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Expression of the *las*B gene encoding an organic solvent-stable elastase in *Pichia pastoris* and potential applications of the recombinant enzymes in peptide synthesis

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ABSTRACT

The *las*B gene encoding a solvent-stable elastase from *Pseudomonas aeruginosa* (PAE) was isolated and heterologously expressed in *Pichia pastoris*, resulting in production of three heterogeneously glycosylated recombinant elastases (rPAEs). rPAEs showed higher solvent-stability and thermostability than native PAE, but these recombinant and native enzymes achieved similar values of specific activity (2393 U/mg and 2427 U/mg for rPAEs and the native one, respectively), apparent K_m (2.55 and 2.48 g/l for rPAEs and the native one, respectively) and k_{cat} (0.0489 and 0.0496/s for rPAEs and the native one, respectively) for casein hydrolysis. While rPAEs and their native counterpart displayed similar substrate specificity in bipeptide synthesis reactions in water-miscible organic solvents, the former gave higher synthesis rates and yields than the latter. The yields and rates of rPAEs-catalyzed bipeptide synthesis reactions substantially varied with the type of solvent, and dimethylsulfoxide (DMSO) was found to be more suitable for these reactions than methanol, ethanol, isopropanol, and *n*-butanol. The optimal reaction conditions for rPAEs-catalyzed Cbz-Ala-Phe-NH_2 synthesis were the presence of 50% (v/v) DMSO, and at pH 8.0 and temperature 20–30 °C.

1. Introduction

The *Pseudomonas aeruginosa* elastase (PAE), also named pseudolysin (EC 3.4.24.26), is a 33 kDa zinc metalloprotease encoded by the *las*B gene. It is synthesized as a preproenzyme of 498 amino acids (aa) consisting of a signal peptide (23 aa), a propeptide (174 aa), and mature elastase (301 aa) [1]. PAE has proved to be useful for peptide synthesis in organic solvents due to its strong stability in both water-miscible and water-immiscible organic solvents [2–4]. It has higher solvent-stability than many commonly used proteases, namely subtilisin, thermolysin, α-chymotrypsin, and *Aspergillus oryzae* protease [4]. Furthermore, it not only has high catalytic activity [5,6] but also can give considerably high equilibrium yields of peptide synthesis reactions in organic solvents [7]. On the other hand, the application of PAE as a catalyst for peptide synthesis is still limited because of two reasons: its host *P. aeruginosa* is an opportunistic pathogen which may cause a variety of diseases including lung infections associated with cystic fibrosis, burn wound infections, urinary tract infections, the lower respiratory tract infections, hospital-acquired pneumonia, and keratitis; and the fermentation of *P. aeruginosa* generates toxic metabolites [8–10]. Thus, expression of PAE in a safe host is essential for promoting its application in peptide synthesis.

With the development of molecular biotechnology, much attention has been paid to the heterologous expression of a recombinant elastase. The *las*B gene was previously successfully cloned and expressed in *Escherichia coli* system [1,11]; however, the expressed recombinant enzyme failed to be secreted into the culture medium, and its expression level was also low and unable to meet the demand of industrial applications. On the other side, the *Pichia pastoris* expression system with the *AOX1* promoter has been applied extensively for decades for secretive production of various recombinant heterologous proteins, yielding high levels of foreign proteins free of endotoxins as well as oncogenic and viral DNA [12,13]. Thus, it may be feasible to express PAE in *P. pastoris* for facilitating its industrial applications.

The objectives of this study were to isolate the *las*B gene from *P. aeruginosa* C11 and heterologously express it in *P. pastoris*, and to investigate the expressed recombinant elastases (rPAEs) for their activity of catalyzing peptide synthesis in organic solvents. Our results suggested that the rPAEs heterologously

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expressed in *P. pastoris* may be promising biocatalysts for peptide synthesis.

2. Materials and methods

2.1. Materials

N-carbobenzoxy-L-alanine (Cbz-Ala), N-carbobenzoxy-L-arginine (Cbz-Arg), N-carbobenzoxy-L-aspartic acid (Cbz-Asp), N-carbobenzoxy-L-threonine (Cbz-Thr), L-leucineamide (Leu-NH_2), and L-phenylalaninamide (Phe-NH_2) were obtained from Shanghai Hanhong Chemical Co., Ltd. DMSO (dimethylsulfoxide), methanol, ethanol, isopropanol, *n*-butanol, isoamyl alcohol, chloroform, methylbenzene, dimethylbenzene, *n*-hexane, and *n*-octane were purchased from Sinopharm (Shanghai, China).

2.2. Construction of the expression vector

P. aeruginosa C11 was previously isolated in our lab [14]. Its genomic DNA was extracted for amplifying the DNA sequence encoding the propeptide and mature protein of PAE via PCR using the forward (5′-GAGAATTCCTGATCGACGTGTCCAAACT-3′) and the reverse primer (5′-AACCTAGGAAGACGGCTTGAGCGACC-3′), which contain the restriction sites (the underlined) of *Eco*RI (Fermentas, Canada) and *Avr*II (Fermentas, Canada), respectively. The PCR amplification was performed under the following conditions: initial denaturing at 96 °C for 5 min; subsequent 25 cycles of denaturing at 96 °C for 40 s, annealing at 58 °C for 30 s, and extension at 74 °C for 2 min; and a final extension at 72 °C for 5 min. The PCR products were analyzed on a 1% (w/v) agarose gel by electrophoresis. The target band about 1.5 kb in length was incised from the gel, purified, and then inserted into a pGH-T cloning vector (Generay, Shanghai, China). The correct insertion of the target DNA fragment was confirmed by sequencing. The inserted DNA fragment was released from pGH-T vector by digestion with *Eco*RI and *Avr*II, and then ligated into the pPIC9K digested by the same enzymes. Successful insertion of the target DNA into pPIC9K was confirmed by sequencing.

2.3. Transformation of the expression vector

The recombinant pPIC9K (Invitrogen) harboring the inserted gene sequence was linearized with *Sac* I (Fermentas, Canada). Next, with Gene Pulser (Bio-Rad), the linearized plasmid was electroporated into competent cells of *P. pastoris* KM71 (Invitrogen), which were prepared according to the manufacturer's instructions. The transformed cells were then plated on minimal dextrose (MD) agar and cultured at 28 °C for 4 days.

2.4. Expression of rPAEs

A *P. pastoris* transformant was cultured in 25 ml of BMGY medium at 28 °C for 24 h. Cells were collected by centrifugation at 5000 × *g* at 4 °C for 5 min and resuspended in 10 ml of BMMY medium. Then the cell suspension was incubated at 22 °C with shaking at 250 rpm for 5 days, and 0.1 ml of methanol was added to the medium per day.

2.5. Purification of rPAEs and native PAE

The culture of a *P. pastoris* transformant was centrifuged at 10,000 × *g* for 5 min; the culture supernatant was then collected, followed by gradually adding solid ammonium sulfate to the supernatant to 80% saturation. The mixture was subsequently centrifuged at 10,000 × *g* for 5 min; the resulting precipitate was collected and then dissolved in 25 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.0). The resultant crude protein solution was loaded into a Sephadex G-75 gel filtration column and eluted with 50 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.0). The fractions with caseinolytic activity were pooled and concentrated by using ultrafiltration spin columns. Then the concentrated sample was added into a DEAE Sepharose Fast-Flow column equilibrated with 10 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.0). Elution was performed with a linear gradient of 10 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.0) containing NaCl at a concentration from 0 M to 0.5 M. Fractions with caseinolytic activity were pooled, concentrated, and then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The native PAE from *P. aeruginosa* C11 was purified as previously described [14].

2.6. Caseinolytic activity assay

A 50 μl-aliquot of the enzyme solution prepared in the above section was added to 2.0 ml of 1% (w/v) casein (Sigma, USA) solution containing 50 mM barbital/HCl (pH 7.0) and incubated at 60 °C for 30 min. The reaction was terminated by adding 2.0 ml of 10% (w/v) trichloroacetic acid, and the mixture was incubated at 37 °C for 20 min and then centrifuged at 10,000 × *g* for 10 min. One milliliter of the resulting supernatant was added to 5 ml of 2% (w/v) Na_2CO_3 containing 0.4% (w/v) NaOH and 1 ml Folin–Ciocalteu reagent, and the mixture was then incubated at 40 °C for 15 min. Its absorbance was read at 750 nm. One caseinolytic activity unit (U) was defined as the amount of enzyme that gives an absorbance equivalent to 1 μg tyrosine liberated per minute under the test conditions.

2.7. SDS-PAGE

The purity and molecular weights of proteins were determined using SDS-PAGE with 12% acrylamide according to the method of Laemmli [15]. The protein molecular weight marker used in SDS-PAGE was purchased from Shanghai Generay Biotech Co., Ltd. (Generay, Shanghai, China). The polyacrylamide gel was stained with Coomassie brilliant blue R-250 (Generay, Shanghai, China).

2.8. Peptide synthesis reactions

Peptide synthesis reactions were performed in the presence of 50% (v/v) various solvents and 50 mM sodium phosphate buffer (pH 7.0). The substrate concentrations were 50 mM for Cbz-Ala, Cbz-Asp, Cbz-Thr, and Cbz-Arg; and 100 mM for Phe-NH_2 and Leu-NH_2. Each reaction contained 22 mg/l rPAEs or native PAE. The reaction mixture was incubated at 30 °C for 2 days for determining the peptide synthesis yield or 1 h for determining the peptide synthesis rate. The theoretical yield of a peptide synthesis reaction was equivalent to the concentration of the carboxyl component (50 mM), the limiting substrate in the reaction, while its actual yield was the concentration of the synthesized bipeptide, which was determined by HPLC. The yield of peptide synthesis (percent yield) was calculated according to the following formula:

$$\text{The yield of peptide synthesis}(\%) = \frac{\text{The actual yield of peptide synthesis}}{\text{The theoretical yield of peptide synthesis}} \times 100\%.$$

2.9. Analysis of high-performance liquid chromatography (HPLC)

Synthesized bipeptides were quantified according to the following method: 0.1 ml of 2 M HCl was added to 1 ml of reaction mixture to terminate the enzymatic reaction; 5 min later, the mixture was diluted 10 times with the eluent of HPLC. An aliquot of the diluted mixture was analyzed using reversed-phase HPLC