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Enhancement of the Activity of L-Aspartase from *Escherichia coli* W by Directed Evolution

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L-Aspartase was modified by directed evolution. After four rounds of error-prone PCR and three rounds of DNA shuffling, an evolved enzyme purified from the final round showed a 28-fold increased k_{cat}/K_m and 4.6-fold decreased K_m. The thermostability and stable pH range were also enhanced. The DNA sequence of the evolved aspartase gene showed seven base changes, resulting in three amino acid changes from the native enzyme: N217K, T233R, V367G. The mechanism of the enhancement of activity was analyzed. © 2000 Academic Press

What is called the directed evolution of enzyme *in vitro*, belongs to "irrational" design of protein that is a new strategy for protein engineering of enzyme. By mimicking natural evolution mechanism (random mutation, recombination and natural selection), an enzyme gene is remade *in vitro* under particular evolutionary conditions created artificially in the absence of any knowledge of spatial structure and catalytic mechanism of enzyme in advance. The mutant enzymes with desired properties are screened for a defined goal [1]. This new strategy can be used to engineer any proteins or enzymes.

L-Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) catalyzes the reversible conversion of L-aspartate to fumarate and ammonia ion [2]. It is an important enzyme in industry. L-Aspartase from *E. coli* W is composed of four identical subunits of molecular weight 52,200 Da. Each monomer of aspartase is composed of three domains. The central domain, containing residues 142–396, has almost all α-helical structure. It is the most conserved domain in the aspartase–fumarase structural family. The central core of the domain is made up of five long helices. In the tetramer, these five helices form a 20-helix cluster, which is important to maintain its active conformation. The X-ray crystal structure reveals that residues from three different subunits are located closely to the active site, with the potential to be involved in catalysis. Lys327, located at a cleft in this region, is the functional residue of this enzyme [3].

In the past, we replaced Lys126 with Arg residue using site-directed mutagenesis and obtained the activity of the mutant enzyme for about 5-fold compared to that of wild-type one [4]. We also obtained a mutant lacking 14 peptide from C-terminus, which has a 1.21-fold enzyme activity [5]. The truncation of the C-terminal region by limited proteolysis [6] and replacement of Cys430 with Trp [7] enhanced 3.3, 3-fold activity respectively. Because the active site of L-aspartase has not been determined and the catalytic mechanism has to be further proved, it is difficult to enhance the enzyme activity by rational design. Therefore, we select the method of directed evolution to modify the L-aspartase gene, in order to obtain the mutants with enhanced enzyme activity.

MATERIALS AND METHODS

Enzymes, reagents and strains. Restriction enzymes, *Taq* DNA polymerase, *Pfu* DNA polymerase, DNase I and silver bead gel extraction kit were purchased from MBI. Reagents and primers were purchased from Sangon, Shanghai, China. DEAE-Sepharose F.F. and Sephacryl S-200 were purchased from Pharmacia.

E. coli J_2 containing plasmid carrying aspartase gene was stored in our laboratory. *E. coli* JRG1476 (*asp*A$^-$) strain was a generous gift of Dr. J. R. Guest (University of Sheffield, Sheffield, England).

Screening method. The Noriyuki Nishimura medium [8] that contains 0.5% L-aspartic acid as the sole nitrogen source, 1% glucose, 0.1% $(NH_4)_2SO_4$, 0.7% K_2HPO_4, 0.3% KH_2PO_4 and 0.01% $MgSO_4 \cdot 7H_2O$ was used for Asp-N screening.

The product of error-prone PCR and DNA shuffling was digested with *Sac*I and *Bam*HI, and then ligated into pUC18 vector digested with the same enzymes. The ligature was transformed into *E. coli* JRG 1476 competent cells. Colonies which grow faster or larger than the control one on the Asp-N screening agar plates are transferred into 5 ml 2YT liquid medium and cultured at 37°C overnight. The activity of crude enzyme of these colonies was measured. The mutants whose aspartase activity were higher than native enzyme were selected as the template for next round of directed evolution. This method provides rapid and efficient screening to identify.

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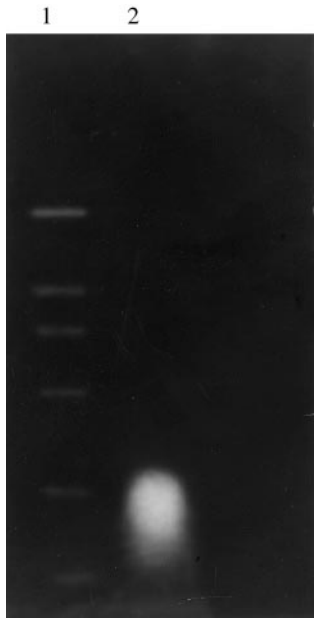

FIG. 1. Identification of the product of DNase I digestion. Lane 1, marker; lane 2, the product of DNase I digestion.

Error-prone PCR. Random mutagenesis was introduced by error-prone PCR [9]. Two primers of P_1 (5′-TCGTGAGCTCTCG-GGTATTCGGTCGATGCAG-3′) and P_2 (5′-TCTGGGATCCTGTAC-GATTACTGTTCGCTTTC-3′) were used to amplify the full-length aspartase sequence. The underlined sequences are *Sac*I and *BamH*I restriction sites for P_1 and P_2 respectively, which allowed the PCR products to be ligated into pUC 18 vector digested with the same enzymes. At the same time, we amplified *asp*A using *Pfu* DNA polymerase as a control.

DNA shuffling. DNA shuffling was carried out as described previously [10] with minor modification. DNase digestion condition was changed and the fragments of 100–200 bp were purified using Silver bead gel extraction kit. In the step of fragment reassembly, *Pfu* polymerase was used to improve the fidelity of PCR.

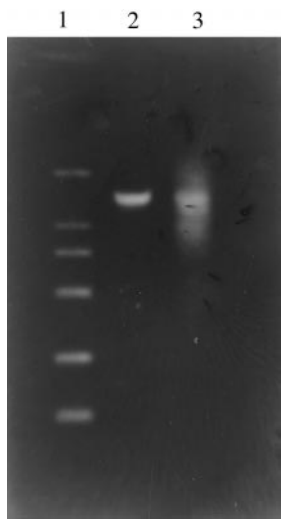

FIG. 2. Identification of the product of fragment reassembly. Lane 1, marker; lane 2, normal PCR; lane 3, the product of fragment reassembly.

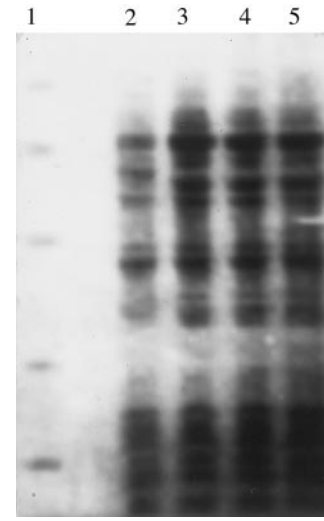

FIG. 3. Gel: 10% SDS–PAGE of wild-type and evolved aspartases. Lane 1, marker; lane 2, *E. coli* JRG 1476; lane 3, control one; lanes 4 and 5, mutant.

Enzyme purification. *E. coli* JRG 1476 cells that carry wild-type or evolved genes were grown in 3 ml Asp-N medium supplemented with ampicillin (100 μg/ml) for overnight. One ml from each culture was then transferred into 100 ml of Asp-N medium supplemented with ampicillin and grown to OD_{600} 0.6. The culture was induced by adding IPTG to final concentration of 1 mmol/liter for 4 h. The cells were then disrupted by French Pressure Cell Press. The denatured protein was removed by nucleoprotamine, $(NH_4)_2$ SO_4 fraction, DEAE-Sepharose F.F., Sephacryl S-200 column chromatography.

Enzyme assay. The activity of aspartase was routinely determined spectrophotometrically at 30°C as described previously by measuring the increase in absorption at 240 nm due to fumarate formation [7].

Sequencing of the evolved L*-aspartase gene.* The 1.6-kb DNA fragment encoding the evolved aspartase was sequenced on DNA sequencer 377 (PE).

FIG. 4. Gel: 7.5% PAGE of wild-type and evolved aspartases. Lane 1, Native aspartase; lane 2, evolved aspartase.

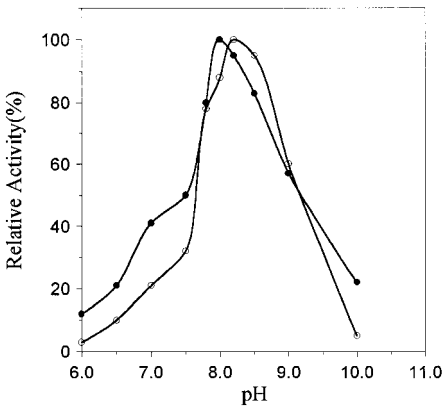

FIG. 5. The optimum pH of native (●) and evolved aspartases (○).

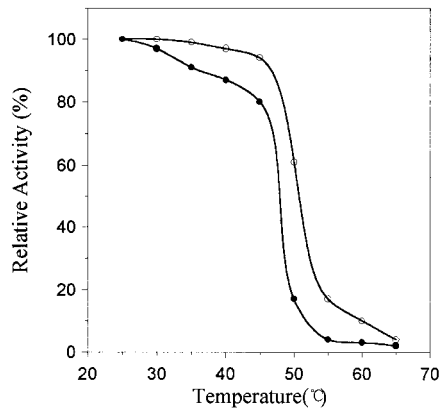

FIG. 7. The thermostability of native (●) and evolved aspartases (○).

RESULTS

Error-prone PCR. For one round of error-prone PCR, 3000 colonies were screened. 40–50 colonies with the highest aspartase activity were selected as the template of the next round of error-prone PCR. The activity of the mutated aspartases from the fourth round of error-prone PCR was slightly higher than the third ones.

DNA shuffling. DNase I digestion was done in the presence of Mn^{2+}. The fragments which were 100–200 bp were confirmed on a 2% agarose gel (Fig. 1). In the step of fragment reassembly, *Pfu* DNA polymerase was used to improve the fidelity of PCR (Fig. 2). One microliter of this reaction was used as template in a 25-cycle PCR. A single band with correct molecular weight was obtained.

A mutant with high aspartase activity was screened from the third round of DNA shuffling. SDS–PAGE showed that the amount of expression of the evolved aspartase was similar to the control one (Fig. 3).

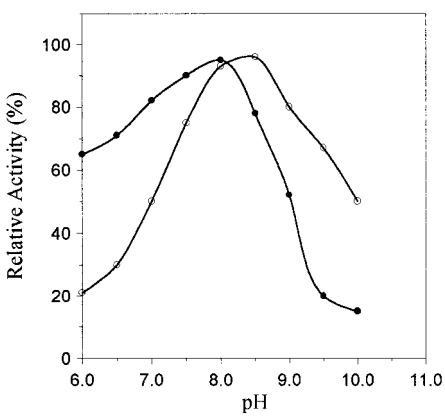

FIG. 6. The pH stability range of native (●) and evolved aspartases (○).

Purification of the wild-type and evolved aspartases. The wild-type and evolved aspartases were purified to homogeneity and confirmed by native PAGE (7.5% gel). The evolved aspartase was very similar to that of wild-type (Fig. 4).

The optimum pH of native and evolved aspartases. As shown in Fig. 5, the optimum pH of wild-type aspartase is 8.0 while the evolved one is 8.2.

The stable pH range of native and evolved aspartases. The stable pH range of evolved enzyme is 7.8–9.3 and the wild-type enzyme is 7.0–8.0 (Fig. 6).

The thermostability of native and evolved aspartases. The evolved aspartase showed an increase of thermostability (Fig. 7). After being incubated for 30 min at 50°C, the wild-type enzyme conserves about 17% of its activity, while evolved one conserves about 61% of that.

Kinetic parameters for evolved aspartase. The Lineweaver–Burk plot was used to obtain kinetic parameters. The k_{cat} and K_m values for the wild-type and evolved aspartases are shown in Table I.

DNA sequence. The DNA sequence of the evolved aspartase gene showed seven nucleotide substitutions and three of them are predicted to cause amino acid changes in the translated aspartase sequence (Table II).

DISCUSSION

Sequencial error-prone PCR does not have apparent effect on enzyme activity because of its lacking of the additive effect of beneficial mutagenesis. Our experi-

TABLE I

Aspartase	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)	K_m (M)
Wild-type	180	1.5×10^5	1.2×10^{-3}
Evolved enzyme	1.09×10^3	4.2×10^6	2.6×10^{-4}

TABLE II

Amino acid	Wild-type	Evolved enzyme
217	AAC Asn (N)	AAG Lys (K)
233	ACA Thr (T)	AGA Arg (R)
278	GTT Val (V)	GTA Val (V)
287	CTG Leu (L)	CTA Leu (L)
367	GTC Val (V)	GGC Gly (G)
371	GCT Ala (A)	GCC Ala (A)
373	TTC Phe (F)	TTT Phe (F)

mental route is to accumulate beneficial mutagenesis by error-prone PCR and then recombine these mutagenesis by high-fidelity DNA shuffling protocol. After four rounds of error-prone PCR and three rounds of DNA shuffling, a mutant with 28-fold aspartase activity was obtained. Our work demonstrated that directed evolution is an efficient method for enhancing L-aspartase activity.

Thr233 is located at the edge of subunit interaction region and near the Lys327 within 15 Å [11]. The evolved Arg233 may bind to the carbonyl of α-carboxylate of the substrate, causing enhanced affinity of aspartase with substrate and stabilizing the carbanionic intermediate. The decreased K_m and enhanced K_{cat} lead to the assignment of a binding role for Arg233. Val367 located at the terminus of the fifth α-helix which is replaced with Gly results in partial disruption of this α-helix, which changes into a turn. However, the basic structure of the five long α-helices has not been disrupted. This is consistent with the conclusion that 20-helix cluster is essential in stabilizing the active conformation of aspartase. In spatial structure, Val367 is located at the edge of binding pocket. The increased flexibility introduced by Gly may be helpful for "induced-fit" of substrate and aspartase. Asn217 is located at the mid of the second α-helix of central domain and its change to Lys has no effect on second structure of aspartase. The side-chain of Lys is of the highest flexibility, even when the conformation of main-chain is rigid. The enhanced thermostability and pH stability of evolved enzyme are assigned to the introduction of side-chain of Lys. The important catalytic residues of the active site, such as Lys327, Ser143, Arg29, are unchanged in the evolved aspartase, implying that the catalytic mechanism of the evolved enzyme remained the same. Further examination of the catalytic mechanism is underway.

Directed evolution of enzyme *in vitro* can rapidly improve enzymatic functions without the need to delineate spatial structure and catalytic mechanism. The analysis of mutations obtained by molecular evolution of proteins provides a new tool for studying structure-function relationships. It is helpful for elucidating the catalytic mechanism of aspartase and is a solid basement for rational designing aspartase randomly in future.

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