 mM dCTP/dTTP, 7 mM Mg^{2+}, 0.3 mM Mn^{2+}, 2 μL each primer (0.4 μM), 25 μL 2 × Taq PCR Mix, the rest was complemented with ddH_2O. The PCR condition was set as the followed: a denaturation step initiated at 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 60 s, and a final extension step carried out at 72°C for 7 min. The final PCR products were evaluated through a 1% agarose gel electrophoresis separation step and later purified by FastPure Gel DNA Extraction Mini Kit. The purified products were ligated into the previously digested expression vector pET-30a(+) (*EcoR* I and *Xho* I) by ClonExpress II One Step Cloning Kit. The recombinant products were transformed into *E. coli* BL21(DE3) by heat shock to construct the utilized random mutagenesis library.

DNA shuffling and the recombinant library construction Three mutants (X3-12D, X4-7E, X5-11G) with higher enzyme activity screened from the error-prone PCR mutant library were served as templates for further DNA shuffling. The *ansA* genes of the three mutants were cloned by Super Pfu DNA polymerase, and same concentration of mixed parental DNA fragments were subjected for DNase I digestion. The digestion conditions were: 0.004 U/μL DNase I digest at 37°C, 2.5 min. Termination of above reaction occurred by a 10-min heating at 90°C. The DNA fragmentation was evaluated using 2% agarose gel electrophoresis and the resulting 50–100 bp fragments were purified. The primer-free PCR reaction system was composed of 25 μL of 50–100 bp fragments (with final concentrations of 10 ng/μL) and 25 μL 2 × Taq PCR Mixture buffer. The PCR was carried out without adding oligonucleotide primers under the following conditions: a pre-degeneration step at 95°C for 3 min, a denaturation step at 95°C for 30 s, an annealing step at 56°C for 30 s, and an elongation step at 72°C for 1 min, after 40 cycles, an elongation step at 72°C for 7 min was pursued. The full-length *ansA* gene was obtained through resulted PCR products, as well as the above mentioned primers and conditions. The mutant *ansA* genes were ligated into a linearized pET-30a(+) expression vector. The recombinant products were transformed in *E. coli* BL21(DE3) to generate the DNA shuffling library.

High-throughput screening of mutants library The suggested procedure of Guo et al. (28) was adopted and modified slightly. In essence, single colonies were picked from the *E. coli* BL21(DE3) cells harboring recombinant plasmids on LB-kanamycin (50 μg/mL) plates and inoculated into individual wells on 96-well plates. Each well contained 600 μL LB broth which was supplemented with 50 μg/mL kanamycin. The plates were incubated at 37°C and 200 rpm for 12 h as the start culture. An *E. coli* BL21(DE3) clone harboring pET-30a(+)-BmansA was used as a positive control in the above studies. A 50 μL turbid broth from each well was transferred into 600 μL fresh LB-kanamycin broth on another 96-well plate and cultured at 37°C, 200 rpm. After 3.5 h of incubation, IPTG was added (100 μg/mL as a final concentration) to each well and the plate was kept at 16°C, 200 rpm for another 16 h. After a short centrifugation at 4500 × *g* for 5 min, harvested bacterial pellets in each well were re-suspended in 100 μL of lysozyme buffer (1.5 mg/mL). The plates were kept in water bath at 37°C for 30 min then froze at −70°C for 30 min and subsequently re-dissolved at 37°C for 20 min. The freeze/thaw cycle was repeated three times. After a 5-min centrifuging at 4500 × *g*, the supernatant was used as crude enzyme(s) for downstream testing. Mutants showing high enzyme activity were re-screened for confirmation utilizing shake-flask preparations.

The expression and purification of recombinant wide-type and mutant enzymes Recombinant strains expressing the native and mutant L-asparaginase were separately inoculated in 100 mL LB-kanamycin (50 μg/mL) broth and shaken at 37°C. The 100 μg/mL of IPTG was added into the culture when OD_{600} reached 0.6. The culture was kept at 16°C for another 16 h for protein expression. Harvested cells were first centrifuged at 8000 × *g* for 10 min and re-suspended in 50 mM phosphate buffered saline (PBS, pH 8.0) supplementing with 300 mM NaCl. The cells were disrupted using ultrasonication for 15 min at low temperature. After centrifuging at 10,000 × *g* for 0.5 h, the supernatant was used as a crude enzyme mixture, which was further purified by nickel-affinity chromatography. Before sample loading, the 2-mL nickel-charged Ni-NTA agarose column (Qiagen, Shanghai, China) was equilibrated with binding buffer (50 mM PBS, 300 mM NaCl, pH 8.0), and elution buffer (50 mM PBS, 300 mM NaCl and 100 mM imidazole, pH 8.0) was used to elute enzyme preparations by gradient elution. Imidazole was removed later by dialysis. All of the enzyme purifications were carried out at low temperature. Protein contents were determined using the Bradford method (35), while protein molecular mass was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) with 5% stacking gels and 12% separation gels.

Enzymatic activity assays The Nessler's reagent method (22) was adopted to determine L-asparaginase activity with slight modifications. The enzymatic reaction solution consisted of 100 μL of enzyme, 100 μL of 189 mM L-asparagine (substrate) and 700 μL of 50 mM PBS buffer (pH 8.0) and was incubated at 37°C for 20 min. Add 100 μL of 25% (v/v) trichloroacetic acid solution to terminate the enzymatic reaction. Then 40 μL of the above reaction supernatant coupled with 100 μL of Nessler's reagent were mixed with 860 μL of deionized water. After 5 min, ammonia release quantity was determined by OD_{436} at room temperature. One International Unit of L-asparaginase activity was defined as the amount of enzyme releasing 1 μmol of ammonia per minute at pH 8.0 under 37°C.

The effect of pH and temperature on enzymatic activity and stability Compared to wild-type enzyme, the optimum pH values of mutants were studied over the range of 5.0–10.0 at 37°C. We defined the highest activity of each variant as 100% to calculate the relative activity at each pH value. In terms of pH stability, each purified enzyme was subjected to 12-h pre-incubation in buffers of different pH values spanning the range 4.0–11.0 at 4°C. Enzyme activity at time = 0 h was defined as a 100% activity and the residual activity were compared to this initial activity.

The optimum temperature for enzymatic activity of each variant was monitored over a temperature range encompassing 20–50°C spectrum. The maximum activity of each variant obtained within the aforementioned range was designated as 100% in order to calculate the relative activity at each temperature point. To evaluate the thermostability of each mutant, purified enzymes were pre-incubated in a water bath ranging from 20°C to 60°C for 20 min before a 15-min ice bath process. Enzyme activity at time 0 was defined as a 100% activity for comparisons. Thermal denaturation curves of enzyme solutions (0.1 mg/mL) and the temperature of the midpoint of the transition (T_m) were monitored by the CD values at 220 nm in 50 mM PBS buffer (pH 8.0) (25).

Substrate specificity and kinetics parameters The substrate specificity of the wild-type (WT) and mutant enzymes was determined through measuring the relative enzymatic activity toward various substrates, including 200 mM L-asparagine, D-asparagine, L-glutamine, L-aspartic acid, D-aspartic acid, and L-glutamic acid. The K_m and V_{max} of purified variants were determined by changing the concentration of L-asparagine over a range encompassing 4–20 mM spectrum at the optimal pH and temperature value. Lineweaver–Burk plot was used to calculate the kinetics parameters K_m, V_{max} and k_{cat} (22).

Molecular modeling To explore the function of randomly introduced mutations in improving overall enzyme activity, three-dimensional structures of WT and mutant enzymes were constructed using the SWISS-MODEL server (http://swissmodel.expasy.org/) (36). The structural model of wide-type L-asparaginase I spanning amino acid sequences 5 through 330 was based on the crystal structure of L-asparaginase from *Thermococcus kodakarensis* (PDB ID: 5ot0.1.A) with 39.94% of sequence identity (37). The effect of interactions among amino acids side residues in WT and mutant variants on enzyme's secondary structure was predicted by PyMOL software (DeLano Scientific LLC, San Carlos, CA, USA).

Analysis of circular dichroism spectroscopy The circular dichroism spectroscopy analysis was applied to estimate changes of the secondary structure between WT and mutants (22,28). After dissolving the purified WT and variants in 50 mM PBS buffer solution (pH 8.0) and adjusting protein concentrations to 100 μg/mL, the scanning was performed under a wavelength spanning the 190–250 nm range. The 50 mM PBS buffer solution (pH 8.0) was used as a blank.

Food application of L-asparaginase I Fresh potatoes were cut into slices (thickness: 2.0 mm) and then rinsed with distilled water to remove attached starch particles from the surface. All the slices were equally divided into three groups and then immersed in (i) distilled water (control group), (ii) 6 IU/mL WT enzyme solution (WT group), and (iii) 6 IU/mL mutant enzyme DD-12G solution (mutant DD-12G group) at 37°C for 1 h. Pre-treated potato slices were then fried at 180°C for 5 min, followed with cooling and drying at room temperature. The extraction and detection of acrylamide in different groups of fried potato chips were performed according to the method of Zhang et al. (2).

For L-asparagine analysis, pre-treated potato slices were dried on the paper before frying. Six grams of potato slices samples was crushed and then extracted using 50 mL of 50% (v/v) ethanol solution in an ultrasonic bath for 0.5 h, and subsequently transferred to the suction filter. The filtrate was concentrated using a rotary evaporator and dissolved in 5 mL of 0.1 M HCl solution. Finally, the product was filtered through a 0.22 μm microporous membrane for further analysis.

The L-asparagine quantitative analysis of samples was determined by the liquid chromatography-tandem mass spectrometry method (LC–MS/MS) using an Eclipse Plus C18 (1.8 μm, 2.1 × 100 mm) column. The samples were eluted at a 0.3 mL/min flow rate with a mixture of 98% 0.05 M sodium acetate and 2% methanol (v/v). L-Asparagine precursor was detected at m/z = 133.2 in positive mode, and daughter ions were detected at m/z = 87.1 and 74.2 in the same mode.

RESULTS AND DISCUSSION

High-throughput screening of mutants library After two rounds of error-prone PCR, a mutant library containing 9680 colonies was constructed. Among them, three mutants (X3D12, X4E7 and X5G11) showed enhanced enzymatic activity (ranging from 6.27 to 9.94 IU/mL) (Table 1). To be noticed, the mutant X4E7 exhibited an 8.75-fold increase in the enzyme activity in comparison to WT counterpart. After sequence analysis, the results revealed that four amino acid mutations existed in mutant X4E7 and X5G11, while only two substitutions in mutant X3D12.

Three selected error-prone PCR mutants (X4E7, X3D12 and X5G11) were further subjected to templates for initiating DNA shuffling experiments. Through two sequential generations of DNA shuffling, a new mutant library containing 4488 colonies was constructed, and two variants (D-9B and DD-12G) were selected with a significant improvement in catalyzing L-asparagine among these colonies. The L-asparaginase activity of D-9B and DD-12G was 21.64 IU/mL and 22.78 IU/mL, respectively, approximately 20.22-fold and 21.33-fold higher than that of WT (1.02 IU/mL) (Table 1). After gene sequencing, results showed four and five amino acid substitutions happened in D-9B and DD-12G, respectively (Table 1). The obtained results suggest the efficiency of the implemented directed evolution strategy. This is obvious in the light of the more multiples increase in the enzymatic activity of the DD-12G clone (obtained in this study through directed evolution) in comparison to the previously reported L-asparaginases derived from *Bacillus subtilis* B11-06 (23) and *B. licheniformis* MTCC429 (24) through a rational design approach.

Purification of wide-type and mutant L-asparaginases The molecular weight of purified WT and mutant L-asparaginases was confirmed by SDS–PAGE. As in Fig. 1, both the mutant D-9B and DD-12G showed same protein bands with the WT enzyme at 42 kDa, which indicated the successful expression of mutants. The

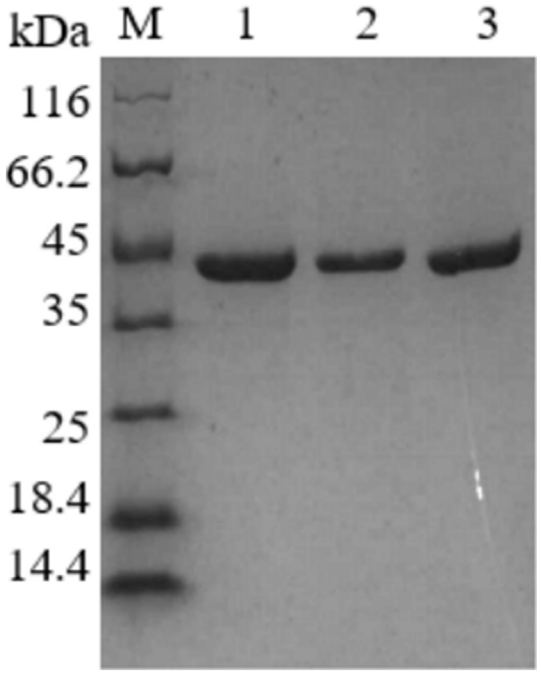

FIG. 1. SDS–PAGE analysis of purified wild-type L-asparaginase I and mutant variants. Samples were analyzed on 12% polyacrylamide gels. Lane M, A protein molecular weight marker; lane 1, purified wild-type L-asparaginase I; lane 2, the purified D-9B isoform; lane 3, the purified DD-12G isoform.

specific activity of the mutant D-9B and DD-12G was 17.28 times and 19.59 times higher than that of WT enzyme, respectively (Table 2). In particular, the specific activity of the mutant DD-12G obtained in this study was 1146.29 ± 3.89 IU/mg, which was higher than that of the optimal mutant L-asparaginase screened by Long et al. (23) and Sudhir et al. (24).

The influence of pH and temperature on the enzymatic activity of obtained variants and their stability The optimum pH of both the mutant D-9B and DD-12G was pH 8.0, which remained the same value as that of the WT enzyme (Fig. 2A). After 12-h pre-incubation in buffers of different pH values (5.0–11.0), approximately 11% and 41% of the initial activity was retained in the WT and mutant D-9B, respectively. Interestingly, mutant DD-12G retained more than 82% of its enzymatic activity under the same conditions, indicating a higher tolerance of this L-asparaginase isoform to broader range of pH (Fig. 2B). This later characteristic of the DD-12G mutant might embed more suitability of this variant for the complex food processing environment (38).

Little changes occurred to optimum temperature of the enzymatic reaction where the value of mutant D-9B and DD-12G shifted to 35°C and 40°C, respectively, compared to WT (30°C) (Fig. 2C). In terms of the thermostability of variants following the 20-min incubation at various temperatures, WT retained 74% of its initial activity at 40°C, while D-9B and DD-12G exhibited around 79% and

TABLE 1. The enzymatic activity of a wild-type L-asparaginase I obtained from *Bacillus megaterium* H-1 and mutants obtained after the error-prone PCR and DNA shuffling enhancement.[a]

Enzyme	Enzyme activity (IU/mL)	Amino acid substitutions
WT	1.02 ± 0.16	None
X3D12	6.27 ± 0.11	V26A, K122N
X5G11	9.68 ± 0.37	S2T, V26A, E30G, D181G
X4E7	9.94 ± 0.23	S2T, V26A, Q229L, G276D
D-9B	21.64 ± 0.45	V26A, E30G, K122N, G276D
DD-12G	22.78 ± 0.21	V26A, E30G, D181G, V245G, G276D

[a] Values represent the means ± standard deviation (n = 3).

TABLE 2. Catalytic properties and T_m of the wild-type L-asparaginase I and the obtained variants.[a]

Isoform	Specific activity (IU/mg)	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}mM^{-1}$)	T_m (°C)
WT	55.66 ± 1.80	28.63 ± 0.62	97.09 ± 0.93	3.39	37.5 ± 0.5
D-9B	1017.68 ± 2.06	26.07 ± 0.52	3460.40 ± 1.21	132.73	36.6 ± 0.5
DD-12G	1146.29 ± 3.89	21.63 ± 0.38	3166.42 ± 1.83	146.39	40.3 ± 0.7

[a] Values represent the means ± standard deviation (n = 3).

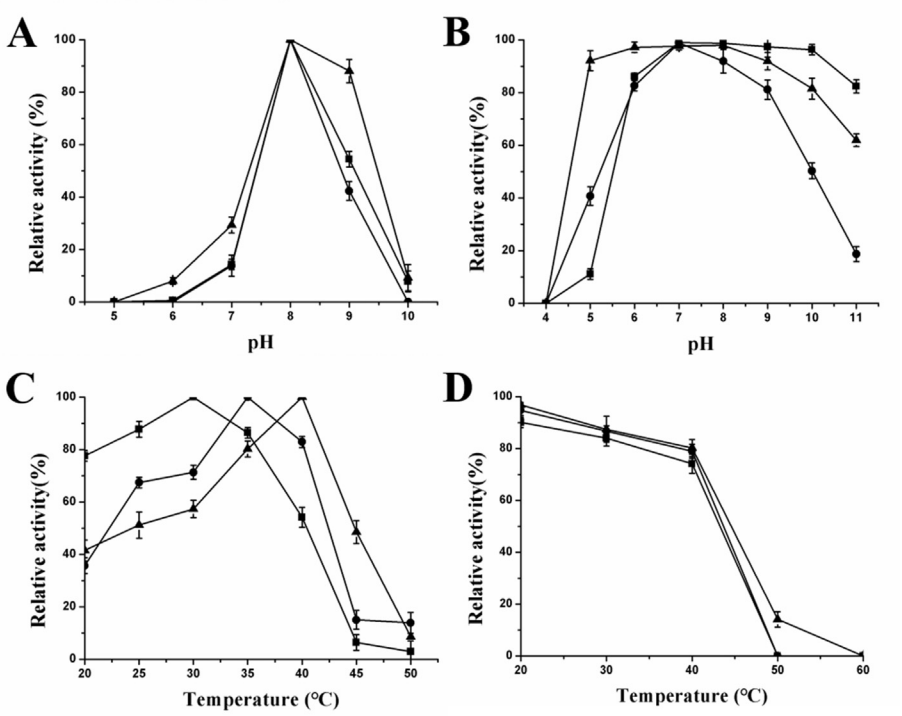

FIG. 2. The effect of pH and temperature on studied enzymatic activity. Squares, wild-type L-asparaginase I; circles, D-8B; triangles, DD-12G. (A) The relative activity of wild-type L-asparaginase I and mutants at different pH values. (B) The residual activity of wild-type L-asparaginase I and mutants after incubation in various buffer preparations for 12 h. (C) The relative activity of the wild-type L-asparaginase I and mutant enzymes at various temperatures in 50 mM phosphate buffer (pH 8.0). (D) Residual activity of wild-type L-asparaginase I and mutant enzymes after incubation at various temperatures in 50 mM phosphate buffer (pH 8.0) for 20 min. Data are expressed as the mean value (±SD) of three independent experiments.

80% activity, respectively. Both the WT and the mutant D-9B were inactivated at 50°C, while the mutant DD-12G remained 14.1% of its original activity (Fig. 2D). In comparison with the WT enzyme (37.5°C), the T_m value of the mutant DD-12G increased by 2.8°C, while the T_m value of the mutant D-9B decreased by 0.9°C (Table 2). In short, the mutant DD-12G displayed a significant improvement both in the optimum temperature and the thermal tolerance, thereby contributing to applications in the baking and frying industries (39).

Substrate specificity and enzyme kinetics parameters of the WT and mutant L-asparaginases The WT and mutant enzymes (D-9B and DD-12G) all remained the specific activity toward L-asparagine, but no enzymatic activity to other substrates, like D-asparagine, L-glutamine, L-aspartic acid, D-aspartic acid, and L-glutamic acid. Furthermore, the WT enzyme and two mutants showed no activity against L-glutamine. This later observation is pivotal for medical applications of these enzymes in order to avoid unintended side effects caused by interferences from L-glutamine (24,40–42).

The kinetics study showed that the K_m value of the mutant D-9B and DD-12G was 26.07 mM and 21.63 mM, respectively, and lower than that of the WT enzyme (28.63 mM). The k_{cat}/K_m for the mutant D-9B and DD-12G was 132.73 $min^{-1}mM^{-1}$ and 146.39 $min^{-1}mM^{-1}$, respectively, which indicated a approximately 38.15-fold and 42.18-fold increase over the WT enzyme (3.39 $min^{-1}mM^{-1}$) (Table 2). Clearly, the catalytic efficiency and the substrate affinity of mutated enzymes were enhanced. The K_m of the two mutants was lower than that of the WT, but still higher than that of previously-reported L-asparaginases (type II) from *B. megaterium* H-1 (0.8 mM) (2) and *Pyrococcus furiosus* (0.012 mM) (21), which cannot meet the K_m requirements of possible variants destined for medical and therapeutic applications (37).

Structure analysis of wide-type and mutant L-asparaginase Since the crystal structure of the *B. megaterium* L-asparaginase has not been resolved yet, the sequence of the WT enzyme was submitted to the SWISS-MODEL server and the closest retrieved homology-based model structure of L-asparaginase from *T. kodakarensis* (with 39.94% identity) was used as the template for our modeling work (37). Thr15 and Thr88 were predicted as the catalytic active sites of the enzyme by ProtComp 9.0 (Softberry Inc., Mount Kisco, NY, USA). Molecular docking was performed using AutoDockTools 1.5.6 software (The Scripps Research Institute, San Diego, CA, USA) to simulate the binding of L-asparagine near Thr15.

A total of six amino acid mutations (V26A, E30G, K122N, D181G, V245G and G276D) occurred in the mutant D-9B and DD-12G were inspected through the generated models. As illustrated by Fig. S1, Thr15, Gln115, Ile116, Thr162, Lys163, Ser238, Ile277 and Try278 were all within a 3 Å distance from the substrate binding site. The improved flexibility of loops near active sites was the major reason for the greater enhancement of enzymatic activity. Several studies have demonstrated that weakening polar interactions such as hydrogen bonds can increase variant's flexibility, a property associated with enhanced activity (25,30,43). As shown in Fig. 3A, Glu30 from the WT enzyme was in the same loop of Thr15, one of the catalytic sites. The mutation E30G decreased the hydrogen bonds from four to three, thereby enhanced the flexibility of loops near active site, increased the entry of the substrate into the active site and improved the catalytic efficiency of two mutant enzymes (Fig. 3B). The substitution K122N in mutant D-9B also made the loss of hydrogen bond between Ser121 and Lys122 (Fig. 3C, D), thus it made a loop formed by residues Thr112-Asp124 more flexible to facilitate the access of substrate into the active site to participate in catalytic reaction (25,43). According to previous studies, the introduction of glycine

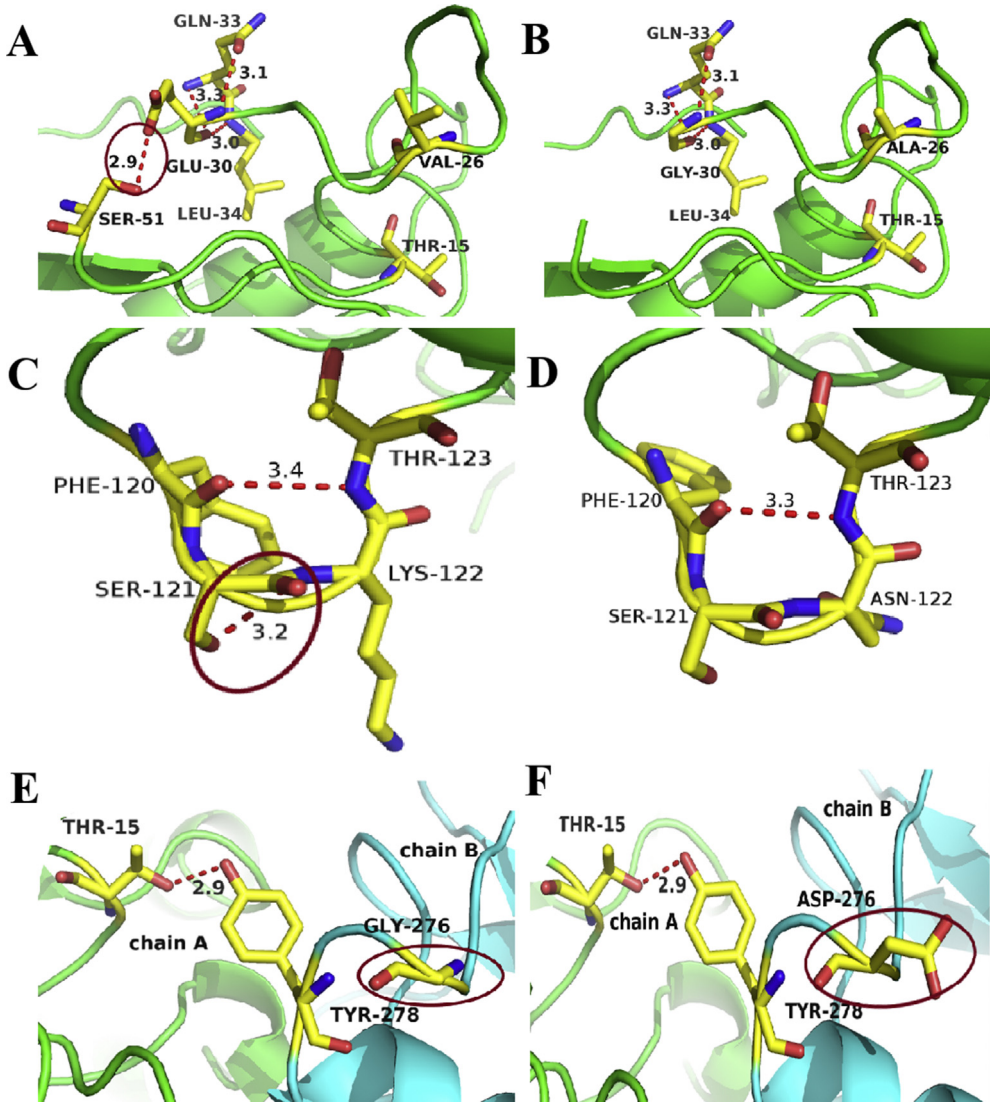

FIG. 3. Homology modeling. Location of amino acid substitutions in the predicted L-asparaginase I. (A) Structural representation of the mutation site 26 and 30 of the wild-type L-asparaginase I. (B) Structural representation of the mutation site 26 and 30 of the mutants. (C) Structural representation of the mutation site 122 of the wild-type L-asparaginase I. (D) Structural representation of the mutation site 122 of the mutant D-9B. (E) Structural representation of the mutation site 276 of the wild-type L-asparaginase I. (F) Structural representation of the mutation site 276 of the mutants.

residue could also strengthen the flexibility of the protein (44). By the substitution of Glu30 and Val245 with two glycine residues at surface loops, the backbone conformation became more flexible due to lacking of β-carbon, thus increasing the loop flexibility and facilitating the catalytic reaction (43,45). Furthermore, mutating hydrophilic residues on the protein surface could trigger changes in regional flexibility in variants (18,46). As illustrated by Fig. 3E, Gly276 and Try278 of chain B located in a surface loop region formed by residues Gln268-Gly282, near the active site Thr15 of chain A. The substitution of hydrophilic residue Asp276 in two mutants might decrease the dense packing of hydrophobic residues and strengthen the loop's flexibility (Fig. 3F), resulting in an open of active site Thr15 for more substrates to enhance enzymatic activity and efficiency (29,42,47).

The substitution D181G occurred in mutant DD-12G may be responsible for the enhanced thermostability. Asp181 was located at a flexible loop, which connected N-terminal and C-terminal domain of each monomer (37). The surrounding amino acids Tyr175, Val176, Ala177, Ile179, Val184 and Tyr186 were all hydrophobic amino acids, whereas aspartate was hydrophilic. The mutation of polar charged residue Asp to Gly in mutant DD-12G strengthened the hydrophobicity of the area, and subsequently improved thermal stability of the enzyme (21,33,48).

The protein secondary structure of the WT enzyme changed little after the mutation of V26A (both the aliphatic amino acids) in both mutants, indicating the less significance of this mutation in improving enzymatic activity or stability (Fig. 3A, B).

Circular dichroism spectroscopy analyses of mutant L-asparaginase I The ovality of each variant was measured in the range of 190–250 nm by circular dichroism spectroscopy and the proportions of protein secondary structures of each isoform were calculated and analyzed (Table 3). In essence, any increase in the number of the β-sheets/turns or the stabilization of α-helixes could enhance protein rigidity, a property related to protein thermal stability (30,49,50). Similarly, increasing the percentage of random coils enhances the flexibility of overall protein structure but decreasing its stability (49,51).

Compared with the WT, the number of α-helixs in mutant D-9B decreased from 20.7% to 20.5% and random coils increased from

TABLE 3. Proportions of secondary structures in the wild-type L-asparaginase I and mutant enzymes.

Secondary structure	Proportion (%)		
	WT	D-9B	DD-12G
α-helixes	20.7	20.5	21.7
β-sheets	23.8	23.9	24.1
Turns	18.5	18.2	19.1
Random coils	37.0	37.4	35.1

37.0% to 37.4%. According to the above analysis, the changes of overall secondary structures were not significant, in accord with our experimental result that thermal stability of mutant D-9B was not significantly changed. In contrast, the number of α-helixes of mutant DD-12G increased from 20.7% to 21.7%, β-sheets increased from 23.8% to 24.1%, and the random coils decreased from 37.0% to 35.1%, which might explain why mutant DD-12G was more thermostable than WT counterpart.

Overall, the generated mutations in the above sites did not alter the secondary structure significantly, but the co-operation of several substitutions improved the enzyme's catalytic properties. One reasonable explanation may be that loops near the catalytic active site become more flexible and mobile by weakening interactions within the N-terminal loops or through the introduction of flexible residues at the relevant positions. This by itself increases the accessibility of substrate to entry into the active site (relating to K_m) and enhances the catalytic efficiency.

L-Asparagine content in pre-treated potato slices and acrylamide content in fried potato chips The effects of WT and mutant L-asparaginase on acrylamide reduction from fried potato chips were evaluated by LC-MS/MS (Fig. S2). After enzymatic treatment using mutant DD-12G, the residual content of acrylamide in fried potato chips was merely 0.127 ± 0.017 mg/kg, which was a significant decrease in comparison with control group (1.105 ± 0.089 mg/kg) and WT enzyme group (0.236 ± 0.041 mg/kg) (Fig. S3). With removal rate of approximately 88.5%, mutant DD-12G enzyme could effectively reduce acrylamide level in potato chips. Besides, a similar downward tendency in L-asparagine content of three groups was found (Figs. S3 and S4). After enzymatic treatment using mutant DD-12G, the residual content of L-asparagine in pre-treated potato slices was merely 18.641 ± 0.713 mg/kg, lower than that of control group (39.067 ± 1.099 mg/kg) and WT enzyme group (25.025 ± 0.921 mg/kg), which indicates that L-asparaginase treatment lead to low acrylamide potato chips due to the reduction of L-asparagine.

The current study represents the first experimental approach to improve the activity of L-asparaginase I from *B. megaterium* H-1. Two mutants D-9B and DD-12G showed substantial increase in enzyme's catalytic activity were screened out by directed evolution. Kinetics studies revealed that the two mutants had better substrate affinity and increased catalytic properties. Moreover, there was a co-evolution of the enzyme's catalytic activity and its thermal stability as a result of the directed evolution approach, which was implemented evident by the obtained mutant DD-12G that possessed both an increased activity and an enhanced thermostability. In conclusion, the results here confirm that the *in vitro* directed evolution of enzymes can feasibly yield mutants with much more desirable industrial properties and it can be an effective vehicle for improving their activities (L-asparaginase as an example).

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