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Enzymatic production of L-ornithine from L-arginine with recombinant thermophilic arginase

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ABSTRACT

In this study, to develop a simple and efficient enzymatic production process for the environment-friendly synthesis of L-ornithine from L-arginine, the *Escherichia coli* BL21 (DE3) strain overexpressing arginase (ARG) from *Bacillus caldovelox* was chosen as the potential biocatalyst. The biochemical properties of the recombinant ARG were characterized and compared with those of the native enzyme. The maximal conversion rate of L-arginine to L-ornithine was 87.1% with a final L-ornithine concentration of 112.3 g/L under the following optimal conditions: 170 g/L L-arginine, 12 g/L whole-cell biocatalyst, 10 μM Mn^{2+}, 60 °C, pH 9.0, and 4 h of incubation. When compared with a recent work, the biocatalytic process described in the present study achieved higher average L-ornithine synthesis rate of 26.2 g/L/h, and thus has great potential for large-scale production of L-ornithine.

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1. Introduction

L-Ornithine, which plays an important role in the urea cycle, is a non-protein amino acid with various bioactive functions and applications in many fields, including health care, drug manufacturing, and chemical industry [1,2]. Ornithine has the ability to detoxify excess ammonia in the human body and thus has significant effect on liver cells [3]. Furthermore, L-ornithine is effective for treating liver diseases, strengthening the heart, and protecting against endotoxin-induced shock, and can promote protein synthesis and catabolism of sugar and fat [4,5]. Therefore, L-ornithine is an ideal nutritional supplement for bodybuilders and athletes. Owing to its multiple functions in health care, L-ornithine has a sizable market worldwide. Hence, a simple, efficient, and energy-saving method for L-ornithine production is needed.

In recent years, several effective methods have been used for the preparation of L-ornithine, such as chemical synthesis methods [6,7], microbial fermentation [8–10], and enzymatic methods [11,12]. In chemical synthesis methods, acrolein, hydrogen cyanide, ammonia, and CO_2 are used as raw materials, and DL-ornithine is obtained after a multi-step reaction [6]. However, these methods could not be widely used owing to their limitations such as low yield, poor purity, and difficulty in product separation [7]. Microbial fermentation is employed for producing L-ornithine from citrulline or arginine auxotrophic microorganisms such as *Corynebacterium glutamicum* and *Arthro bactericitreus* [8,10]. The auxotrophs resistant to mycophenolic acid or ornithinol are capable of producing higher yields of L-ornithine than conventional auxotrophs, with the highest L-ornithine yield reaching 50 g/L [10]. The use of fed-batch fermentation could further increase the production of L-ornithine to 70 g/L in 60 h [9]. However, the complex composition of the fermentation broth could make subsequent product separation difficult. Hence, some researchers have turned to enzymatic synthesis of L-ornithine involving arginase (ARG; E.C. 3.5.3.1) [11–13]. Purified ARG is rarely used for L-ornithine production [12,13], and 72.7 g/L L-ornithine could be obtained in 10 h. However, enzyme isolation and purification can be relatively expensive and time-consuming. Recently, L-ornithine was produced by using whole-cell (recombinant ARG) biotransformation in the presence of surfactants, with the L-ornithine yield reaching 111.52 g/L after 15 h [11]. However, whole-cell biotransformation always has the problem of cell permeability, and addition of permeability reagent may lead to difficulties in subsequent product separation. Therefore, a simple and effective method for L-ornithine production is required.

In the present manuscript, the ARG-encoding gene from the thermophilic bacterium *Bacillus caldovelox* was expressed in *Escherichia coli* for the conversion of L-arginine to L-ornithine,

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and an attempt was made to shorten the reaction time and solve the problem of cell permeability without adding any permeability reagent. The kinetic parameters of the recombinant thermophilic ARG and the effects of biotransformation conditions were investigated.

2. Materials and methods

2.1. Materials

The expression plasmid pET-28a (+) and the host strain *E. coli* BL21 (DE3) were obtained from Novegen (Masdison, WI). The T vector, restriction enzymes (*Nco*I and *Hind*III), T4 DNA ligase, plasmid mini-preps kit, and agarose gel DNA purification kit were supplied by TaKaRa Biotechnology (Otsu, Japan). L-Ornithine, L-arginine, and phthaldialdehyde were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Expression in E. coli

The published sequence of the ARG gene from *B. caldovelox* was modified by using codon optimization. The synthesized ARG gene was inserted into the multicopy plasmid pET-28a (+) by introducing the *Nco*I and *Hind*III restriction sites, and then the obtained plasmid pET28a (+)-ARG was transformed into *E. coli* BL21 (DE3). Luria-Bertani (LB) medium (10 g/L peptone, 5 g/L yeast extract, and 10 g/L NaCl; pH 7.0) was used for seed culture. The seed culture was incubated on a reciprocal shaker (200 rpm) at 37 °C in a 250-mL flask containing 25 mL of LB medium (supplemented with 0.1 g/L kanamycin sulfate) for 6 h. Fermentation was conducted in a 500-mL flask containing 50 mL of Terrific Broth (TB) medium (24 g/L yeast extract, 12 g/L peptone, 5 g/L glucose, 2.31 g/L KH_2PO_4, 16.43 g/L K_2HPO_4, and 0.1 g/L kanamycin sulfate; pH 8.0) at 37 °C and 200 rpm in a rotary shaker. When the optical density at 600 nm (OD_{600}) reached 0.6, IPTG was added to the culture at a final concentration of 0.1 mM for induction. The culture of recombinant strain without IPTG induction was used as the control.

2.3. Enzyme purification and SDS-PAGE analysis

The recombinant ARG was purified by using a facile purification procedure comprising a heat-treatment step [14]. The intact cells were collected and resuspended in lysis buffer (100 mM Tris, 50 mM $MnCl_2$, pH 7.5) and lysed by sonication (power 285 W, ultraphonic 4 s, pause 4 s, total 20 min). After centrifuged at 200,000 × *g* for 1 h at 4 °C, the obtained supernatant was rapidly heated to and incubated at 70 °C for 15 min. The insoluble material was removed by centrifugation at 30,000 × *g* for 15 min at 4 °C. Solid ammonium sulphate was added to the supernatant to 30% saturation and the solution was centrifuged at 30,000 × *g* for 10 min at 4 °C. The supernatant was made 50% saturated by further addition of ammonium sulphate and the solution was centrifuged as before. The pellet was solubilized in 200 mM Tris buffer (pH 7.5) and centrifuged as before. The supernatant was dialysed against 20 mM Tris, pH 7.5, overnight at 4 °C. The purified enzyme was obtained after dialysis and further centrifugation. The SDS-PAGE analysis was performed with 5% and 10% polyacrylamide gel slabs for concentration and separation, respectively (Bio-Rad Laboratories, Hercules, CA). After electrophoresis, the gels were stained with Coomassie brilliant blue R-250, and the protein bands in the gels were visualized after destaining the gels in destaining solution.

2.4. Measurement of ARG activity

To measure the ARG activity, the reaction mixture containing 20 mM L-arginine and enzymes was incubated with 50 mM Tris–HCl buffer (pH 9.0) in a final volume of 2 mL at 60 °C. The reaction was stopped after 15 min by adding 1.5 M hydrochloride. The L-ornithine concentration in the mixture was measured as described by Chinard [15], and L-ornithine monohydrochloride was used as the standard. One ARG unit corresponds to the amount of enzyme that could generate 1 μmol L-ornithine per minute from L-arginine by hydrolysis. The protein concentration was determined by the Bradford method [16], with bovine serum albumin as the standard.

2.5. Determination of the kinetic parameters of the recombinant ARG

2.5.1. Determination of optimal pH and pH stability

The optimal pH and pH stability of the recombinant ARG were determined at a pH range of 6.0–12.0 by using different solutions, including 50 mM sodium phosphate buffer (pH 7.0, 7.5), 50 mM Tris–HCl buffer (pH 8.0, 8.5, 9.0), 50 mM glycine–NaOH buffer (pH 9.5, 10.0, 10.5), 50 mM sodium bicarbonate buffer (pH 10.5, 11.0, 11.5), and 50 mM KCl–NaOH buffer (pH 12.0). For measuring the pH stability of the recombinant ARG, the enzyme was incubated at the indicated pH for 12 h at 4 °C and then the enzyme activity was measured as described previously. The relative activities were expressed as the percentage of maximum enzyme activity.

2.5.2. Determination of optimal temperature and temperature stability

The optimal temperature of the recombinant ARG was determined by measuring the enzyme activity at various temperatures (30–90 °C) as described earlier. To measure the temperature stability of the recombinant ARG, the enzyme activity was determined after incubating it at the indicated temperature for 0.5 h. The activity of ARG at each time point was normalized as the percent of enzyme activity at time zero at each corresponding temperature.

2.5.3. Effect of metal ions on enzyme activity

To determine the effect of metal ions on the recombinant ARG, the enzyme activity was measured as described earlier in the presence of various metal ions (Cu^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, Fe^{2+}, Mg^{2+}, and Zn^{2+}) at a concentration of 0.1 mM. The relative activity determined in the absence of metal ions at 60 °C for 15 min was taken as 100%.

2.5.4. Determination of K_m and V_{max}

The kinetic parameters of the recombinant ARG were determined by using reaction mixtures containing variable amounts of L-arginine (2–40 mM) in Tris–HCl buffer (50 mM, pH 9.0) at 60 °C. The K_m and V_{max} values were calculated by the Lineweaver–Burk plotting method.

2.6. Production of recombinant ARG in E. coli

The frequently used TB medium was employed to determine the ARG activity, and the conditions for the expression of the recombinant ARG were examined.

2.7. Production of L-ornithine by transformation of L-arginine with ARG

The transformation reaction was optimized under the following conditions: 25 mL of 0.1 M carbonate buffer solution (pH 9.0) in a 250-mL flask on a rotary shaker (150 rpm) at 60 °C. To optimize L-ornithine production, different concentrations of L-arginine (120–200 g/L), intact cells (0–21 g/L, wet cell weight), and Mn^{2+} (10^{-2}–10^{-6} M) were employed and the reactions were analyzed.

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