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BIOCATALYSIS

Construction and evaluation of a novel bifunctional phenylalanine–formate dehydrogenase fusion protein for bienzyme system with cofactor regeneration

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Abstract Phenylalanine dehydrogenase (PheDH) plays an important role in enzymatic synthesis of L-phenylalanine for aspartame (sweetener) and detection of phenylketonuria (PKU), suggesting that it is important to obtain a PheDH with excellent characteristics. Gene fusion of PheDH and formate dehydrogenase (FDH) was constructed to form bifunctional multi-enzymes for bioconversion of L-phenylalanine coupled with coenzyme regeneration. Comparing with the PheDH monomer from *Microbacterium sp*., the bifunctional PheDH–FDH showed noteworthy stability under weakly acidic and alkaline conditions (pH 6.5–9.0). The bifunctional enzyme can produce 153.9 mM L-phenylalanine with remarkable performance of enantiomers choice by enzymatic conversion with high molecular conversion rate (99.87 %) in catalyzing phenylpyruvic acid to L-phenylalanine being 1.50-fold higher than that of the separate expression system. The results indicated the potential application of the PheDH and PheDH–FDH with coenzyme regeneration for phenylpyruvic acid analysis and L-phenylalanine biosynthesis in medical diagnosis and pharmaceutical field.

Keywords Bifunctional enzyme · Biocatalyst · Biosynthesis · Cofactor regeneration · Phenylalanine dehydrogenase · Formate dehydrogenase

Introduction

In biotechnology and molecular biology field, enzymes owing two or more combined activities with advisable stability have been wide applied [29]. Although the diversity of natural enzymes provides some candidates with bifunctional activity, most fusion enzymes caused by the fusion of individual enzymes in vitro were constructed based on a well-defined structure and evolutionary characteristics [5, 29]. Plenty of artificial fusion enzymes showing noticeable performance have been reported [1, 9, 11, 20, 28, 35], and they were simply built by means of end-to-end fusion [6, 12, 34] or using a linker to tether the whole genes which encode the intact functional proteins [9, 20]. Gene fusion is a key technique which facilitates gene function research and biochemical studies. Overlap PCR, an effective method of gene fusion, has been extensively used in introduce insertions, point mutations, deletions or replacements into any locus of a gene and construct fusion protein [23, 29].

Chiral amino acids as crucial chiral drug intermediates are mainly biosynthesized by amino acid dehydrogenases [4]. Phenylalanine dehydrogenase (EC 1.4.1.20, PheDH) catalyze the conversion of phenylpyruvate to L-phenylalanine in reductive amination with NADH as coenzyme [2, 14, 33]. The PheDH has received much attention as it is a key biocatalyst in biosynthesis of the phenylalanine in food industry and of pharmaceutical peptides in pharmaceutical industry [25, 31]. The enzyme has also been widely used in the diagnostic kits as biosensors to detect the blood serum of neonates for PKU (phenylketonuria) detection [13, 27,

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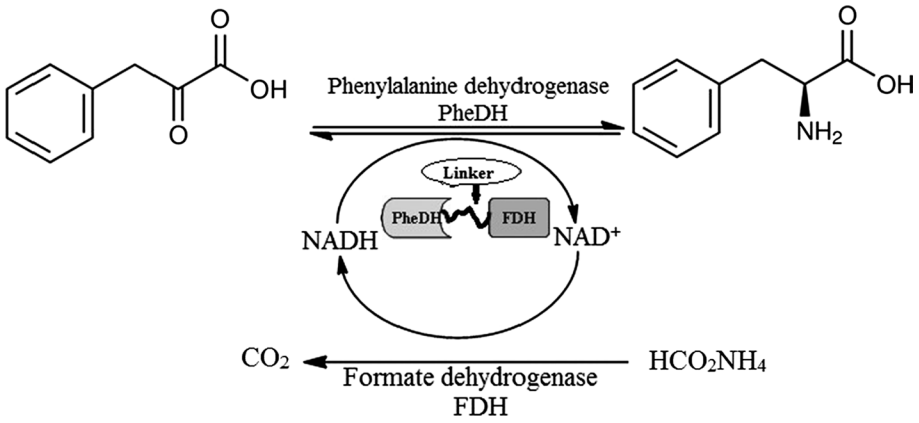

Scheme 1 The co-enzyme regeneration system for quick phenylpyruvic acid analysis and L-phenylalanine biosynthesis

32]. However, the consumption of coenzyme in the reductive amination reaction is very expensive while the co-enzyme regeneration can be used to reduce the costs of co-factor, suggesting the significance of constructing a co-enzyme regeneration system.

The functional fusion of two or more enzymes with respect to enzymatic catalysis and reaction kinetics would offer several advantages exceeding individual enzymes. It had been demonstrated that proper linker peptides, which were inserted between individual enzymes, could reduce folding interference from one to each other for the fusion enzyme to function as independently as possible [8]. Thus, in this study, a linker peptide (GGGGS) was used to tether a novel PheDH from marine bacteria *Bacillus halodurans* and a formate dehydrogenase (FDH) from *Candida boidinii* for constructing a co-enzyme regeneration system (Scheme 1) to quickly analyze phenylpyruvic acid and high-efficiency production of L-phenylalanine. In addition, the performance of the fusion enzyme, a bifunctional fusion enzyme composed of PheDH and FDH, was characterized, assessed and compared with the performances of the separately expressed proteins.

Materials and methods

Strains, vectors, enzymes and reagents

The vectors, pET-28a-*pdh* containing the PheDH gene (1140 bp, Gene ID: 893554) from *Bacillus halodurans* and the pET-28a-*fdh* containing the FDH gene from *Candida boidinii* were constructed in our laboratory (data unpublished); and the sequence information of the FDH gene from yeast *Candida boidinii* (ATCC 32195) was obtained as previously reported [18]. *Escherichia coli* DH5α and *Escherichia coli* BL21 (DE3) were used as host strains for cloning and heterologous expression, respectively.

All enzymes were recruited from TaKaRa Co., Ltd. (Dalian, China). Agarose Gel DNA Fragment Recovery Kit Ver.2.0 and Purification Kit Ver 2.0 were purchased from TaKaRa Co., Ltd. (Dalian, China). Phenylpyruvate acid, L-phenylalanine, D-phenylalanine and NAD(H) were purchased from Sigma-Aldrich Co. (Shanghai, China). All the other chemicals were chromatographically pure or analytically graded and used without further purification, and they were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Construction of bifunctional fusion enzymes

For construction of PheDH–FDH fusion enzymes, the *pdh* gene was amplified from the pET-28a-*pdh* with two primers, BnPDH-H-F1 (GGAATTCCATATGATGCTAACG AAAACGCCAACTGTCAC, containing *Nde*I sites, underlined) and BnPDH-H-R1 (*GCTACCACCACCACC*TTT ACGTAAGTTCCATTTCGGCCGG, containing liker sequence, italic), as forward and reverse primers, respectively. The two primers, FDH-H-F2 (*GGTGGTGGTGGT AGC*ATGAAAATTGTCCTGGTCCTGTATG, containing liker sequence, italic) and FDH-H-R2 (CCCAAGCTTTT ACTTTTTATCGTGTTTGCCATAG, containing *Hind*III sites, underlined), as forward and reverse primers, respectively, were used to amplify the *fdh* gene with the pET-28a-*fdh* as template. The resulting fragments were mixed and it was used as the template to amplify the fusion gene using the two primers, BnPDH-H-F1 and FDH-H-R2. Then, the PCR products were purified for construct pET-28a-*pdh-fdh* to express the fusion enzyme in *Escherichia coli* BL21 (DE3). The PCR product was cloned on the pET-28a (+) vector by ligation using T4 ligase at the restriction enzyme cutting sites of *Nde*I and *Hind*III. The recombinant plasmids, pET-28a-*pdh-fdh*, were then transformed into *Escherichia coli* DH5α and incubated at 37 °C. The expected fusion genes (about 2.2 kb) which encode the fusion enzymes PheDH–FDH were verified by DNA sequencing.

Expression and purification of fusion enzyme

The recombinant plasmids, pET-28a-*fdh*, pET-28a-*pdh* and pET-28a-*pdh-fdh* were transformed into *Escherichia coli* BL21 (DE3) competent cells to overexpress PheDH, FDH and PheDH–FDH, respectively. Then, these bacteria were cultured in 200 mL of LB liquid medium containing kanamycin (100 μg/mL) at 37 °C, isopropyl-b-D-thiogalactopyranoside (IPTG) (0.1 mM) was added for induction until the optical density of bacterial culture at 600 nm (OD 600) reached about 0.6–0.8. After 8 h incubation at 20 °C with shaking at 200 rpm, the bacterial cells were collected by centrifugation, resuspended twice in 100 mM HEPES (pH 7.0) and broken by High-Pressure Homogenizer (Niro Soavi, Germany). After centrifugation at 12,000 rpm for 30 min, the supernatant, his-tagged enzyme was purified by an AKTA Prime system equipped with a 10-mL Ni-IDA column (GE Healthcare, USA). Finally, the results of expression and purification of the enzyme were checked by 12 % SDS-PAGE according to the method of Laemmli [7] and the pure protein concentration was determined by Bradford Protein Assay Kit.

Enzyme assay

PheDH activity was measured by NADH degradation or generation with phenylpyruvate or phenylalanine as substrate, respectively, as previously described by Hamid Shahbaz Mohamadi and Eskandar Omidinia [26] with moderate modifications. PheDH activity for oxidative deamination was assayed at 25 °C by the increase of NADH (monitored at 340 nm) with L-phenylalanine or D-phenylalanine as substrate. The reaction mixture (1.0 mL) contained 4 mM NAD^+, 40 mM glycine–KCl–KOH buffer (pH 10.4), 20 mM D-phenylalanine or L-phenylalanine, and moderate enzyme (0.01 mg/mL, the final concentration). The PheDH activity for the reductive amination was determined at 25 °C by measuring the consumption of NADH at 340 nm ($\varepsilon = 6220\ M^{-1}cm^{-1}$) with a Spectra MaxM5 Microplate Reader (Thermo) in the reaction mixture (1.0 mL) containing 50 mM glycine–KCl–KOH buffer (pH 10.4), 20 mM sodium phenylpyruvate, 100 mM $NH_3{\cdot}H_2O$–NH_4Cl buffer, 0.09 mM NADH with moderate enzyme (0.01 mg/mL, the final concentration). Similarly, FDH activity was measured by detecting the production of NADH in the oxidative ammonium formate at 340 nm. One unit (U) of enzyme activity was defined as the quantity of enzyme catalyzing the consuming or formation of 1 μmol NADH per min under standard conditions. Specific activity was recorded as units/mg protein.

The kinetic parameters of PheDH were assayed by varying the concentration of phenylpyruvate or phenylalanine (2–50 mM) at a fixed NADH (0.1 mM) while the kinetic parameters of FDH were determined through varying the concentration of formate (2–50 mM) at a fixed NAD^+ (0.2 mM). All reactions were performed under the standard assay conditions (Except special statement) and the above reactions were repeated three times. The values of kinetic parameters K_m and k_{cat} were determined using Lineweaver–Burk double-reciprocal plot.

Measurement of fusion enzyme stability

For the part of the reductive amination or oxidative deamination, the optimal pH and temperature of PheDH were 7.0 and 60 °C, and the optimum condition for the FDH was 10.0 and 60 °C (data unpublished). The thermal stability of the PheDH–FDH and free enzymes was determined under the optimal pH by pre-incubating the enzymes at the temperatures from 37 to 80 °C for 1 h and the residual enzyme activity was measured as described above. The pH stability of the enzymes was assayed at 4 °C for 24 h in different buffer systems (pH 6.0–9.5), and then the remaining activity was measured under the standard methods. The buffer at pH 6.0–9.5 was 0.2 M $NH_3{\cdot}H_2O$–NH_4Cl buffer (pH 6–11.0) for the PheDH of the reductive amination and FDH. The stability of the oxidative deamination of the PheDH–FDH was performed with the same methods, except the buffers were 0.2 M barbital sodium–hydrochloric acid buffer (pH 6.0–9.0) and 0.05 M glycine–sodium hydroxide buffer (pH 8.6–11).

Enzymatic production of L-phenylalanine by the bifunctional enzyme complex

The transformed bacteria, carrying pET-28a-*fdh*, pET-28a-*pdh* and pET-28a-*pdh-fdh*, respectively, were inoculated, induced, cultivated, collected, centrifuged (8000 rpm, 10 min), and washed with phosphate buffer (0.2 M, pH 8.0) three times, collected (each was 0.79 mg, wet weigh) and the thallus was stored at −80 °C for 12 h. After that, pET-28a-*pdh* containing pET-28a-*fdh* and pET-28a-*pdh-fdh* was thawed at 40 °C and resuspended with $NH_3{\cdot}H_2O$–NH_4Cl buffer (1.5 M, pH 8.0). The conversion tests were implemented in a total volume of 20 mL reaction systems (I, II, III and IV) containing NADH (5 mM), 1.5 M $NH_3{\cdot}H_2O$–NH_4Cl buffer (pH 8.0), 1.0 M $COONH_4$ and phenylpyruvate acid. The elements of reaction systems (I, II, III and IV) were similar, except for containing a distinct concentration of phenylpyruvate acid for each system (25, 50, 100 and 300 mM, respectively, and equal to 4.13, 8.25, 16.5 and 49.5 g/L). The enzymatic reactions were performed at 40 °C, 200 rpm for assaying the optimum concentration of the substrate. 100–300 μL was sampled during 0–72 h. Meanwhile, pH was adjusted to 7.0–9.0. To detect the pure enzyme catalytic ability, the conversion tests were

Table 1 Apparent kinetic parameters for the coupled or native PDH and FDH

Enzyme	Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
PDH-His	Phenylpyruvic acid	0.34 ± 0.1	10.3 ± 0.7	30.56
PDH–FDH-His		0.87 ± 0.21	5.03 ± 0.3	5.75
PDH-His	L-phenylalanine	6.88 ± 0.41	2.49 ± 0.28	0.36
PDH–FDH-His		5.78 ± 0.68	0.008 ± 0.001	0.002
FDH-His	$COONH_4$	10.1 ± 1.03	2.18 ± 0.22	0.22
PDH–FDH-His		21.7 ± 0.81	0.34 ± 0.09	0.02

The PDH and PDH–FDH had no activity with the D-Phe as substrate. Data represent the mean ± standard deviation of triplicate samples

implemented in a total volume of 3 mL reaction systems containing NADH (2 mM), 0.5 mM $NH_3{\cdot}H_2O$–NH_4Cl buffer (pH 8.0), and 8.3 or 18.6 mM phenylpyruvate acid under the same condition.

The samples were detected by high-performance liquid chromatography (HPLC) using Agilent 1200 HPLC with a Chirex 3126 column (150 × 4.6 mm, Chirex 3216 purchased from Phenomenex) at 35 °C; eluent: 2 % $CuSO_4$ aqueous solution:isopropanol = 95:5 (v/v), flow rate: 1.0 mL/min; detection wavelength: 254 nm [19]. The concentration of L-phenylalanine or D-phenylalanine was quantified by the peak areas of L-phenylalanine or D-phenylalanine with pure L-phenylalanine or D-phenylalanine as a standard. The conversion of phenylpyruvate acid was calculated as follows: molecular conversion (%) = $M_f/M_t \times 100$, M_f and M_t were the final concentration of L-phenylalanine and the original substrate concentration of phenylpyruvate acid, respectively. The enantiomeric excess (e.e.) of L-phenylalanine was calculated as follows: e.e. (%) = $(A_1 - A_2)/(A_1 + A_2) \times 100$, A_1 and A_2 were the peak areas of L-phenylalanine and D-phenylalanine, respectively [21].

Results

Expression and purification of PheDH–FDH fusion protein

The recombinant plasmids, pET-28a-*fdh*, pET-28a-*pdh* and pET-28a-*pdh-fdh*, were transformed and expressed in *Escherichia coli* BL21 (DE3). After purification by the AKTA Prime system, the recombinants were harvested and exhibited to a single band, with the purified PheDH–FDH presenting the expected molecular mass (82.6 kDa) (Fig. S1b) while free PheDH and FDH exhibiting 41.8 and 40.3 kDa, respectively (Fig. S1a). The size of the fusion enzyme was akin equal to the sum of two free enzymes [17], indicating that the bifunctional fusion protein was successfully constructed.

Kinetic parameters

Kinetic parameters were determined according to the Michaelis–Menten plot. Kinetic parameters, K_m, k_{cat} and k_{cat}/K_m, of the PheDH, FDH and PheDH–FDH are presented in (Table 1). The K_m values of fusion PheDH–FDH for phenylpyruvic acid or formate was much higher than that of free enzymes. The catalytic constant k_{cat} and catalytic efficiency constant k_{cat}/K_m of fusion PheDH–FDH for phenylpyruvic acid, L-phenylalanine or $COONH_4$ were lower than that of free enzymes. This phenomenon that fused enzyme showed lower activity than the free enzyme had been reported [24]. However, kinetic parameters of K_m of fusion PheDH–FDH for L-phenylalanine displayed lower than that of native PheDH. In fact, the bifunctional fusion enzyme of PheDH–FDH displayed a higher overall productivity rate in producing L-phenylalanine than that of free enzymes, suggesting the advantages of bifunctional fusion enzyme. And the PheDH–FDH was no activity when using D-phenylalanine as substrate, suggesting that the fusion enzyme has positive enantio-selectivity.

Measurement of fusion enzyme stability

For the part of the reductive amination, the purified PheDH–FDH showed significant stability under less than 40 °C. After 1 h incubation at pH 7.0 (Fig. 1a), the PheDH–FDH retained approximately 80 % of its maximal activity under less than 40 °C, but less than 10 % over 50 °C. Like the parent, the purified PheDH–FDH showed prominent stability under weakly acidic and alkaline conditions (pH 6.5–9.0), retained about 80 % of the maximum activity over a pH range from 6.5 to 9.0 for 24 h at 4 °C, and exceeded 40 % of the maximal activity at pH 6.0 and 9.5 (Fig. 1b).

The purified PheDH–FDH showed better prominent stability than that of its parent under weakly acidic and alkaline conditions (pH 6.5–9.5), and retained about 80 % of the maximum activity over a pH range from 6.5 to 9.5 for 24 h at 4 °C while the purified PheDH retained less activity under the same condition in the oxidative deamination

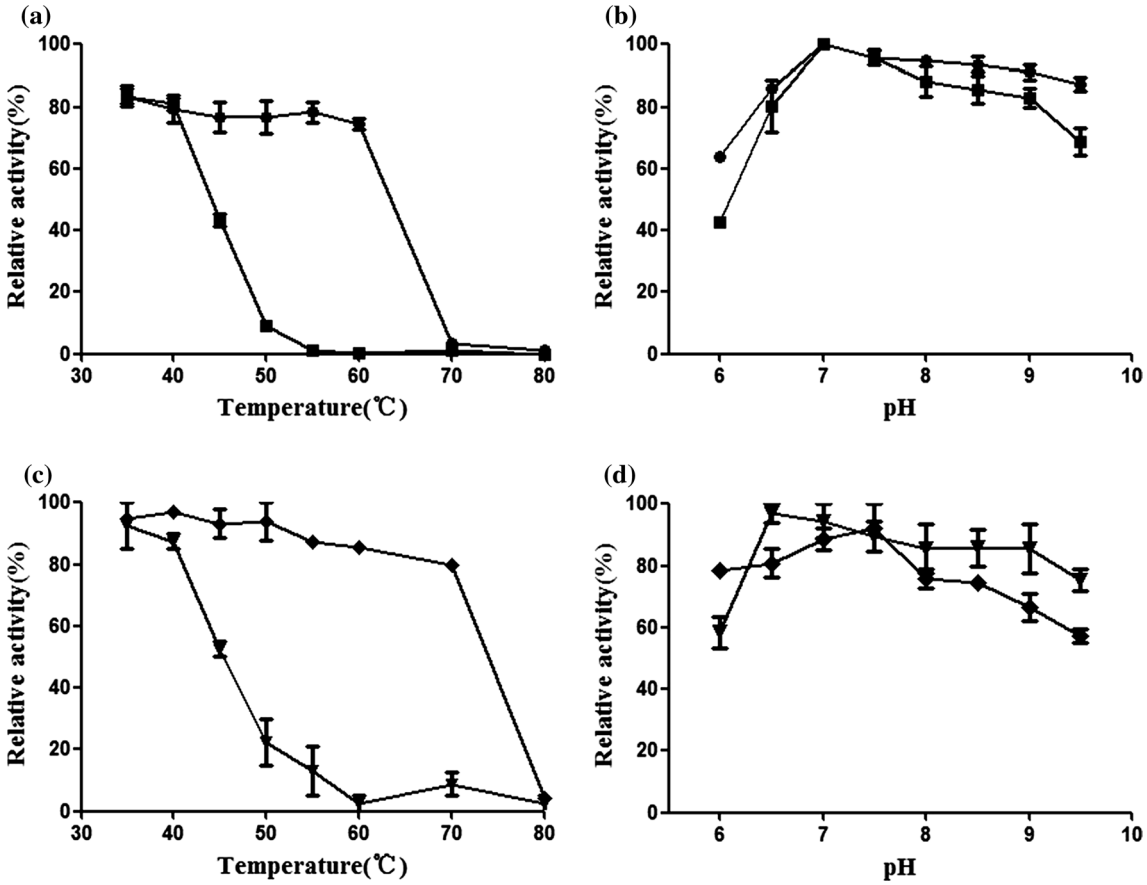

Fig. 1 Effects of temperature and pH on PheDH–FDH and PheDH stability. **a** Effect of temperature on the stability of the PheDH (*black circles*) and PheDH–FDH (*black squares*) of reductive amination. At the optimal pH 7.0, the purified enzyme was pre-treated at a different temperature for 1 h. The activity of the enzyme without pre-incubation was defined as 100 %. **b** The pH stability of the PheDH–FDH and PheDH was determined by incubating the enzymes at a different pH at 4 °C for 24 h of reductive amination. Then assays were conducted in the standard conditions and the enzyme activity without pre-treatment was taken as 100 %. **c** Effect of temperature on the stability of the PheDH (*Black squares*) and PheDH–FDH (*black triangle*) of oxidative deamination. At the optimal pH 7.0, the purified enzyme was pre-treated at a different temperature for 1 h. The activity of the enzyme without pre-incubation was defined as 100 %. **d** The pH stability of the PheDH–FDH and PheDH was determined by incubating the enzymes at a different pH at 4 °C for 24 h of oxidative deamination. Then assays were conducted in the standard conditions and the enzyme activity without pre-treatment was taken as 100 %. *Error bars* represent the standard deviation

(Fig. 1d). Similarly, the purified PheDH showed significant stability under the condition at high temperature (40–70 °C) while the purified PheDH–FDH showed significant stability when the temperature was lower than 40 °C. After 1 h incubation under pH 7.0 (Fig. 1c), the PheDH–FDH retained approximately 82 % of its maximal activity under less than 40 °C, but less than 10 % over 60 °C. Without any stabilizer, the purified PheDH–FDH with $COONH_4$ as the substrate retained more than 60 % of its maximum activity over temperature ranges from 37 to 45 °C, but less than 10 % over 55 °C (Fig. 2a). The purified PheDH–FDH with $COONH_4$ as the substrate retained approximately 80 % of the maximum activity over a pH range from 7 to 10.5 for 24 h at 4 °C (Fig. 2b).

Comparison of the fusion enzyme and individual enzymes

To further appraise the performance, the yield of L-phenylalanine generated by the PheDH–FDH was compared with the yield of the separate enzyme system. Enzymatic reactions were applied and the L-phenylalanine was detected by HPLC. The molar conversion of pure separate enzymes system was 97.93 % (1.36 g/L, at the 2nd h) and 74.30 % (2.14 g/L, at the 2nd h) at 8.3 mM and 17.0 mM, respectively (Fig. 3a, b). The molar conversion of pure fusion enzyme system was 83.50 % (1.05 g/L, at the 2nd h) and 67.05 % (1.86 g/L, at the 2nd h) at 8.3 and 17.0 mM, respectively (Fig. 3b), indicating their significant potential

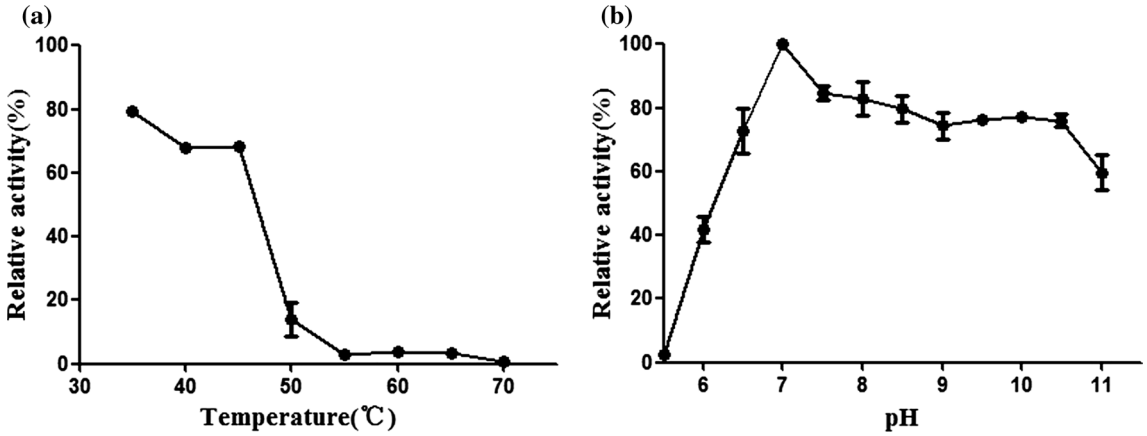

Fig. 2 Effects of temperature and pH on PheDH–FDH stability with $COONH_4$ as the substrate. **a** Effect of temperature on the stability of the PheDH–FDH (*black circles*). At the optimal pH 7.0, the purified enzyme was pre-treated at a different temperature for 1 h. The activity of the enzyme without pre-incubation was defined as 100 %. **b** The pH stability of the PheDH–FDH was determined by incubating the enzymes at a different pH at 4 °C for 24 h. Then assays were conducted in the standard conditions and the enzyme activity without pre-treatment was taken as 100 %. *Error bars* represent the standard deviation

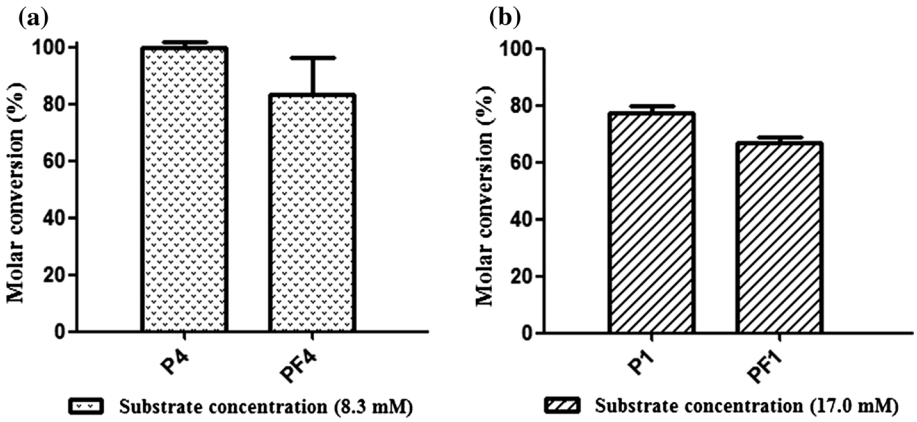

Fig. 3 The molecular conversion of the bifunctional enzyme and separate expression system with pure enzymes. **a** The concentration of substrate (phenylpyruvic acid) was 8.3 mM. **b** The concentration of substrate (phenylpyruvic acid) was 17.0 mM. *Error bars* represent the standard deviation

in synthesis of L-phenylalanine. The optimum concentration of substrate (phenylpyruvic acid) of separate expression system (containing pET-28a-*fdh*, and pET-28a-*pdh*) was intended to be 100 mM based on the molar conversion at 25, 50, 100 and 300 mM were 62.88 % (at the 12th h), 43.50 % (at the 72nd h), 66.80 % (at the 1st h), 52.03 % (at the 1st h), respectively (Fig. S2 and Fig. 4). The concentration of L-phenylalanine was 2.01, 4.15, 11.10 and 25.92 g/L in the reaction systems I, II, III and IV, respectively. Similar, the optimum concentration of substrate (phenylpyruvic acid) of pET-28a-*pdh-fdh* system was determined to be 100 mM based on the molar conversion at 25, 50, 100 and 300 mM, which were 39.11 % (at the 72nd h), 74.30 % (at the 24th h), 99.87 % (at the 12th h), 51.29 % (at the 1st h), respectively (Fig. S3 and Fig. 4). The concentration of L-phenylalanine was 1.42, 6.16, 16.82 and 25.56 (153.9 mM) g/L in the reaction systems I, II, III and IV, respectively. The e.e. of L-phenylalanine was calculated as follows: e.e. $(\%) = (A1 - A2)/(A1 + A2) \times 100$, $A1$

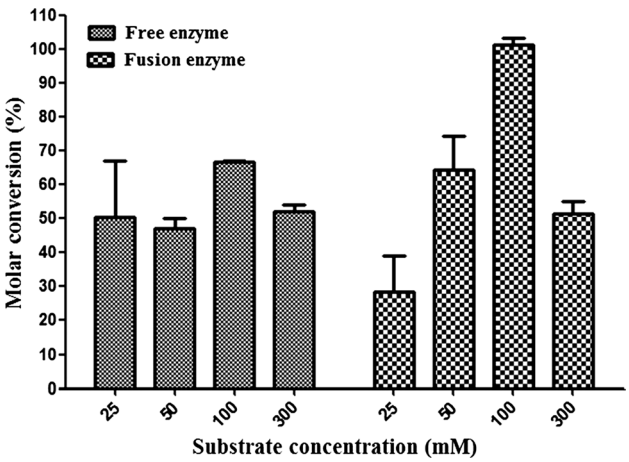

Fig. 4 The maximum molecular conversion of the bifunctional enzyme and separate expression system. The concentration of substrate (phenylpyruvic acid) in the enzymatic reaction systems I, II, III and IV were 25, 50, 100 and 300 mM, respectively. *Error bars* represent the standard deviation

and *A*2 were the peak areas of L-phenylalanine and D-phenylalanine, respectively. The value of e.e. was more than 99 % in all above reactions, suggesting the native PheDH and bifunctional PheDH–FDH have excellent performance of enantiomers choice. Obviously, the conversion yield of the fusion enzymes was significantly higher than that of the separately expressed enzymes.

Discussion

Artificial fusion enzyme with multi-function broadens the application range of native enzymes, and it had been applied to cell signals, expression, localization and ligand interaction [29]. The fusion enzyme, showing better performance compared with the individual, was employed in the biotechnology field in the synthesis of ethylene and oligosaccharide [10, 20, 36], and bifunctional enzymes as a potential biocatalyst and a monitoring facility have been widely applied in molecular biology and biotechnology fields. L-phenylalanine, a crucial intermediate, was mainly used for synthesis of enantiomerically non-natural amino acids, as drug precursors, Alzheimer's disease and aspartame in food and pharmaceutical industries [4, 15]. As PheDH plays a primary role in the biosynthesis of L-phenylalanine, coenzyme addition was bound to increase costs, suggesting the importance of constructing a coenzyme regeneration system for biosynthesis of L-phenylalanine. The specific linker, GGGGS, may influence a proper distance when the two moieties to be fused to affect the enzyme activity [22], and proper linker peptides can work on reducing the folding interference for the two portions of a fusion enzyme to keep independently as soon as possible [22]. In this paper, the results clearly demonstrated that the bifunctional PheDH–FDH, possessing the ability of coenzyme regeneration and L-phenylalanine biosynthesis, was constructed. The potential commercial value of propargylglycine made it attractive to use PheDH as a catalyst to chirality modification pure L- and D-enantiomers [30]. The PheDH–FDH which was constructed in this work showed no activity with the D-phenylalanine as substrate, indicating that it has preferable enantiomers choice.

To the best of our knowledge, this is the first report of build, assessment and characterization of a bifunctional fusion enzyme by tethering a novel PheDH from *Bacillus halodurans* and an FDH from *Candida boidinii* for coenzyme regeneration and application in L-phenylalanine biosynthesis. Like *Microbacterium sp*. PheDH for which approximately half the enzyme activity was lost after incubation at 65 °C for 1 h [3], the PheDH–FDH showed less than 20 % of its maximal activity from 55 to 80 °C (Fig. 1a, c). The weak activity of the artificial fusion protein was mainly caused by the structural instability of fusion enzymes [17]. Like the parent, the PheDH–FDH showed prominent stability under weakly acidic and alkaline conditions (pH 6.5–9.5) (Figs. 1b, d, 2b).

The optimum concentration of substrate (phenylpyruvic acid) for both the separate expression system (containing pET-28a-*fdh*, and pET-28a-*pdh*) and the fusion enzyme (pET-28a-*pdh-fdh*) was intended to be 100 mM (Fig. 4, S2 and S3). After 11 h, the L-phenylalanine concentration decreased significantly in the III and IV reaction systems, possibly because of the mass consumption of the coenzymes in the forward reaction; thus, the reaction would be performed in the reverse reaction. Without adding pET-28a-*fdh*, the molar conversion of pET-28a-*pdh* enzyme system was less than 10 % from 0 to 72 h (data not show).

The analysis of the result suggests that the pET-28a-*pdh-fdh* has a higher conversion rate with time saving and cost reducing benefits, indicating it has a high potential for industrial production. While the catalytic efficiency of PheDH–FDH was about 0.19-fold lower than that of native enzymes, the molecular conversion rate in the bifunctional fusion enzyme system was 1.50-fold higher than that of separate expression system, suggesting that the bifunctional PheDH–FDH showed high performance in coenzyme regeneration and L-phenylalanine accumulation. This phenomenon may be caused by the formation of the substrate channel, making the product of the first reaction to be quickly transferred to the second enzyme, which leads to the reduction of the time of diffusion and thus increasing the overall reaction rate [16]. The amount of phenylpyruvic acid can be measured by the fusion enzyme, which indicated the PheDH–FDH bienzymes coupled with coenzyme regeneration system can be used to detect PKU.

In conclusion, a novel bifunctional fusion enzyme, PheDH–FDH, with excellent performance was constructed, characterized and applied in coenzyme regeneration and the enzymatic synthesis of L-phenylalanine. The PheDH–FDH showed prominent stability than the *Microbacterium sp*. PheDH under weak acidic and alkaline conditions. The resulting fusion enzyme PheDH–FDH expressed in a single host showed better performance than that of the separately expressed enzymes. The molecular conversion rate of the bifunctional enzyme was 1.50-fold higher than that of the separate expression system. The PheDH and fusion PheDH–FDH with coenzyme regeneration have the potential application for phenylpyruvic acid analysis and the enzymatic production of L-phenylalanine in food and pharmaceutical industries.

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Compliance with ethical standards

Conflict of interest The authors have declared no conflict of interest.

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