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Biochemical and Biophysical Research Communications 331 (2005) 107–112
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The hybrid enzymes from α-aspartyl dipeptidase and L-aspartase

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Received 20 March 2005
Available online 30 March 2005

Abstract

With combinative functionalities as well as the improved activity and stability, the novel hybrid enzymes (HEs) from the heterogeneous enzymes of α-aspartyl dipeptidase (PepE, monomer) and L-aspartase (L-AspA, tetramer) were constructed successfully by gene random deletion strategy. The wild-type hybrid enzyme (WHE) and the evolved hybrid enzyme (EHE) were selected, respectively, upon the phenotype and the enzyme activity. The relative activity of the WHE tested was about 110% of the wild-type PepE and 26% of the wild-type L-AspA, whilst the activity of EHE was about 340% of the PepE and 87% of the L-AspA. In comparison to its individual wild-type enzymes, the EHE exhibited an improved thermostability, when examined at the enzyme concentration of 10^{-7} mol/L, but the WHE showed a reduced thermostability. The activity of the EHE was about 3-fold compared to that of the WHE. The current results give a good example that the hybridization of enzymes could be attained between the monomer and multimer enzymes. In addition, they also indicate that construction hybrid enzyme from evolved enzymes is feasible.

Keywords: α-Aspartyl dipeptidase; L-Aspartase; Hybrid enzyme; Gene random deletion; In vitro selection

Hybrid enzyme has long been the active field that biochemists make the best ever efforts to engineer enzymes of improved functionalities. The desired properties for hybrid enzyme were obtained by in vitro selection. PepE is a monomeric enzyme, consisting of 229 amino acids, with a calculated molecular weight of 24,768 Da. It is a new serine protease and soluble in cytoplasm. Its catalytic site is the special Ser-His-Glu triad [1]. It specifically hydrolyzes the dipeptide at the α-carboxyl group of N terminus. However, it shows a little or no activity to Asp-X dipeptide whose C terminus or N terminus is blocked, as well as to other polypeptides except Asp-Gly-Gly [2,3]. On the other hand, L-AspA (L-aspartate ammonia-lyase, EC 4.3.1.1) is an important industrial enzyme that catalyzes the reversible conversion of fumarate and NH_4^+ to L-aspartate. L-AspA from *Escherichia coli* W is composed of four identical subunits with a molecular weight of 52,200 Da. Each subunit has 477 amino acids constituting three domains, D1, D2, and D3. The central core of D2 forms five long helices that are slightly bent and are nearly parallel to each other. The five-helix bundles of D2 from four subunits form a stable structure of a 20-helix cluster in the tetramer [4]. The inter-subunit contacts maintain the active conformation of L-AspA [5].

Upon directed evolution in our laboratory, we had succeeded in the evolution of PepE and L-AspA, respectively, with improved functionalities. We adopted a new strategy to evolve PepE under the pressures of both catalytic activity and thermostability, following in vitro selection. The catalytic activity and thermostability of the evolved enzyme (EE), pepEM3074, were enhanced dramatically, with a 47-fold increased k_{cat}/K_m compared to that of the wild-type enzyme. The pepEM3074 retained 80% relative activity after incubated at 60 °C

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.03.140

for 30 min [6]. Also, we evolved the L-AspA with a 28-fold increased activity compared to the wild-type enzyme [6–9]. Meanwhile, we constructed D1–D2–D2–D3 of monomeric L-AspA by duplicating the central domain and introducing a random hexapeptide between the repeated D2 units. Through the conjunction of the random hexapeptide loop, the monomeric L-AspA, drAsp017, acquired a 21.3% relative activity of the wild-type enzyme [10].

PepE is a monomer, while L-AspA is a tetramer, and their sequence homology is low. Several hybrid enzymes, such as ITCHY [11], SCRATCHY [12], TV-ITCHY [13], CP-ITCHY [13], THIO-ITCHY [14], SHIPREC [15], RACHITT [16], RID (random insertion/deletion) [17], and ADO [18], have been reported using recombining genes with low or even lack of sequence homology. In order to explore the possibility of constructing the hybrid enzymes from the heterogenous PepE and L-AspA, so as to develop new enzymes with the combinative functions, we constructed using the gene random deletion strategy, respectively, two hybrid enzyme libraries: a wild-type hybrid enzyme library using the wild-type PepE and L-AspA as well as an evolved hybrid enzyme library using the evolved PepE (EPepE) and L-AspA (EAspA). Upon in vitro selection, we obtained the wild-type hybrid enzyme (WHE) and the evolved hybrid enzyme (EHE), respectively.

Materials and methods

Preparation of the hybrid enzyme library. The pUC plasmids that carry the wild-type or evolved PepE (in pUC19) or L-AspA (in pUC18) were previously constructed in our laboratory [6,7]. Since pUC19 and pUC18 are identical in genetic structure except the orientation of their multi-cloning sites (MCS), the *Bam*HI, *Eco*RI, and *Aat*II in the two plasmids were specifically designed so that, the digested products from those two plasmids may be reconstructed into a complete pUC plasmid carrying the hybrid enzyme gene.

The plasmids carrying the enzyme genes were digested, respectively, with *Bam*HI/*Aat*II for pUC19 and *Eco*RI/*Aat*II for pUC18, producing a shorter fragment carrying the gene of PepE and a longer fragment carrying the gene of L-AspA. These two fragments were purified and digested with *Exo*III (MBI Fermentas, Hanover) at 200 U/μl in a 200 μl reaction buffer (50 mM Tris–HCl, pH 7.6, 1 mM $MgCl_2$, and 1 mM DTT). The reaction mixture was incubated at 30 °C for 10 min to generate base deletion pool. *Exo*III was then removed using the EZ Spin Column DNA Extraction Kit UNIQ-10 (Sangon, Shanghai) and the fragments were purified. Since *Exo*III digests recessed 3′ terminus only, it digested *Bam*HI/*Eco*RI restriction site and had no effect on the *Aat*II site. The digested large and small fragments were then ligated at the *Aat*II site using T_4 DNA ligase (TaKaRa, Dalian) to get linear fragments. Subsequently, the resulting fragments were purified and digested with S1 Nuclease (TaKaRa, Dalian) at 37 °C for 30 min producing blunt-ended fragments. Finally, the fragments carrying the hybrid enzyme genes were re-encircled via T_4 DNA ligase into a complete pUC plasmid (pUCHE) containing the library of hybrid enzymes for future selection.

Selection of the hybrid enzyme. The hybrid enzyme gene library was transformed into *E. coli* JRG 1476 $AspA^-$ cells and plated onto the Asp-N selection medium (0.5% L-Asp, 1% glucose, 0.1% $(NH_4)_2SO_4$, 0.7% K_2HPO_4, 0.3% KH_2PO_4, and 0.01% $MgSO_4 \cdot 7H_2O$). Since the cells were $AspA^-$ strain that utilized L-AspA as the sole nitrogen source, the growing colonies were only those capable of expressing L-AspA. Subsequently, the growing colonies were inoculated onto the medium spread with 200 μl of 0.77 mM Asp-pNA, a substrate for which the PepE-expressing colonies hydrolyzed it to produce yellow color. Through selection of both phenotype and catalytic activity, the colonies containing the plasmids that carried hybrid enzyme genes for both PepE and L-AspA were selected. For the expression of hybrid gene in right orientation, restriction site *Sal*I was added to 5′ terminus of the gene using PCR during library construction. For further analysis of the function of the hybrid enzyme, pUCHE was digested with *Sal*I/*Pst*I and the purified hybrid enzyme genes were cloned into pBV220 for the enzyme expression. The selected colonies were transferred into 5 ml of 2× YT liquid medium and cultured at 30 °C until the OD_{600} reached around 1.0, inducing at 42 °C. The hybrid enzymes were purified for activity assay [7,9].

Activity assay. The activity of the hybrid enzyme was determined using the method as described [6,7,10], The thermostability of the hybrid enzyme was measured according to the method as described [10].

Results and discussion

Construction of the hybrid enzyme library

The construction procedure of the hybrid enzyme library by gene random deletion is outlined in Fig. 1. The pUC plasmids carrying the genes of either the PepE and L-AspA or their evolved forms were digested, respectively, with *Bam*HI/*Aat*II and *Eco*RI/*Aat*II,

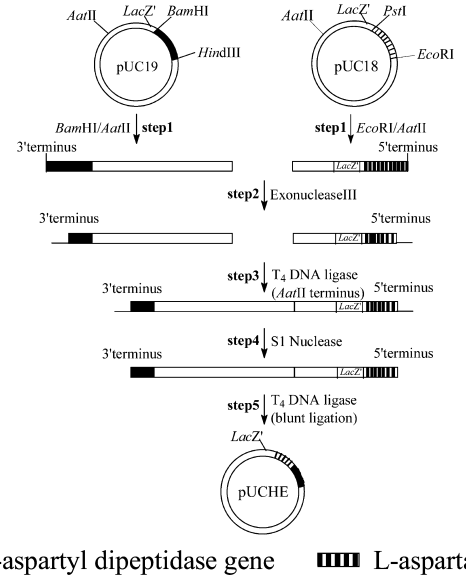

Fig. 1. A schematic strategy for construction of the hybrid enzyme library. Step 1: the pUC plasmids carrying separately the gene of PepE and L-AspA or their evolved forms were digested, respectively, with *Bam*HI/*Aat*II and *Eco*RI/*Aat*II. Step 2: *Exo*III digested the recessed 3′ terminus of the *Bam*HI- and *Eco*RI-digested fragments to generate the linear deletion library. Step 3: the *Aat*II-ends of fragments were ligated. Step 4: S1 nuclease digestion made the cohesive terminus blunt. Step 5: the linear fragments were ligated with T_4 DNA ligase to generate the re-circulated library plasmids of the hybrid enzyme.

generating a longer fragment without *LacZ′* gene and a shorter fragment with *LacZ′* gene. Since exonuclease III (*Exo*III) only recognizes the recessed 3′ ends, the *Bam*HI and *Eco*RI digested ends would be cut by *Exo*III, producing a linear deletion library upon controlling the condition and the time of reaction. Next, the fragments in the library were ligated at the *Aat*II-cutting ends following the digestion of S1 nuclease to blunt up all the remaining termini. Finally, the fragments were ligated creating the circulated plasmid library of the hybrid PepE/L-AspA or their evolved hybrid enzymes.

In vitro selection of the hybrid enzymes

Development of an efficient and accurate method is essential for high-throughput selection of the genetically engineered enzyme. In this study, we applied the method of combined selection of phenotype and the enzyme activity. First, the plasmids containing the established hybrid enzyme gene library were transformed into $AspA^-$ cells growing on the Asp-N culture medium for the selection of L-AspA activity. Then, the plasmids prepared from the grown colonies were plated onto the cultural medium containing Asp-pNA to measure the PepE activity according to the color changes. Finally, the recombinant plasmids from the selected colonies were examined with restriction enzyme digestion and the identified recombinants were expressed, purified, and characterized for hybrid enzymes. Through in vitro selection of 4300 independent colonies each for the two hybrid enzyme libraries, the WHE and EHE, was obtained, respectively. Fig. 2 shows enzyme digestion analysis of the selected hybrid enzyme genes. The sizes of the *Sal*I/*Pst*I digestion fragments of the selected hybrid enzyme genes and the corresponding wild-type genes are consistent with each other, indicating that the selected hybrid enzyme genes maintain the essential constitution of the wild-type genes.

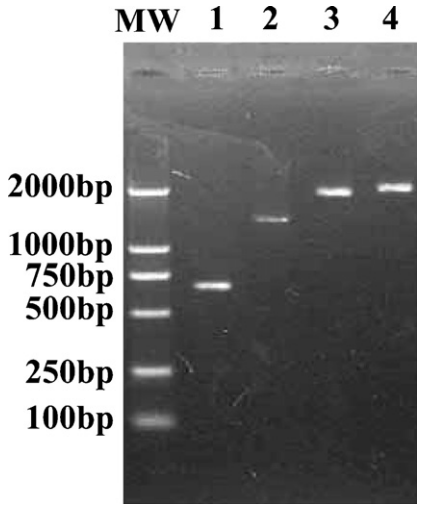

Fig. 2. Digestion analysis of WHE gene and EHE gene from the selected plasmids on a 1.0% agarose gel with Tris–acetate/EDTA as the running buffer. MW, molecular weight DL-2000; lane 1, the wild-type PepE gene (ca. 831 bp); lane 2, the wild-type L-AspA gene (ca. 1431 bp); lane 3, the selected WHE gene (ca. 1.9 kbp); and lane 4, the selected EHE gene (ca. 2.0 kbp).

In order to examine the expressed products of those selected hybrid enzyme genes, the digested WHE and EHE genes were cloned into pBV220, respectively, and the expressed proteins were analyzed on SDS–PAGE (Fig. 3). The results indicated that the molecular weights of the WHE and EHE mono-subunits were 73 and 74 kDa, respectively. Their molecular weights were further refined using the size-exclusion column chromatography (see Fig. 4), which revealed that the molecular weights of WHE and EHE were about 282 and 309 kDa, respectively. These results suggested that the WHE and EHE are likely oligomeric enzymes of four subunits.

Catalytic activity and thermostability

The purity of WHE and EHE was verified with a 12% SDS–PAGE. The kinetic parameters of the hybrid

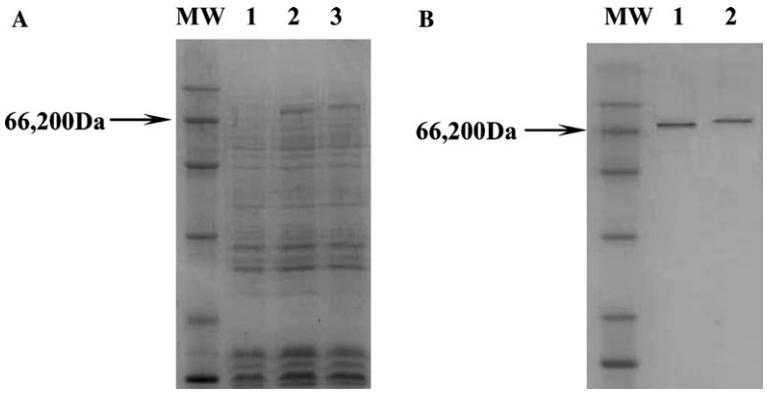

Fig. 3. Analysis of the expressed WHE and EHE (in *E. coli* JRG 1476, pBV220 as vector) on a 12% SDS–PAGE. MW, protein molecular size marker (the bands from top to bottom are 97,400, 66,200, 43,000, 31,000, 20,100, and 14,400 Da bands, respectively). (A) Lane 1, the control (the plain *E. coli* JRG 1476); lane 2, the expression product of the WHE gene; and lane 3, the expression product of the EHE gene. (B) Lane 1, the purified WHE; lane 2, the purified EHE.

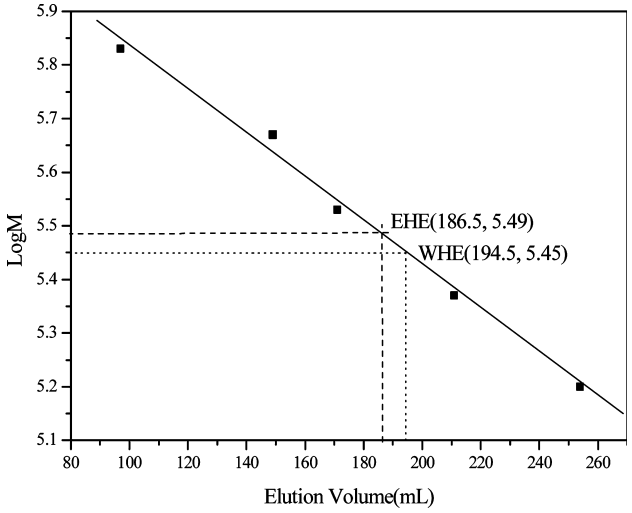

Fig. 4. The size-exclusion column chromatography of WHE and EHE (Sephadex G-200). Column condition: 1.8 × 80 cm; flow rate: 20 ml/h. The following proteins were selected as standards to calibrate the column (molecular weight given in parentheses): aldolase (158 kDa), catalase (232 kDa), fibrinogen (341 kDa), ferritin (470 kDa), and thyroglobulin (670 kDa).

Table 1
Comparison of the kinetic parameters for PepE, EPepE, and HEs

Enzymes	K_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m (s^{-1} M^{-1})	Relative activity (%)
PepE	2.2	3.9×10^{-3}	5.6×10^{2}	100
EpepE	57.2	2.2×10^{-3}	2.6×10^{4}	4640
WHE	2.1	3.4×10^{-3}	6.2×10^{2}	110
EHE	4.4	2.3×10^{-3}	1.9×10^{3}	340

Table 2
Comparison of the kinetic parameters for L-AspA, EAspA, and HEs

Enzymes	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m (s^{-1} M^{-1})	Relative activity (%)
L-AspA	1.8×10^{2}	1.2×10^{-3}	1.5×10^{5}	100
EaspA	1.1×10^{3}	2.7×10^{-4}	4.1×10^{6}	2730
WHE	2.4×10^{2}	6.2×10^{-3}	3.9×10^{4}	26
EHE	8.2×10^{2}	6.4×10^{-3}	1.3×10^{5}	87

enzymes were determined and compared with those of the wild-type PepE and L-AspA. The results in Tables 1 and 2 show that EHE had a relative activity of nearly three times higher than that of WHE. In comparison with their corresponding wild-type enzymes, the thermostability of WHE (see Figs. 5A and B) tested was in general declined to varying extent for either PepE or L-AspA activities. But, EHE exhibited a better thermostability over WHE for both the wild-type enzyme activities.

The selected HE genes were sequenced. Deduction of those sequences revealed that the WHE gene was 1989 bp, in which 33 bp (corresponding to the 11 amino acids of PepE at C terminus) and 72 bp (corresponding to 24 amino acids of L-AspA at N terminus) were deleted. Similarly, EHE lost four amino acids at C terminus of the EPepE and 21 amino acids at N terminus of the EAspA, respectively.

A structure model for the subunit of WHE was simulated using Swiss-MODEL (see Fig. 6). A partial structure of PepE was grafted onto the N terminus of L-AspA based on the sequence and the model structure analyses. However, the spatial structure of L-AspA, in particular its central domain D2, was not destroyed. This had ensured the formation of four subunit oligomeric enzyme. Compared with both wild-type PepE and L-AspA, the

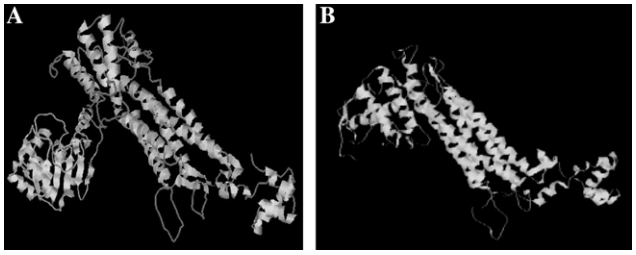

Fig. 6. Comparison for subunit structure of WHE and wild-type L-AspA. (A) The subunit of WHE; (B) the subunit of L-AspA. The helices, strands, and loop are shown as ribbons, arrows, and lines, respectively.

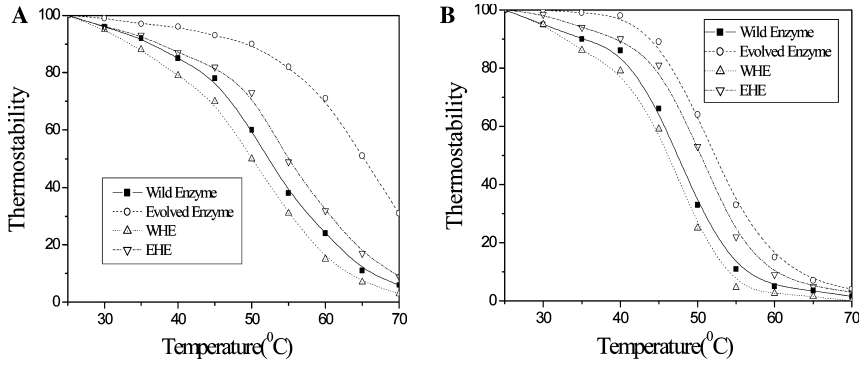

Fig. 5. The thermostability of WHE and EHE. The enzymes were incubated at different temperatures for 30 min, and then the remaining catalytic activities were detected under standard enzyme assay conditions. The relative activity is represented as a percentage of maximum activity. (A) Thermostability of wild-type enzyme, EPepE, WHE, and EHE for the PepE. (B) Thermostability of wild-type enzyme, EAspE, WHE, and EHE for the L-AspA.

relative activity of WHE was 110% (that is, the activity of monomeric PepE was 27.5%) and 26%, respectively, and the relative activity of EHE was 340% (equaled to about relative 85% activity of monomeric PepE) and 87%, respectively. In general, the hybrid enzyme constructed by gene random deletion has homogeneous region with corresponding wild-type enzymes, that is, there is similarity or similar folding pattern between the two enzymes. Therefore, the less similarity both the enzymes have, the lower activity the hybrid enzyme possibly possesses, even no activity, vice versa [19], which is a possible phenomenological reason for the activity of hybrid enzyme being lower than those of wild enzymes or starting enzymes. While, because of the directed evolution for PepE and L-AspA, the enzyme activity as well as thermostability of EHE were higher than that of both WHE and wild-type enzyme. Owing to each subunit still having catalytic activity after the monomer changed into an oligomer, the PepE activities of both WHE and EHE were higher than that of wild enzyme, that is, the cumulative results made the PepE activity of hybrid enzymes enhance significantly. The activity of EHE was about 3-fold compared to that of WHE, which was possible due to the fact that the formation of EHE nearly equaled the fusion of two evolved genes. As mentioned before, in EHE, only four amino acids of C terminus in PepE were deleted, in contrast, 21 amino acids of N terminus in L-AspA were deleted. Moreover, the deleted amino acid residues had little effect on the activity. For the flexibility of conformation in active center [20], with the hybridization of the two evolved enzymes takinf place, the optimized balance between the rigidity (for stability) and the flexibility (for activity) was rebuilt through the apparent trade-off [21], which reshaped the active center gradually [22,23] and improved the adaptability of EHE to environment evidently.

Conclusion

Constructing hybrid enzyme from α-aspartyl dipeptidase and L-aspartase or their evolved enzymes, specifically for hybrid method for heterogeneous enzymes, especially for monomeric and oligomeric enzymes, was provided. At the same time, monomeric enzyme turned into oligomeric enzyme with enhanced stability or activity. On the other hand, the strategy described here may serve as a model system for the directed evolution of enzyme with a 20-helix cluster or the similar structure as fumarase–L-aspartase family. In addition, the hybrid enzyme we constructed can be used in the synthesis of the precursor for aspartame. Synthesizing aspartate from fumarate and NH_4^+, and then taking advantage of the catalytic action of α-aspartyl dipeptidase, the precursor of aspartame was produced. The cooperation was improved by hybrid enzyme, as well, the reactions of multi-step and multi-enzyme were simplified, and multi-steps were converted into one-step. It cannot only reduce the synthesis cost, but also accelerate the reaction rate and have great significance in industry.

Acknowledgments

We thank Dr. J.R. Guest (University of Sheffield, UK) for providing *E. coli* JRG 1476 ($AspA^-$) strain. We thank Dr. Dongyun Hao, Dr. Zhengyun Zhao, and Dr. Zhonghua Yan for comments and valuable discussions on the manuscript.

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