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Directed Evolution of α-Aspartyl Dipeptidase from *Salmonella typhimurium*

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Model-free approaches (error-prone PCR to introduce random mutations, DNA shuffling to combine positive mutations, and screening of the resultant mutant libraries) have been used to enhance the catalytic activity and thermostability of α-aspartyl dipeptidase from *Salmonella typhimurium*, which is uniquely able to hydrolyze Asp-X dipeptides (where X is any amino acid) and one tripeptide (Asp-Gly-Gly). Under double selective pressures of activity and thermostability, through two rounds of error-prone PCR and three sequential generations of DNA shuffling, coupled with screening, a mutant pepEM3074 with approximately 47-fold increased enzyme activity compared with its wild-type parent was obtained. Moreover, the stability of pepEM3074 is increased significantly. Three amino acid substitutions (Asn89His, Gln153Glu, and Leu205Arg), two of them are near the active site and substrate binding pocket, were identified by sequencing the genes encoding this evolved enzyme. The mechanism of the enhancement of activity and stability was analyzed in this paper.

***Key Words:* α-aspartyl dipeptidase; protein engineering; double selective pressures; directed evolution.**

α-Aspartyl dipeptidase, first isolated from *Salmonella typhimurium* by Carter *et al.* (1) in 1984, is a highly specific enzyme that acts only on dipeptides containing N-terminal Asp residues. It has weak activity toward C-blocked dipeptides. No activity was seen toward larger peptides with N-terminal aspartate, a N-blocked Asp-X dipeptide or a peptide with an internal Asp residues, with one exception (Asp-Gly-Gly) (2, 3). α-Aspartyl dipeptidase gene whose nucleotide sequence was determined by Conlin *et al.* in 1994 contains an open reading frame of 687 bp that predicts a 24,768 Da protein and a promoter with a cyclic AMP receptor protein (CRP) site. Extensive work demonstrated that α-aspartyl dipeptidase was the first number of a novel family of serine proteinases with a glutamate in the unusual catalytic triad that contained Ser-His-Glu (4).

With exquisite substrate specificities, α-aspartyl dipeptidase offers the potential advantages for organic synthesis of the precursors of dipeptide sweeteners, the derivatives of N-terminal L-Asp dipeptides, and other physiologically active peptides by direct reversal of the hydrolytic process, particularly in the presence of organic media (5). However, the practical application of α-aspartyl dipeptidase to dipeptide synthesis has been limited, mostly due to its relatively poor stability and catalytic activity under the conditions of industrial production. Obtaining high enzyme activity and stability is important for synthetic application. Because the catalytic mechanism and molecular basis of stability of α-aspartyl dipeptidase is poorly understood, it is difficult to enhance its properties by rational design. Therefore, to obtain the mutants with enhanced enzyme activity and stability, we modified α-aspartyl dipeptidase by the approach of directed evolution of enzyme *in vitro*, which belongs to "irrational" design for protein engineering (6). Directed evolution of enzyme *in vitro* is a useful strategy of protein engineering to explore the functions of enzymes artificially by mimicking key processes of natural evolution mechanism (random mutation and recombination coupled with screening). A significant advantage of this method is that neither structural information nor catalytic mechanism is required to guide the evolution of enzymes. Here we show that the activity and stability of α-aspartyl dipeptidase were increased significantly by directed evolution.

MATERIALS AND METHODS

Materials and Plasmid

Restriction endonucleases: *Bam*HI and *Pst*I were purchased from MBI (Canada). *Taq* and *Pfu* DNA polymerase were from Promega

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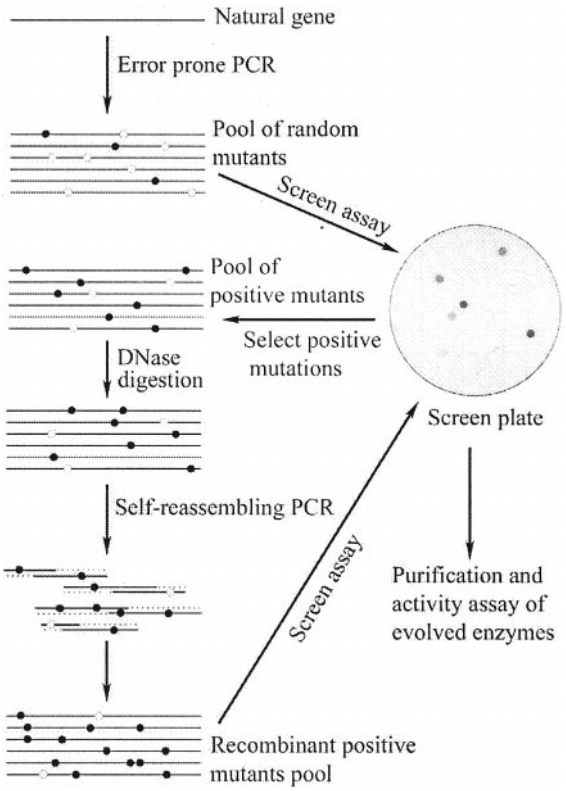

FIG. 1. Experimental strategy of directed evolution. Positive (●) and negative (○) mutations were introduced to α-aspartyl dipeptidase gene by error-prone PCR, and the positive mutants were selected as templet DNA to initiate self-assembly PCR to form a large library of positive recombinants. The promising isolates of reassembled genes containing the different combinations of mutation were detected for the next round of DNA shuffling. After two rounds of random mutagenesis and three sequential generations of DNA shuffling, the mutants were analyzed in detail.

(U.S.A.). DNase I, DNA Marker DL-2000 and λ DNA/*Hin*dIII were bought from TaKaRa (Japan). L-Aspartic acid *p*-nitroaniline (Asp-pNA) was purchased from BECHEM AG (Sweden). DNA recovery Kit (silver beads) was from Sangon (China). DEAE-Sepharose Fast Flow and Sephacryl S-100HR were bought from Pharmacia (U.S.A.). All other chemicals were of analytical grade.

The plasmid of pBV220 and strain DH5α were gifts from professor Zhiqing Zhang of National Key Lab of Virus Genetic Engineering of China. The plasmid pBVPE containing α-aspartyl dipeptidase gene was constructed and stored in our lab.

Purification and Kinetic Parameter Determination of Wild Type and Mutant

Wild α-aspartyl dipeptidase and its mutant obtained by directed evolution were purified as described (7). The enzyme concentration was determined by the Lowry method. Initial rates of hydrolysis of Asp-pNA, specific activity, k_{cat} and K_m of enzymes were determined as described by Zhang *et al.* (7).

The optimum pH and thermostability of evolved enzyme were determined and compared with that of wild enzyme.

Directed Evolution

Random mutagenesis. Plasmid pBVPE containing the 820-bp wild-type α-aspartyl dipeptidase gene and leading sequence (including CRP site, TAAT box and SD sequence) were digested by *Bam*HI and *Pst*I. The 820-bp fragments were purified in low-melting-point agarose and served as the templet for error-prone PCR. Random mutations were introduced into the amplification product during error-prone PCR (8). Primers NT (5′-GACGGATCCGCGACCGCGCTTTGCCTG-3′) and CT (5′-GTTCTGCAGGCCTGGGATTAAAAGCG-3′) contains *Bam*HI and *Pst*I restriction site. The mutagenesis frequency was controlled to the desired level (1–2 amino acid substitution) by changing the concentration of Mg^{2+}. 40 pmol of each primer and 5 ng template DNA were added to 100 μl error prone PCR system (0.2 mM of each dNTP/2.2 mM $MgCl_2$/50 mM KCl/10 mM Tris (pH 9.0)/0.1% Triton X-100/5 U *Taq* DNA polymerase). A PCR program is: 95°C for 5 min; add *Taq* DNA polymerase; 94°C for 60 s, 55°C for 60 s, 72°C for 60 s (cycling 32 times), and 72°C 10 min. The product of amplification was recovered on a 1.0% low-melting-point agarose gel. The removal of free primers from the PCR product was found to be very important.

DNA shuffling and recombination library construction. DNase I digestion to generate random fragments in the presence of Mn^{2+} was carried out based on the method described by Lorimer *et al.* (9, 10). DNA shuffling was operated using the method of Stemmer (11, 12). The product of self-reassembling PCR was diluted 40 times and used as the templet of high-fidelity PCR described by Huimin Zhao and Arnold (13). PCR conditions: 40 pmol of each primer, 1× *Pfu* buffer, 0.2 mM each dNTP and 5 U *Pfu* DNA polymerase. The PCR program of is: 94°C for 5 min, 72°C for 2 min, 10 cycles of 94°C for 60 s, 53°C for 60 s, 72°C for 60 s, followed by another 20 cycles of 94°C for 60 s, 58°C for 60 s, 72°C for 60 s + 10 s/cycle, and finally 72°C for 10 min. This program gave a single band at the correct size. Purified PCR product was digested by *Bam*HI–*Pst*I and ligated with plasmid pBV220 to create recombination library of pBVPEM. The resulting plasmid library was transformed into *E. coli* DH5α.

Screen assay. First, the transformed strains were grown in LB plate containing 50 μg/ml ampicillin. The individual colonies were transplanted onto another plate containing 200 μl 0.77 mM Asp-pNA on the surface. The transplanted colonies were cultured on the screen plate at 30°C for 10 h and then induced at 42°C for 4 h. The colonies with yellow color were marked and the homologous colonies were selected as the pool of crude screening. The crude colonies were cultured in 2 ml LB medium at 30°C for 15 h and were induced at 42°C for 4 h and then centrifuged at 5000 rpm for 5 min. The pellet of bacteria was resuspended and diluted to OD_{600} = 1.0 with 50 mM imidazole (pH 7.0). 10 μl diluted bacteria was added to 990 μl of 0.77 mM Asp-pNA/50 mM imidazole (pH 7.0) and was checked using ΔOD_{405}. The spare of bacteria was incubated at 60°C for 30 min, and then the remaining activity was checked. The strains that have both higher α-aspartyl dipeptidase activity and thermostability were selected for examination of specific activity. Dozens of strains with the highest activity and thermostability were selected as the parents for next round of directed evolution.

Sequencing of α-aspartyl dipeptidase variant genes. Selected plasmids pBVPEM were digested by *Bam*HI and *Pst*I and the α-aspartyl dipeptidase gene was cloned into Bluescript SKII+ phagemid at the same site. Single-strain phagemid containing a mutant α-aspartyl dipeptidase gene was prepared and sequenced on DNA Sequencer 377 (PE).

RESULTS

Directed Evolution

Directed evolution *in vitro* is a new and highly effective strategy in the field of protein engineering. It allows to engineering of all kinds of enzymes, even with little knowledge of spatial structure or catalytic mechanism. In this paper, random mutations were introduced to α-aspartyl dipeptidase gene first by error-

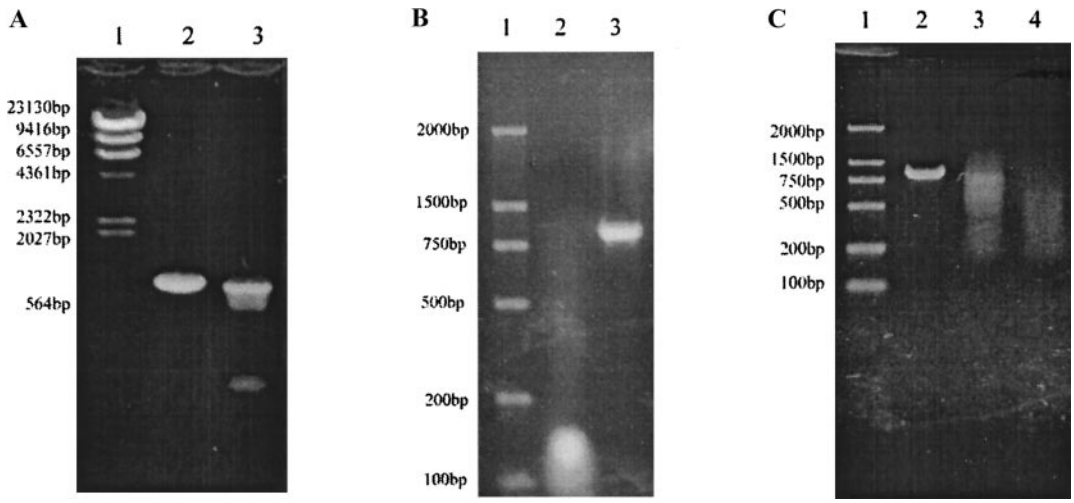

FIG. 2. The results of error-prone PCR and DNA shuffling. (A) Error-prone PCR products of α-aspartyl dipeptidase gene. 1, λ DNA/*Hin*dIII MW marker; 2, PCR products of target DNA; 3, error-prone PCR products. (B) Product of DNase I digestion. 1, DNA ladder DL-2000; 2, the product of DNase I digestion; 3, α-aspartyl dipeptidase gene. (C) Detection of self-reassembly PCR. 1, DNA ladder DL-2000; 2, the product of high-fidelity PCR; 3, the product of self-reassembly PCR (after 52 cycles); 4, the product of self-reassembly PCR (after 20 cycles).

prone PCR and the genes carrying positive mutation were selected as a pool for the source of DNA to initiate DNA shuffling (Fig. 1). Under double selective pressures of both specific activity and thermostability, through two rounds of error-prone PCR and three sequential generations of DNA shuffling, an evolved α-aspartyl dipeptidase pepEM3074, with 47-fold higher activity than that of the wild-type enzyme, was obtained.

Error-prone PCR and DNA shuffling. The frequency of mutation was controlled to 1- to 2-amino-acid substitution in each gene by limiting the concentration of Mg^{2+} to 2.2 mM in error-prone PCR (Fig. 2A). After DNase I digestion, 50- to 150-bp fragments were recovered to ensure the best recombination frequency (Fig. 2B). DNA polymerase *Pfu* was used in the self-reassembly PCR to reduce the introduction of a new mutation. The length of self-reassembled fragments was increased during DNA shuffling and after 52 cycles the main band of DNA fragments was increased to approximately 800 bp (Fig. 2C). The recombination DNA of proper size was amplified with *Pfu* DNA polymerase.

Screening assay. The most important step of any directed evolution experiment is the development of a rapid method for screening large numbers of variants for the desired features. The screen is required to be sensitive enough to identify mutations conferring even a small enhancement to achieve the desired result by subsequently combining these improved properties. To minimize the hard work and the length of time of the large-scale screen, we designed a three-step screen method: the first step is the crude screening on an Asp-pNA plate; the second step is measuring the enzyme activity of bacterium; and the last step is purifying the evolved enzyme and measuring specific activity. In the crude screen, thousands of mutant strains can be screened at the same time rapidly and most inactive or low active mutants were eliminated to minimize the library. At the second step, the evolved strains that have higher activity than their parents were selected. At last 15–30 colonies with the highest activity were picked at each round and used as a pool for the source of DNA to initiate the next round of DNA shuffling. This three-step screening assay makes it much easy to deal with a mutation library of thousands clones.

Specific activity and kinetic parameters of evolved enzyme. After two rounds of random mutagenesis and three sequential generations of DNA shuffling, a mutant pepEM3074 with 26.9-fold increased k_{cat} and 43.2% decreased K_m was screened. An additional four mutants, PepEM3017, PepEM3033, PepEM3095, and PepEM3112, have increased k_{cat}/K_m of 40.0-, 25.6-, 34.9-, and 28.1-fold respectively. The wild-type and

TABLE 1

The Kinetic Parameters of Mutants and Wild-Type Enzyme

Enzyme	$k_{cat} \times 10^{-1}$ (s^{-1})	K_m (mM)	$k_{cat}/K_m \times 10^{-2}$ ($s^{-1} \cdot mM^{-1}$)	Relative activities (%)
Wild-type	2.17	3.98	5.45	100
PepEM3017	50.0	2.29	218	4000
PepEM3033	57.9	4.12	140	2560
PepEM3074	58.3	2.26	258	4690
PepEM3095	44.4	2.33	190	3490
PepEM3112	56.5	3.70	153	2810

TABLE 2

Base and Amino Acid Substitutions in pepEM3074

Base site	Base	Position in codon	Amino acid	Amino acid
429	A → C	1	89N	Asn → His
621	C → G	1	153Q	Gln → Glu
737	G → C	3	191P	Silent
778	T → G	2	205L	Leu → Asp

mutant α-aspartyl dipeptidases were purified and checked in 13% SDS–PAGE. The kinetic parameters of mutants were determined and compared with the wild-type (Table 1).

The Comparison of DNA Sequence, Thermostability, and Optimum pH

The sequence of the evolved α-aspartyl dipeptidase gene showed a total of four DNA base substitutions and three of them are predicted to cause amino acid changes (see Table 2).

Directed evolution has increased the thermostability of α-aspartyl dipeptidase significantly. At 60°C, for example, the evolved enzyme has a $t_{1/2}$ about 6-fold that of its wild-type counterpart (Fig. 3B). After incubation for 30 min at 60°C, the native enzyme conserved about 25% of its activity, while the evolved enzyme retained about 80% of its activity (Fig. 3A).

The pH dependence of k_{cat} of reactions catalyzed by pepEM3074 was changed inconspicuously. The optimum pH of the evolved enzyme pepEM3074 is 7.0, while the optimum pH of wild-type is 7.2 (Fig. 3C).

DISCUSSION

Experimental Strategy of Directed Evolution

Directed evolution *in vitro* is a new and highly effective strategy in the field of protein engineering. The principle is to remake enzyme gene *in vitro* under particular evolutionary conditions created artificially in the absence of any knowledge of spacial structure and catalytic mechanism of enzyme by mimicking natural evolution mechanism (random mutation, recombination, and natural selection). The expected properties of the enzymes that have been evolved for millions of years in nature can be easily obtained through several rounds of directed evolution. Not only can profitable mutations be rapidly accumulated by directed evolution, but also it is a promising approach to combine two or more improved properties evolved separately.

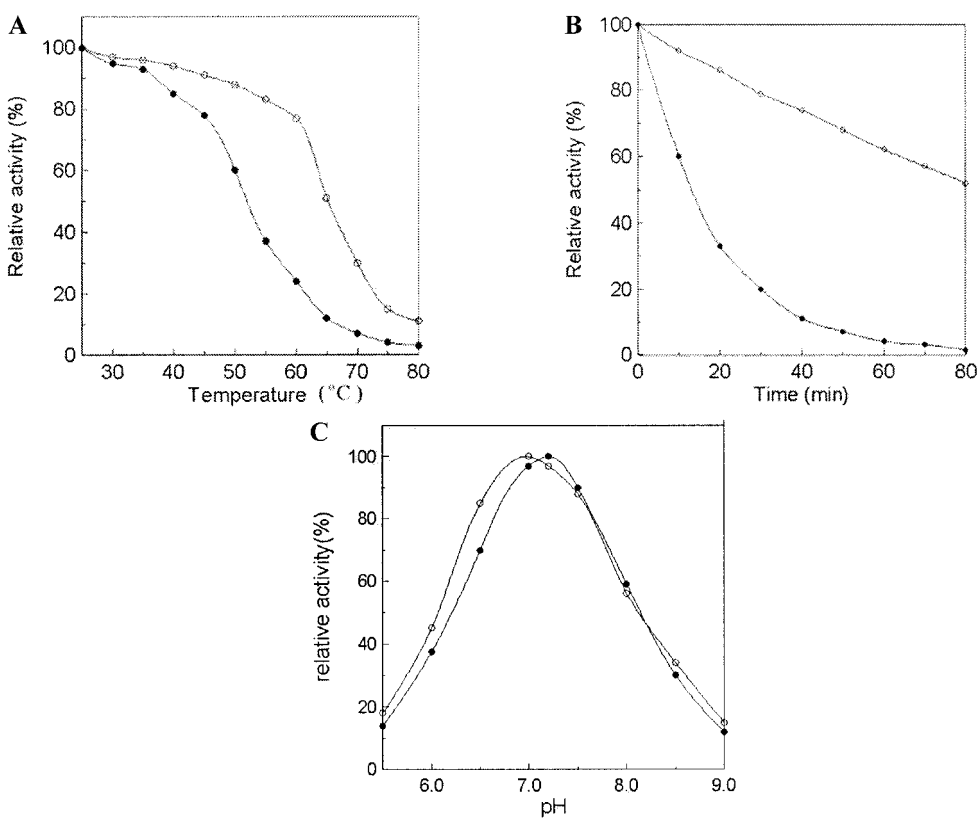

FIG. 3. Comparison between wild type (●) and evolved (○) enzymes. (A) Wild type and evolved enzymes were incubated at various temperature for 30 min, followed by detection of their residual activity. (B) The enzyme was incubated at 60°C and determined at the indicated time. (C) The relative activity of wild type and evolved enzymes at different pH.

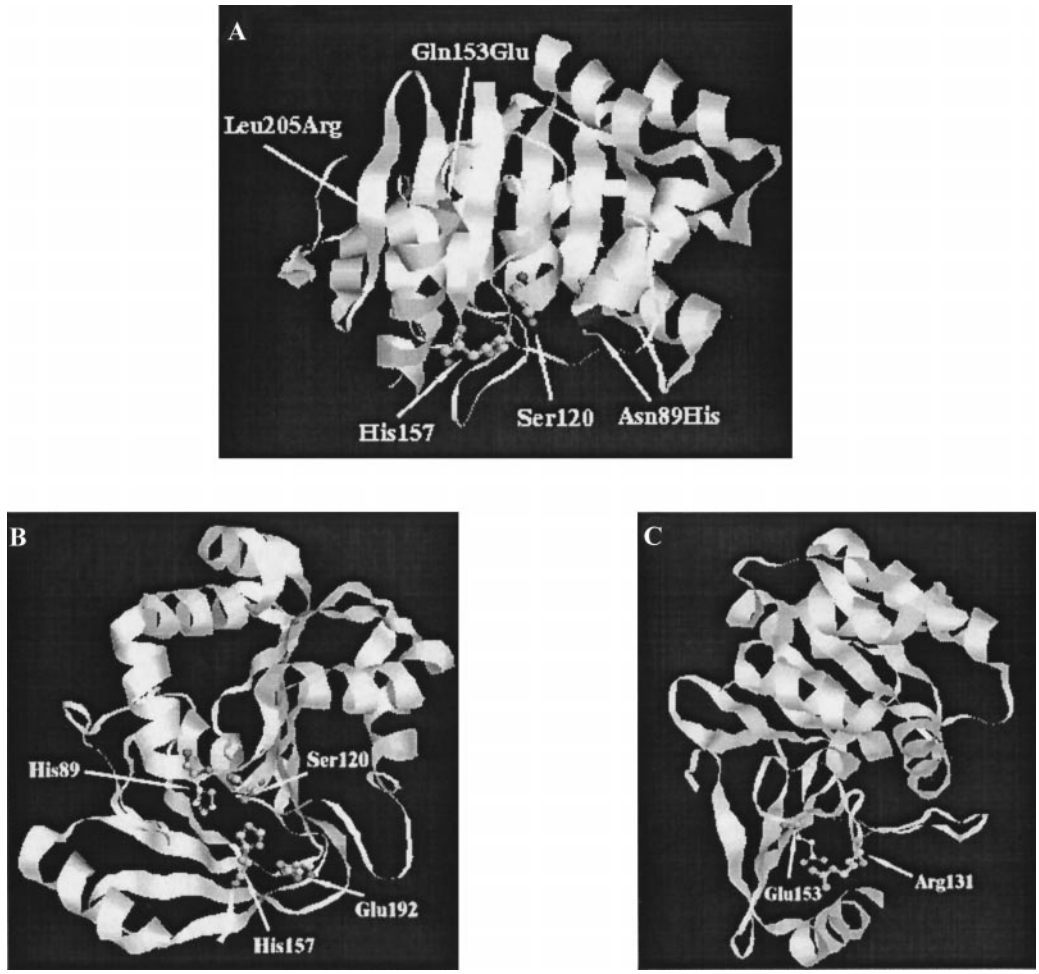

FIG. 4. Models of mutant PepEM3074 based on the structure of wild type α-aspartyl dipeptidase. (A) The positions of mutations (Asn89His, Gln153Glu and Leu205Arg). (B) The spatial positions of His89 and the active site. (C) The spatial positions of Glu153 and Arg131.

The most important element of any directed evolution experiment is to develop a rapid and large-scale screening assay sensitive enough to ensure that even a small enhancement of activity can be observed. To minimize the amount of work and the length of time of large-scale screening, we designed a three-step screening method in which thousands of mutant strains can be screened at the same time rapidly.

α-Aspartyl dipeptidase is useful in the synthesis of the precursors of dipeptide sweeteners and other physiologically active dipeptides, but the activity and stability of the wild type enzyme is not satisfying enough to be applied to industrial production. One goal of our experiments is to enhance the expected properties of wild-type enzyme by directed evolution—evolving the enzyme by error-prone PCR, DNA shuffling, and screening. In addition, the following sequence and structure researches of the evolved enzyme will offer more valuable information for structure–function relationship of α-aspartyl dipeptidase.

Analysis of the Effects of the Mutations in pepEM3074

Figure 4A shows the positions of the amino acid substitutions in a three-dimensional structure model generated using the known structure of wild-type α-aspartyl dipeptidase resolved by Miller *et al.* (4). In spatial structure, Asn89His is very close to the amino acid residues of active center; Gln153Glu is only four amino acid residues apart from His157, the second catalytic triad residues, while Leu205Arg is far from the active site.

Residue 89 is located in loop connecting the forth α-helix and the fourth strand of an eight-stranded β-sheet, the larger of the two β-sheets of α-aspartyl dipeptidase. Interestingly, this residue is the nonconserved amino acid in GGXT motif (residues 87–90, where X represents any amino acid residue), which is critical for the conformation of the active site and can be widely found in ORFs with little or no overall sequence similarity to aspartyl dipeptidase (14, 15). It

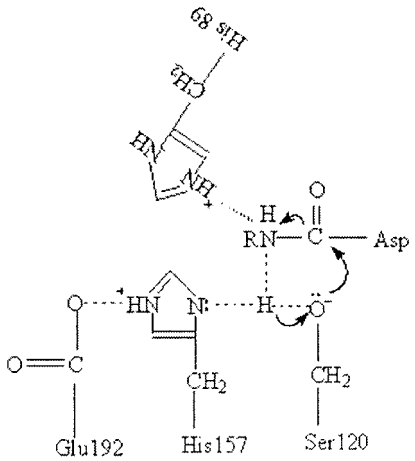

FIG. 5. Hypothesis explaining how His89 in the evolved α-aspartyl dipeptidase accelerates the reaction rate at the active site.

appears, therefore, the substitution of Asn with His at this position will affect the properties of activity and binding substrate significantly, while not disrupting the necessary elements of secondary structure (Fig. 4B). Hydrogen bonding of Nδ1 of His157 to the β-carboxyl group of Glu192 increases the nucleophilicity of the Nϵ2 of the histidine, which interacts with the hydroxyl group of Ser120 by hydrogen bond. Consequently, the oxygen atom of the side chain of Ser120 becomes a powerful nucleophile that can attack the carbon atom of the carbonyl group in the substrate to form a tetrahedral intermediate. His89 in pepEM3074 probably helps stabilize the transition state to accelerate the rate-determining step. For example, the imidazole ring of His89, as general acid, can provide a proton to stabilize the carbanionic intermediate during transition state (Fig. 5), which will account for the increased k_{cat} of the evolved aspartyl dipeptidase. Additionally, being very close to the substrate binding pocket, Asn89His may cause subtle changes in the binding and orientation of substrate and leads to enhanced affinity of enzyme with substrate.

Gln153, which is located at the terminus of the first strand of the smaller β-sheet and only four residues away from the active His, is replaced with Glu in the evolved enzyme. This substitution possibly subtly adjusts Nϵ2 of His157 into an orientation more favorable for hydrogen bonding to hydroxyl group of Ser120. Moreover, carboxyl group of Glu153 in pepEM3074 probably forms internal ion pair(s) with the positive guanidine of Arg131 (Fig. 4C). If so, the suppression of the added salt bridges in pepEM3074 will be beneficial to thermostability by stabilizing the secondary structure of the surrounding peptides, in which several residues, such as His157, Asn134, and Asp135, are situated in the active site or interact with the N-terminal amino group of the substrate.

The role of the remotely located Leu205Arg is difficult to explain. However, this mutation can exert it influence on the aspartyl dipeptidase structure over large distances in a manner that is hard to predict. Further examination of the catalytic mechanism is underway.

ACKNOWLEDGMENT

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