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Enzymatic production of agmatine by recombinant arginine decarboxylase

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ABSTRACT

In this study, a novel process for enzymatic production of agmatine from L-arginine using recombinant arginine decarboxylase (ADC) was established. The *speA* gene encoding ADC was expressed in *Escherichia coli* BL21 (DE3) in a soluble and active form, and recombinant ADC exhibited a maximum specific activity of 0.53 U mg^{-1} following optimization of culture conditions using an orthogonal array experiment. Up to 14.3 g l^{-1} of agmatine was obtained from 20 g l^{-1} of L-arginine in 6 h under optimum conditions (3.5 g l^{-1} intact cells, 4 mM Mg^{2+}, 30 mM pyridoxal-5′-phosphate (PLP), pH 7, 37 °C). This represents a significant improvement in a method for production of agmatine.

1. Introduction

Agmatine is a polyamine that induces a variety of physiological and pharmacological effects and possesses great therapeutic potential. In the central nervous system, agmatine binds to various receptors and is considered a novel neurotransmitter [1,2]. This compound also elicits antinociceptive, antidepressive and anxiolytic properties [3–5]. In the cardiovascular system, recent studies indicated that agmatine can decrease heart rate and blood pressure [6,7], and is therefore a potential treatment for cardiovascular diseases such as hypertension, ischemia, diabetes, atherosclerosis and angiogenesis [8]. In addition, agmatine is an effective regulator of cellular proliferation [9] and has been used successfully for preventing inflammation [10]. Due to its multiple functions in the health care industry, agmatine has a sizable market worldwide, and a simple, efficient, environmentally friendly method for agmatine production is needed.

At present, the industrial production of agmatine is reliant on chemical synthesis, with 1,4-butanediamine or 1,4-butylenedibromide as starting materials, and agmatine sulfate or hydrochloride is obtained after a multi-step process involving nitroguanidine, deoxidation, deprotection or substitution, aminolysis and guanidine reaction [11,12]. However, there are problems associated with the large-scale industrial production of agmatine using chemical synthesis: the reaction steps are difficult and they generate toxic intermediates that pose a significant environmental hazard. Development of a biotechnological approach to agmatine production is therefore highly desirable, and an enzymatic method could improve productivity, reduce energy consumption, improve working conditions and mitigate pollution [13].

Agmatine can be produced via decarboxylation of L-arginine by the enzyme arginine decarboxylase (ADC; EC 4.1.1.19). ADC is a pyridoxal-5′-phosphate (PLP)-dependent enzyme that is widespread in microorganisms, plants and animals [14–16]. It has been found in two forms: biodegradative and biosynthetic. The biodegradative form is encoded by the *AdiA* gene, which is induced in some strains of *Escherichia coli* when grown in acidic conditions containing arginine. An important role of this enzyme is the regulation of pH via the consumption of protons [17]. Biosynthetic ADC is encoded by the *speA* gene, which is expressed in *E. coli* grown in minimal media at neutral pH. This enzyme is unique since it requires both Mg^{2+} and PLP as cofactors for activity [18,19]. Biosynthetic ADC catalyzes the decarboxylation of L-arginine to form agmatine, although reports on the enzymatic synthesis of agmatine are scarce.

In this study, the *speA* gene was cloned and ADC was overexpressed in *E. coli* under optimized conditions resulting from an

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orthogonal array experiment. Under optimal conditions, agmatine could be produced from L-arginine in an efficient, cost-effective manner. The novel method developed in this study could form the basis of a much-improved industrial agmatine production process utilizing ADC biotransformation.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

The expression vector pET-20b(+) and the expression host *E. coli* BL21 (DE3) were purchased from Novagen (Madison, WI, USA). The restriction enzymes *Nco*I and *Hind*III and DNA extraction and plasmid Miniprep kits were purchased from TaKaRa (Dalian, China). Primer synthesis and DNA sequence analysis were performed by Sangon (Shanghai, China). Isopropyl-D-thiogalactopyranoside (IPTG), PLP and agmatine sulfate were purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

The *E. coli* BL21 strain was used for cloning. The strain was routinely grown in lysogeny broth (LB, 10 g l^{-1} peptone, 5 g l^{-1} yeast extract and 10 g l^{-1} NaCl; pH 7.0) at 37 °C in 250 ml baffled flasks on a rotary shaker at 200 rpm. Growth was followed by measuring the optical density at 600 nm. The growth medium was supplemented with ampicillin (100 μg ml^{-1}) as required, and expression was induced with the addition of 0.4 mM IPTG.

2.2. Expression of ADC

The *speA* gene was amplified by PCR using *E. coli* BL21 genomic DNA as a template. The sequences of primers used for cloning were 5′-CATGCCATGGCAATGTC TGACGACATGTCTATG-3′ and 5′-CCCAAGCTTG TTACTCATCTTCAAGATAAGTA-3′ (*Nco*I and *Hind*III restriction sites are underlined). The amplified DNA fragment was ligated into the *Nco*I and *Hind*III sites of pET-20b(+) to yield pET-20b(+)-speA. This plasmid was transformed into *E. coli* BL21, and the transformed expression host was selected on LB agar containing ampicillin. A positive colony was used to inoculate LB medium in a 250 ml flask containing 100 μg ml^{-1} ampicillin and culturing was performed at 37 °C with shaking at 200 rpm. When the culture density at 600 nm reached 0.6, IPTG was added at a final concentration of 0.4 mM and induction continued for 4 h. The culture of recombinant strain without IPTG induction was used as the control.

2.3. Enzyme purification and SDS-PAGE analysis

The recombinant ADC was purified by using a modified purification procedure of Wu and Morris [18]. The intact cells were collected and resuspended in 50 mM Tris–HCl buffer (pH 7.5), and sonicated by Ultrasonic Cell Disruptor (power 285 W, ultraphonic 4 s, pause 4 s, total 10 min). The cell debris was removed by centrifugation at 20,000 × *g* for 45 min at 4 °C, and solid ammonium sulfate was added to the supernatant to 39% saturation (pH 6.0) and the solution was centrifuged at 8000 × *g* for 15 min at 4 °C. The supernatant was made 53% saturated by further addition of ammonium sulfate and the solution was centrifuged as before. The precipitate was solubilized in 100 mM Tris–HCl buffer and centrifuged as before. The supernatant was dialysed against 10 mM Tris–HCl, pH 7.5, overnight at 4 °C. The dialysate was centrifuged at 20,000 × *g* for 20 min and the supernatant was rapidly heated to and incubated at 62 °C for 10 min. The insoluble material was removed by centrifugation at 20,000 × *g* for 15 min at 4 °C. The purified enzyme was obtained after further dialysis and centrifugation. The SDS-PAGE analysis was performed with 5% and 10% polyacrylamide gel slabs for concentration and separation, respectively (Bio-Rad Laboratories, 100 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. ADC activity assay

ADC activity assay mixtures (2 ml) contained 50 mM Tris–HCl pH 7.5, 4 mM $MgSO_4$, 1 mM PLP, 0.1 mM dithiothreitol, and 10 mM L-arginine. Reactions were initiated by the addition of 200 μl of crude cell extract and incubated for 15 min at 40 °C. Reactions were stopped with the addition of 1/5 volume of 40% trichloroacetic acid. The concentration of the agmatine product was determined by HPLC as described previously [20,21] with modifications as described below. One unit of ADC activity is defined as the amount of enzyme required to produce 1 μmol of agmatine per min from L-arginine at 40 °C and pH 7.5. The specific activity is expressed as activity units per mg of protein.

HPLC analysis of agmatine was performed by automatic pre-column derivatization with *o*-phthalaldehyde (OPA) and separated on an Acclaim 120 C18 column (250 mm × 4.6 mm × 5 μm). Fluorescent derivatives were detected by excitation at 330 nm and emission at 465 nm. Samples (8 μl) were mixed with 4 μl of OPA derivatization reagent (30 mg OPA in 1 ml methanol, 53 μl ME and 9 ml 0.5 M potassium borate buffer pH 9.2) and stored at −20 °C for no more than 7 days before use. The polar eluent was 10 mM KH_2PO_4 pH 5.3 (buffer A), and the nonpolar eluent was a 5:3:1 (v/v/v) mixture of acetonitrile, methanol and 10 mM KH_2PO_4 (buffer B). The gradient was applied as follows: 0 min 80% A, 4 min 73% A, 8 min 50% A, 12 min 30% A, 16 min 25% A, 20 min 20% A, 24 min 40% A, 28 min 60% A, 32 min 80% A. A flow rate of 1 ml min^{-1} was maintained throughout.

2.5. Protein concentration determination

Protein concentration was determined by the Bradford method [22], using BSA as the reference standard.

2.6. Biochemical characterization of recombinant ADC

2.6.1. Determination of optimal pH and temperature

The optimal pH and pH stability of recombinant ADC were investigated in the pH range 6.0–9.0 using 0.2 M sodium phosphate buffer (pH 6.0–7.0) and 50 mM Tris–HCl buffer (pH 7.0–9.0). For determination of pH stability, ADC was incubated at the indicated pH for 2 h at 4 °C and enzyme activity was measured as described above. The relative activities were expressed as a percentage of the maximum enzyme activity. The optimal temperature was determined by measuring enzyme activity between 20 °C and 80 °C, and the effect of temperature on enzyme stability was evaluated over the same temperature range for 60 min. Samples were withdrawn at specific time intervals and the residual relative activity was measured.

2.6.2. Effect of metal ions on enzyme activity

To determine the effect of metal ions on recombinant ADC, the enzyme activity was measured as described above in the presence of various metal ions (Cu^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Mg^{2+} and Zn^{2+}) at a concentration of 0–8 mM. The activity in the presence of 0.1 mM Mg^{2+} at 40 °C for 15 min was taken as 100%.

2.6.3. Determination of K_m and V_{max}

The kinetic parameters of the recombinant ADC were determined by using reaction mixtures containing variable amounts of L-arginine (5–50 mM) in Tris–HCl buffer (50 mM, pH 7.5) at 40 °C.