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ENZYMATIC SYNTHESIS OF L-TRYPTOPHAN FROM D,L-2-AMINO-Δ2-THIAZOLINE-4-CARBOXYLIC ACID AND INDOLE BY *Pseudomonas* sp. TS1138 L-2-AMINO-Δ2-THIAZOLINE-4-CARBOXYLIC ACID HYDROLASE, *S*-CARBAMYL- L-CYSTEINE AMIDOHYDROLASE, AND *Escherichia coli* L-TRYPTOPHANASE

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L-Tryptophan (L-Trp) is an essential amino acid. It is widely used in medical, health and food products, so a low-cost supply is needed. There are 4 methods for L-Trp production: chemical synthesis, extraction, enzymatic synthesis, and fermentation. In this study, we produced a recombinant bacterial strain pET-tnaA of *Escherichia coli* which has the L-tryptophanase gene. Using the pET-tnaA *E. coli* and the strain TS1138 of *Pseudomonas* sp., a one-pot enzymatic synthesis of L-Trp was developed. *Pseudomonas* sp. TS1138 was added to a solution of D,L-2-amino-Δ2-thiazoline-4-carboxylic acid (DL-ATC) to convert it to L-cysteine (L-Cys). After concentration, *E. coli* BL21 (DE 3) cells including plasmid pET-tnaA, indole, and pyridoxal 5'-phosphate were added. At the optimum conditions, the conversion rates of DL-ATC and L-Cys were 95.4% and 92.1%, respectively. After purifying using macroporous resin S8 and NKA-II, 10.32 g of L-Trp of 98.3% purity was obtained. This study established methods for one-pot enzymatic synthesis and separation of L-Trp. This method of producing L-Trp is more environmentally sound than methods using chemical synthesis, and it lays the foundations for industrial production of L-Trp from DL-ATC and indole.

L-Tryptophan (L-Trp) is an essential amino acid and a low-cost supply is needed [1]. L-Trp is used in feed additives, therapeutic products, health foods, sleeping pills, etc. Furthermore, the possibility of using L-Trp to treat schizophrenia and alcoholism is being investigated [2].

There are 2 primary approaches for industrial production of L-Trp: chemical synthesis and microbial methods; the latter includes enzymatic synthesis and fermentation [2]. Chemical synthesis can produce only the mixture of D, L-forms of amino acids, and an additional optical resolution step is necessary to obtain the biologically active L-isomers. Because of the high production costs associated with this resolution step, only a few amino acids are manufactured by chemical synthesis [3]. Although recent progress in chemical synthesis has made it possible to use chiral catalysts to produce L-isomers directly from prochiral precursors [4], the technology for this asymmetric synthesis is not yet commercially viable [3]. The chiral reagents used in the resolution make chemical synthesis less environmentally friendly than microbial methods, and the costs are higher, too. The fermentation methods used are precursor-conversion fermentation and direct fermentation. These generally suffer from low productivity and feedback inhibition, and the strains used are not readily available. It is therefore important to obtain high-yielding strains by mutation [2]. For example, a regulatory mutant of *Corynebacterium glutamicum* has been reported to produce L-Trp directly from sugars. The productivity was 12.8 g/l [5]. Although the method has the advantage of using cheap starting materials, the productivity will still have to be improved. Application of biotechnology should improve fermentation methods, and greatly decrease the production costs of many amino acids [2]. Compared with precursor and fermentation methods, enzymatic methods use cheap, readily available starting materials, and relatively small amounts of by-products are formed [6]. Genetic engineering could be used to produce recombinant strains containing the appropriate enzymes, thereby increasing the amounts of products synthesized from the substrate. In the various methods proposed, a biocatalyst, in the form of an isolated enzyme or whole cells, has been used either in free or immobilized form. Deeley et al. [7] reported the nucleotide sequence of tryptophanase from *E. coli* K-12. Matsui et al. [8] constructed one Trp-producing recombinant strain of *Brevibacterium lactofermentum* using the engineered trp-operons, the yield was 7.5 g/l.

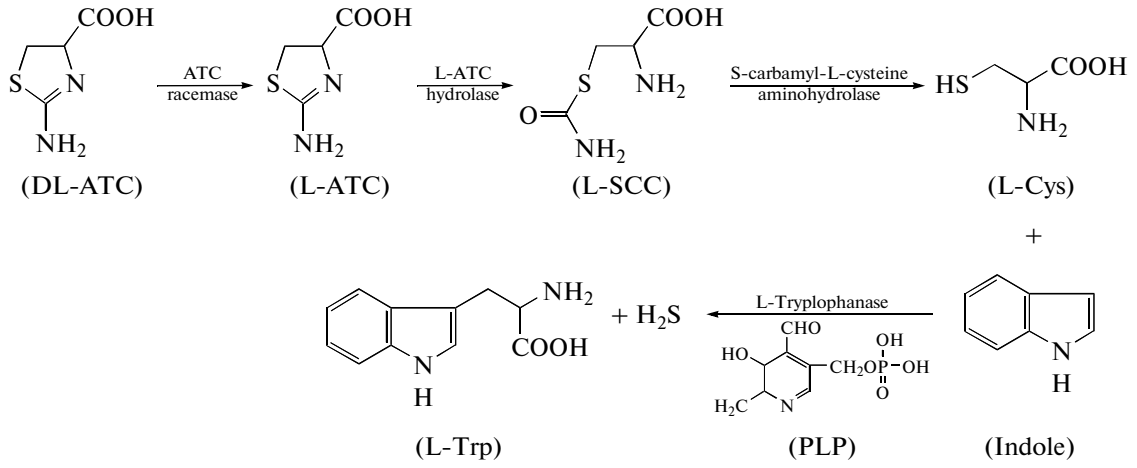

Fig. 1. The principle of enzymatic synthesis of L-Trp with DL-ATC and indole as substrates.

Tryptophanase (EC 4.1.99.1) is a bacterial pyridoxal-5'-phosphate (PLP)-dependent enzyme. It catalyzes α,β-elimination and β-substitution reactions of L-Trp and of some other natural and synthetic amino acids. It is of particular interest because of its possible use for the synthesis of L-Trp and physiologically active analogs of L-Trp [9]. It has been reported [10] that tryptophanase can be used to produce L-Trp with L-Ser, L-Cys and *S*-methyl-L-cysteine as substrates if indole is present in the catalytic system. Shimada et al. [11] reported that L-Trp can be produced from D-Ser, with tryptophanase as the substrate, in the presence of diammonium hydrogen phosphate.

It is known that bacteria convert D,L-2-amino-Δ2-thiazoline-4-carboxylic (DL-ATC) to L-Cys via 2 pathways: the *N*-carbamyl-L-cysteine pathway [12, 13], and the *S*-carbamyl-L-cysteine (L-SCC) pathway [14]. In our previous work, the L-SCC pathway was confirmed in *Pseudomonas* sp. TS1138 [15]. It was found that the *tsB* gene encoded an L-2-amino-Δ2-thiazoline-4-carboxylic hydrolase (L-ATC), which catalyzed the conversion of L-ATC to L-SCC, while the *tsC* gene encoded an L-SCC amidohydrolase, which made L-SCC converting to L-Cys catalytically [16].

In this study, we report the first one-pot enzymatic synthesis of L-Trp, using DL-ATC and indole as substrates (Fig. 1). The *Pseudomonas* sp. TS1138 strain, which produces ATC racemase, L-ATC hydrolase, and L-SCC amidohydrolase, was used to convert DL-ATC to L-Cys. The products of the three genes were involved in the conversion process. Then we constructed a high-level expression system for tryptophanase in *E. coli*, which could be applied to L-Trp synthesis from indole and L-Cys. The chemical substrates DL-ATC and indole were used instead of the L-Ser, L-Cys, or *S*-methyl-L-cysteine used in previously reported studies. The L-Ser precursor was more expensive, and the L-Cys source was less environmentally friendly, which was usually obtained mainly by hydrolysis of hair. We developed a method of L-Trp production using D,L-ATC and indole as the substrates and an L-Trp separation method. This is an important green method of L-Trp production, and it lays the foundations for industrial production of L-Trp from D, L-ATC and indole.

MATERIALS AND METHODS

Materials. *Pseudomonas* sp. TS1138 was isolated from industrial wastewater and stored in our laboratory. *E. coli* JM109 (*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thiA*(*lac-proAB*) F' [*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*Δ*M*15]) and BL21(D3) were purchased from Stratagene (La Jolla, USA) and stored in our laboratory. pET-21a (+) vector was purchased from Novagen (Madison, WI, USA). DL-ATC was obtained from the Tianjin Chemical Reagent Co. (Tianjin, China). The polymerase chain reaction (PCR) fragment recovery kit, pMD 18-T vector, restriction endonucleases, and T4 DNA ligase were purchased from TaKaRa (Dalian, China). Pyridoxal 5'-phosphate, L-Cys, and L-Trp were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Other chemicals used in this study were of analytical grade and commercially available.

Detection and analysis of samples. DL-ATC, L-Cys, L-Trp, and products were identified using a precolumn derivatization method. 100 μl of 10 mM Na_2CO_3 solution (pH 9.0) or 25 mM amino acid, or product sample was placed in separate 2 ml plastic tube. 200 μl of a 1% acetone solution of 18 mM 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) was added to each tube. The molar ratio of FDAA to amino acid was

1.4 : 1. The solutions were mixed and heated on a hot plate at 40°C for 1 h with frequent mixing. After cooling to room temperature, 20 μl of 2 N HCl was added to the reaction mixture. After mixing, 20 μl samples were removed and injected for HPLC. The chromatographic conditions were: a C18 column (Phenomenex Luna 5μ, 100A, 250 × 4.6 mm; Phenomenex Inc., Torrance, CA, USA), with A-phase water containing 0.1% trifluoroacetic acid (TFA), and B-phase acetonitrile (containing 0.1% TFA) as the mobile phase; gradient elution: 0.0 min, 55% A-phase. The process performed for 11.0 min to 47% A-phase at room temperature, detection wavelength 340 nm and flow rate 1 ml/min.

Because of the presence of an indole ring, L-Trp had a maximum absorption at 225 nm, and could be analyzed by HPLC with a UV detector. The chromatographic conditions were: C18 column (Phenomenex Luna 5μ, 100A, 250 × 4.6 mm; Phenomenex Inc., Torrance, CA, USA); mobile phase: methanol/1 mM potassium dihydrogen phosphate (30 : 70); room temperature, detection wavelength 225 nm and flow rate 1 ml/min.

Cloning and expression of *E. coli* tryptophanase. The *E. coli* tryptophanase gene was amplified using a pair of primers: tnaA1 (5'-CCG GAA TTC ATG GAA AAC TTT AAA CAT CTC C-3') and tnaA2 (5'-CCC AAG CTT TTA AAC TTC TTT CAG TTT TGC GG-3'). Chromosomal DNA of *E. coli* JM109 was used as the template. The PCR conditions were as follows: 95°C, 5 min; 94°C, 1 min; 56°C, 1 min; 72°C, 1 min 20 s, 30 cycles; 72°C, 10 min. The amplified fragments were purified and cloned into the *Eco*R I and *Hin*d III sites of pET21a(+). The resulting plasmids were designated as pET-tnaA and transformed into *E. coli* BL21(DE3) cells, named pET-tnaA. The cells were grown at 37°C in Luria broth (LB) medium containing 100 mg/ml of ampicillin to an OD_{600} of 0.6, and then protein production was induced with 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C for 4 h. The BL21/pET-tnaA *E. coli* cells were collected by centrifugation at 5.000 *g* for 10 min at 4°C, and then washed twice with TE buffer (20 mM Tris-HCl, 1 mM EDTA, pH 8.0). The washed cells were resuspended in 2 ml of TE buffer containing 10% glucose and then lyzed by sonication on ice (400 W, 3 s with 3 s breaks for 5 min). The cell wall debris was removed by centrifugation at 12000 *g* for 10 min. The supernatants were used for the enzymatic activity analysis. Proteins in the supernatant were analyzed by SDS-PAGE, and the gel was stained with coomassie brilliant blue. Standard protein markers (TaKaRa, Dalian, China) were applied for molecular weight determination.

Enzyme assay. The assay of tryptophanase activity was carried out by the Ujimaru method [17] with little modification. An assay reaction mixture (0.3 ml) containing 20 μl 0.2 mg/ml pyridoxal 5'-phosphate, 10 μl 5 mM reduced glutathione, and 270 μl of tryptophanase solution was prepared, and then mixed with 1 ml of toluene. After incubation at 37°C for 5 min, 100 μl of L-Trp (5.0 mg/ml) were added; the mixture was then incubated for 10 min at 37°C. The reaction was stopped by addition of 3 ml of 0.1 M *p*-dimethylaminobenzaldehyde solution (*p*-dimethylaminobenzaldehyde was dissolved in the mixture solution of ethanol–sulfuric acid 948 : 52). After 30 min, the OD_{570} was measured using a BIO-RAD680 Microplat Reader (Bio-Rad, USA). One unit of tryptophanase activity was defined as that amount which formed 1 pmol of indole per min under the assay conditions.

Preparation of zymogen cells. *Pseudomonas* sp. TS1138 was cultured at 30°C in ATC medium (%): DL-ATC – 0.2, glucose – 2.0, yeast extract – 0.5, NaCl – 0.15, K_2HPO_4 – 0.3, $(NH_2)_2CO_3$ – 0.2, $MgSO_4 \cdot 7H_2O$ – 0.05, $FeSO_4 \cdot 7H_2O$ – 0.001, pH 7.4) for 14 h, and then washed twice with PBS buffer (20 mM NaH_2PO_4/Na_2HPO_4, 150 mM NaCl, pH 7.4) to obtain zymogen cells, which contained ATC hydrolase and *S*-carbamyl-L-cysteine amidohydrolase. *E. coli* BL21/pET-tnaA was grown at 37°C in an LB medium; 100 μg/ml of ampicillin were used as the selection marker. After being induced as described above, the cells were collected and washed twice with PBS. The zymogen cells contained tryptophanase.

Conversion conditions and analysis of DL-ATC. The optimal conditions for conversion of DL-ATC to L-Cys were determined, namely: temperature, pH, reaction time and concentration of cells, substrate and hydroxylamine. The DL-ATC and L-Cys were detected by the precolumn derivatization/HPLC method described above.

Optimization of L-Trp production. The concentration of BL21/pET-tnaA *E. coli* cells was optimized from 1.0 to 50 g/l and 20 g/l of *E.coli* was chosen for the L-Trp production experiments. On that basis, the conditions for conversion of L-Cys to L-Trp were determined, namely: temperature, pH, reaction time, concentration of PLP and substrate. The L-Cys and L-Trp were detected by the precolumn derivatization/HPLC method described above.

One-pot preparation. L-Trp was produced by a one-pot method, using the optimized conditions. 2.1 l of reaction solution were prepared, and DL-ATC was converted to L-Cys using the *Pseudomonas* sp. TS1138 enzyme system. The reaction solution was then concentrated to 1.0 l using membrane filtration system (LNG-AF-101, Shanghai, China). The *Pseudomonas* sp. TS1138 cells were removed, and the recombinant pET-tanA strain of *E.coli* was added to catalyze the conversion of L-Cys to L-Trp.

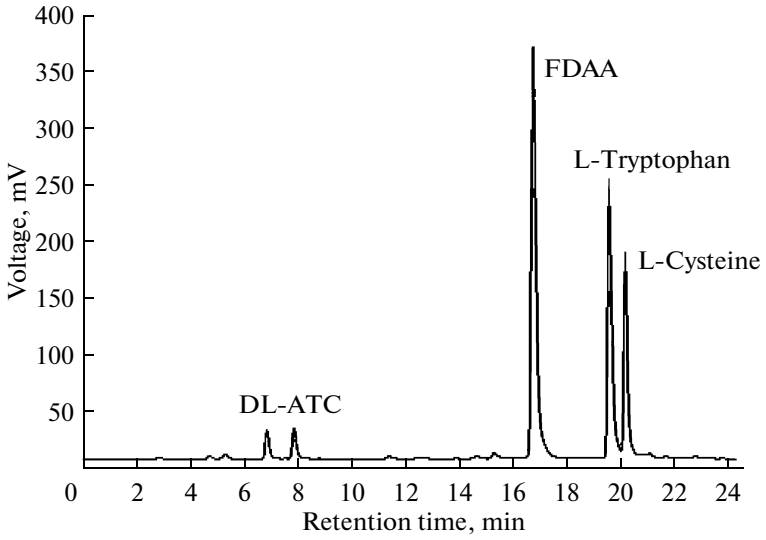

Fig. 2. Test HPLC analysis of the amino acids studied.

Separation of L-Trp. After the conversion, 9 types of macroporous resin, namely AB-8, ADS-17, ADS-21, ADS-F8, D3520, NKA-II, NKA-9, S8, and X-5, were screened for use in purifying L-Trp. The adsorption rates of DL-ATC, L-Cys, indole, and L-Trp were measured. S8 was chosen for removal of indole, and NKA-II was chosen for isolation of L-Trp. The pH of 500 ml of the conversion solution was adjusted to 5.0 using HCl. Then it was added to the column (60 cm × 5 cm) containing macroporous S8 resin, the outflow liquid was collected and added to the column (60 cm × 5 cm) of macroporous NKA-II resin. After careful washing, 50% ethanol was used to elute the column and the fractions were detected using an HPLC/UV detector. The products were collected, concentrated, identified, and quantified using the precolumn derivatization/HPLC method described above.

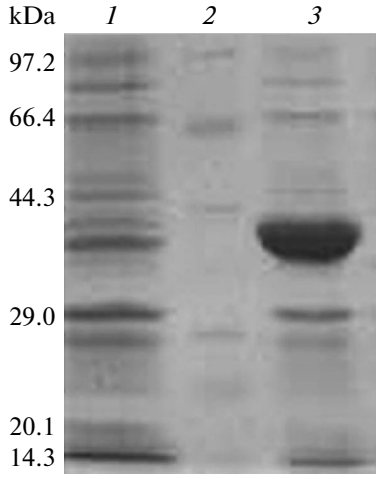

Fig. 3. SDS-PAGE analysis of the expression level of tryptophanase of *E.coli*.
1 – protein standards; *2* – crude cell extracts of *E. coli* BL21 (DE3) harboring pET-21a (+); *3* – crude cell extracts of *E. coli* BL21 (DE3) harboring pET-tnaA.

RESULTS AND DISSUSSION

HPLC analysis of amino acids. Amino acids and DL-ATC contain amino groups, therefore an FDAA precolumn derivatization method can be used for analysis of amino acids and DL-ATC. The test results are shown in Fig. 2. It was possible to detect and separate FDAA, DL-ATC, L-Trp, and L-Cys under the same conditions; the results show that this was a suitable method. To purify and directly detect L-Trp, a UV detector was used because L-Trp contains one indole ring, which could be detected at 225 nm. We have therefore also established a detection method for L-Trp. The precolumn derivatization/HPLC method was used to detect DL-ATC, L-Cys, and L-Trp. The UV/HPLC method was used to monitor L-Trp in the separation process and was found to be an effective method.

Tryptophanase cloning and expression. The PCR was used to clone an L-tryptophanase gene, *tnaA*; this gene had a high sequence homology (99.9%); it was the same size (1416 bp) and had the same protein sequence as the gene from *E. coli* K12. After expression, the lysate of *E. coli* was analyzed by SDS-PAGE. The *E. coli* strain with pET-21a (+) was used as negative controls. The recombinant plasmids, pET-tnaA, were expressed in *E. coli* BL21 (DE3). As shown in Fig. 3, compared with *E. coli* BL21 (DE3) with pET-21a (+) (lane2), the lysate of *E. coli* harboring pET-tnaA (lane3) showed one additional protein band with a

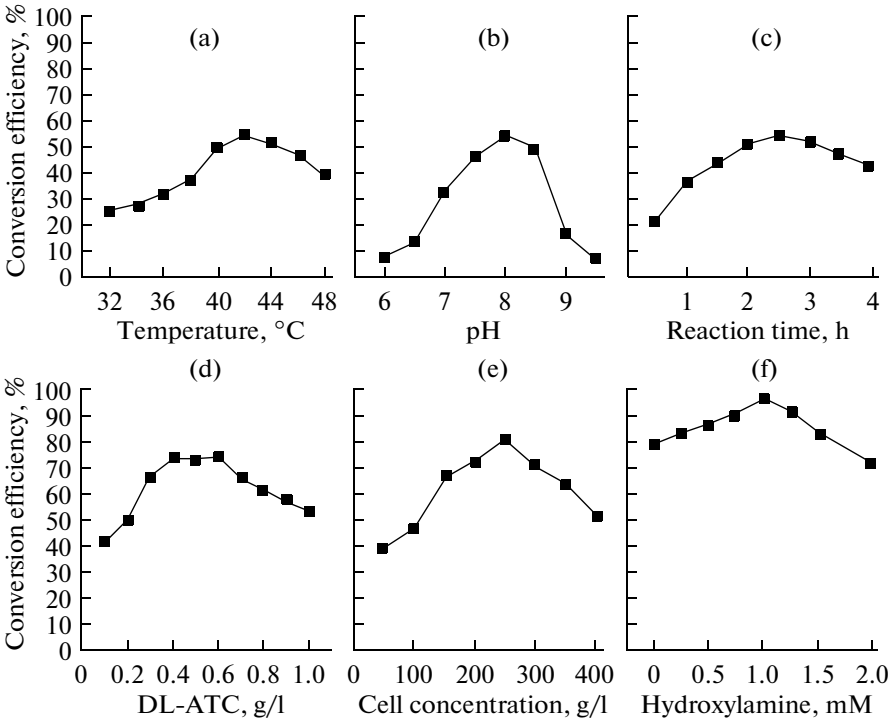

Fig. 4. Optimization of conversion conditions from ATC to L-Cys.
a – conversion temperature; b – conversion pH; c – reaction time; d – concentration of DL-ATC; e – zymogens cell concentration; f – concentration of hydroxylamine inhibitor.

molecular weight of about 39 kDa, which coincided with the expected molecular weight of the gene product of *tnaA*. This observation suggested that *tnaA* might encode the functional protein. After enzyme assay, and the tryptophanase activity from recombinant *E. coli* strain was 3912.6 U/g.

Optimization of conversion of ATC to L-Cys. To improve the ATC conversion rate, temperature, pH, reaction time, concentration of substrate, cells and hydroxylamine were investigated. As Fig. 4 shows, the optimum conditions were 45°C, pH 8.0, 2.5 h, 6 g/l DL-ATC, 20 g/l of bacterial wet weight, and 1 mM hydroxylamine. At the optimum conditions, the conversion rate of ATC was 95.6%. ATC racemase was an important enzyme in this process, and it could effectively improve the conversion rate by transforming D-ATC to L-ATC. *Pseudomonas* sp. TS1138 contained this enzyme and could meet the allosteric requirements of DL-ATC. Because L-cysteine desulfhydryl enzyme could hydrolyze and reduce L-Cys, it was very important to inhibit this enzyme activity. Hydroxylamine was used as inhibitor for that. However, because this compound could partially inhibit the activity of enzymes in the reaction system involved in L-Cys synthesis, it was important to determine the optimum hydroxylamine concentration; the best concentration was found to be 1 mM.

Optimization of conversion of L-Cys to L-Trp. To improve the conversion rate of ATC, the temperature, pH, reaction time, concentration of coenzyme and substrates were investigated. As Fig. 5 shows, the corresponding optimum conditions were 45°C, pH 8.0,

Conversion rate analysis of DL-ATC and L-Cys

No.	Conversion rate of DL-ATC, %	Conversion rate of L-Cys, %	Production of L-Trp, g	Production of purified L-Trp, g	Yield of L-Trp, %
1	94.3	90.1	14.96	10.31	68.9
2	96.7	92.9	15.82	10.09	63.8
3	95.2	93.3	15.64	10.54	67.4
Mean	95.4 ± 1.21	92.1 ± 1.74	15.47 ± 0.45	10.32 ± 0.23	66.7 ± 0.03

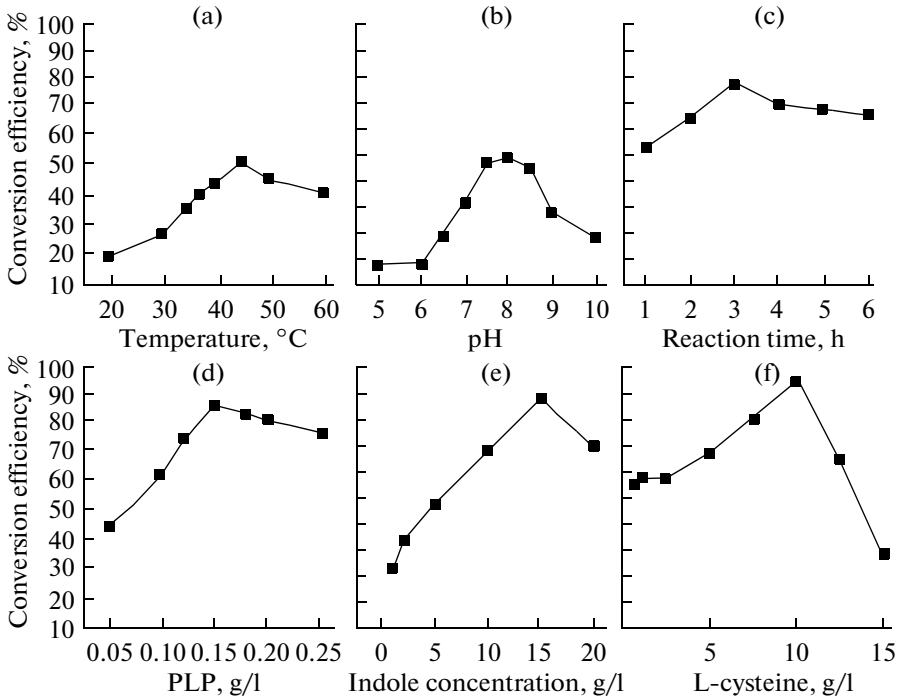

Fig. 5. Optimization of conversion conditions from L-Cys to L-Trp.
a – conversion temperature; b – conversion pH; c – reaction time; d – concentration of PLP coenzyme; e – concentration of indole; f – concentration of L-Cys.

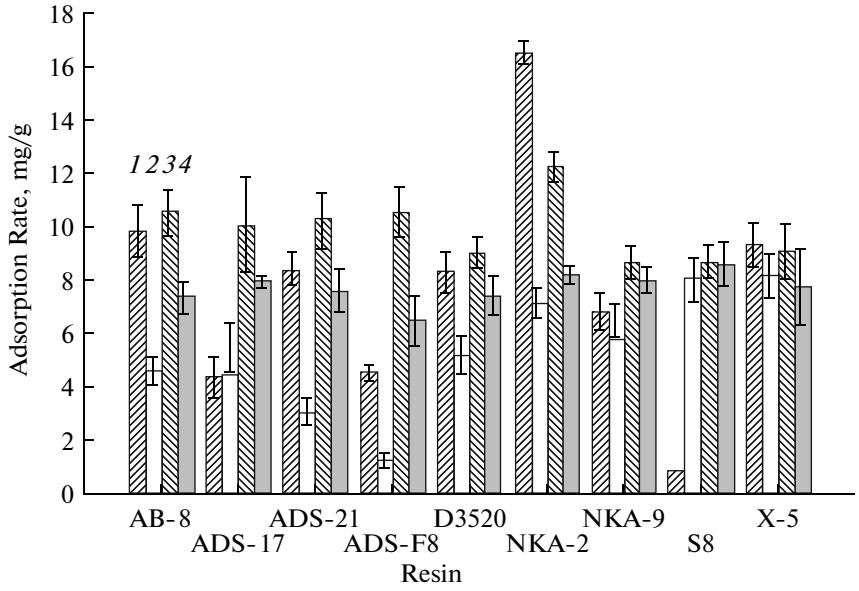

Fig. 6. Adsorption capacity of macroporous resins for L-Trp (*1*), DL-ATC (*2*), L-Cys (*3*) and indole (*4*).

2.5 h, 0.15 g/l of PLP, 15 g/l of indole, and 10 g/l of L-Cys. At the optimum conditions, the L-Cys conversion rate was 92.7%. L-Trp could be effectively produced from indole and L-Ser or L-Cys using L-tryptophanase. The coenzyme PLP was essential in this process, and sufficient coenzyme was needed to improve the combined PLP/L-tryptophanase activity.

L-Trp production and separation. Enzymatic synthesis of L-Trp, using DL-ATC and indole as substrates, was performed using the *E. coli* with a high L-tryptophanase expression level and the *Pseudomonas* sp. TS1138 cells. After catalytic conversion of DL-ATC to L-Cys by *Pseudomonas* sp TS1138 and concentration of the reaction solution, *E. coli* pET-

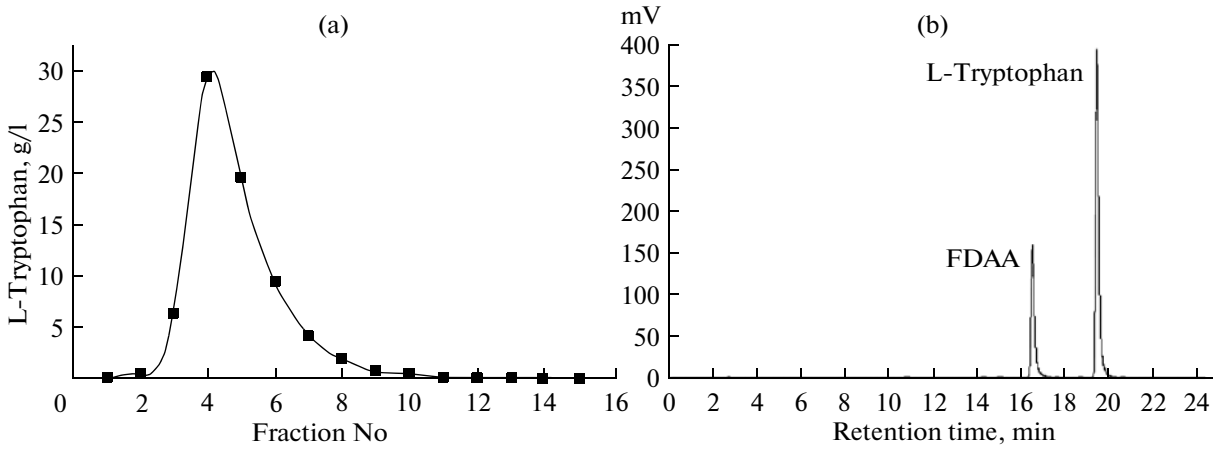

Fig. 7. Purification and identification of L-Trp. a – L-Trp eluting curve from NKA-II resin column; b – HPLC identification of L-Trp.

tnaA was used to convert L-Cys to L-Trp. Under the optimum conditions described above, the conversion reaction was performed 3 times in one pot. The results are shown in Table; the mean conversion rates of DL-ATC and L-Cys were 95.4% and 92.1%, respectively. Quantitative analysis by HPLC, showed that the mean production of L-Cys and L-Trp produced was 9.96 g and 15.47 g, respectively. The results were almost identical with the optimization results.

To remove residual DL-ATC, L-Cys, and indole from the reaction solution, 9 types of macroporous resins, namely AB-8, ADS-17, ADS-21, ADS-F8, D3520, NKA-II, NKA-9, S8, and X-5, were analyzed. The S8 resin could effectively adsorb indole, DL-ATC and L-Cys, and it was used to remove them. NKA-II resin at pH 5 was used to adsorb L-Trp (Fig. 6). After eluting with 50% ethanol and vacuum concentration drying, 10.32 g of L-Trp were obtained; the yield was 66.7% (Table). Every fraction was detected by HPLC/UV detector and converted to concentration of L-Trp. Then the eluting curve was drafted and shown as Fig. 7a. HPLC analysis using L-Trp as the standard (purity: ~ 100%) was performed. In L-Trp production experiments the ratio of area under the peak was defined as the degree of purification. The result revealed that the purity of product was 98.3% (Fig. 7b).

In this study, we established a process for producing L-Trp from DL-ATC via 2 steps involving different enzymatic reactions. First, the enzymatic synthesis of L-Cys from DL-ATC was achieved using the *Pseudomonas* sp. TS1138 strain. The product solution was mixed with indole and used as the substrate for the synthesis of L-Trp using the *E. coli* pET-tnaA cells. The procedure was a one-pot method. DL-ATC is a relatively cheap compound, so if this method could be successfully applied to industrial production, the enzymatic route for synthesis of L-Trp would have more commercial value, and would result in significant economic benefits.

In the enzymatic synthesis of L-Trp, its separation and purification are the main difficulties. In this study, to overcome them, 9 types of resin were investigated. The results showed that indole, DL-ATC and L-Cys could be strongly adsorbed by most of the resins, and that S8 did not absorb L-Trp. However, we found that NKA-II macroporous resin had different adsorption capacities for them at pH 5.0, and it could be used in the separation of L-Trp. L-Trp was isolated by screening with S8 and NKA-II macroporous resins. The product purity was 98.3%, which verified the feasibility of the separation, and showed that it provided a good basis for enzymatic production of L-Trp.

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