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Production of L-DOPA by Thermostable Tyrosine Phenol-lyase of a Thermophilic *Symbiobacterium* Species Overexpressed in Recombinant *Escherichia coli*

LEE, SEUNG-GOO, HYEON-SU RO, SEUNG-PYO HONG, EUN-HWA KIM, AND MOON-HEE SUNG*

Microbial Conversion Research Unit, Applied Microbiology Research Division, Korea Research Institute of Bioscience & Biotechnology, KIST, P.O.Box 115, Yusong, Taejon 305-600, Korea

A thermostable tyrosine phenol-lyase gene of a thermophilic *Symbiobacterium* species was cloned and overexpressed in *Escherichia coli* in order to produce the biocatalyst for the synthesis of 3,4-dihydroxyphenyl-L-alanine (L-DOPA). The substrates used for the synthetic reaction were pyrocatechol, sodium pyruvate, and ammonium chloride. The enzyme was stable up to 60°C, and the optimal temperature for the synthesis of L-DOPA was 37°C. The optimal pH of the reaction was about 8.3. Enzyme activity was highly dependent on the amount of ammonium chloride and the optimal concentration was estimated to be 0.6 M. In the case of pyrocatechol, an inactivation of enzyme activity was observed at concentrations higher than 0.1 M. Enzyme activity was increased by the presence of ethanol. Under optimized conditions, L-DOPA production was carried out adding pyrocatechol and sodium pyruvate to the reaction solution intermittently to avoid substrate depletion during the reaction. The concentration of L-DOPA reached 29.8 g/l after 6 h, but the concentration didn't increase further because of the formation of byproducts by a non-enzymatic reaction between L-DOPA and pyruvate.

Microbial tyrosine phenol-lyase (EC4.1.99.2) is a pyridoxal-dependent enzyme that catalyzes the cleavage of tyrosine and 3,4-dihydroxyphenyl-L-alanine (L-DOPA) to phenol (or pyrocatechol), pyruvate, and ammonia (4). Reverse reaction of the cleavage of L-tyrosine has attracted a great deal of attention and is being used for the synthesis of L-tyrosine and L-DOPA (2-5). The amino acid L-DOPA is an useful drug in the treatment of Parkinson's disease.

Enzymatic synthesis of L-DOPA using tyrosine phenol-lyase was first introduced by Japanese scientists using free cells of *Erwinia herbicola*, achieving a productivity of 58.5 g/l in two days (5). Kupletskaya (6, 7) showed that the *Citrobacter freundii* cells also produced 42 g/l of L-DOPA in the same period.

In most cases, the inactivation of the biocatalyst by pyrocatechol has been emphasized, and different procedures have been used to stabilize the biocatalyst. Enei *et al.* (5) kept the concentration of pyrocatechol at a predetermined level during the reaction by feeding in pyrocatechol at intervals. Kupletskaya *et al.* (7) used pyrocatechol in a mixture with sodium borate, which formed a stable complex with pyrocatechol. Although the biocatalyst was stabilized by using the pyrocatechol-borate complex, the rate of L-DOPA synthesis was found to be seriously reduced by the presence of sodium borate. In continuous reactors, Para and Baratti (9, 10) reported that *Erwinia herbicola* cells immobilized in a viable state maintained their activity for 62 days.

As an effective method of obtaining a thermostable enzyme with greater industrial usefulness, thermophilic microorganisms have attracted much attention. It is generally accepted that thermophiles usually produce more thermostable enzymes than mesophiles because of both molecular evolution and growth at high temperature. In this context, we isolated a thermophilic *Symbiobacterium* species as a thermostable tyrosine phenol-lyase (TTPL) producer from nature. The thermostable tyrosine phenol-lyase (TTPL) gene was cloned and overexpressed in *Escherichia coli* to be used for the synthesis of L-DOPA in this work.

*Corresponding author
Key words: Tyrosine phenol-lyase, thermostable enzyme, 3,4-dihydroxyphenyl-L-alanine(L-DOPA), thermophilic *Symbiobacterium* species

MATERIALS AND METHODS

Materials

All chemicals used in this work were commercial products. Pyrocatechol was purchased from Wako Chemicals (Japan). Sodium pyruvate was purchased from Musashino Chemical Lab (Japan). Other chemicals were purchased from Sigma (USA).

Plasmids and Microorganism

From 2,000 thermophiles isolated from nature, one thermophile producing thermostable tyrosine phenol-lyase (TTPL) was isolated. The isolate was investigated for biochemical and physiological properties and identified as a thermophilic *Symbiobacterium* species. A TTPL gene was cloned from the thermophilic *Symbiobacterium* species, sequenced, and amplified by polymerase chain reaction using an N-primer and C-primer prepared based on the determined DNA sequence for the production of the recombinant TTPL (Fig. 1). The amplified gene was purified on agarose gel electrophoresis, ligated to vector pTrc99A and designated as pHLT1. The plasmid pHLT1 was transformed into *E. coli* JM 105.

E. coli cells harboring the TTPL gene were cultivated in LB-medium (trypton 1.0%, yeast extract 0.5%, and NaCl 1.0%) containing ampicillin at 100 mg/liter. Induction of the *trc* promoter was carried out by 1 mM IPTG when the turbidity of the culture broth reached about 0.6 at A_{600}.

Preparation of Crude-enzyme Solution

E. coli cells grown in LB-medium were harvested by centrifugation at 5,000×g for 20 min and washed with 50 mM potassium phosphate buffer (pH 8.0). The pellet was suspended in the same buffer containing 0.1 mM pyridoxal-5'-phosphate (PLP) and 10 mM 4-amino-phenyl-methansulfonylfluoride. Cells were disrupted by sonification with a Branson Sonifier (Branson Ultrasonics Co., CT, USA). The cell lysate was centrifuged at 20,000×g for 60 min to remove cell debris, and the supernatant was dialyzed against 20 mM potassium phosphate buffer (pH 8.0) containing 0.05 mM PLP. The dialyzed solution was heated at 60°C for 20 min to remove heat-labile *E. coli* proteins and cooled on ice. The protein aggregates were removed by centrifugation at 5,000×g for 20 min and the supernatant was stored at -20°C to be used as crude-enzyme.

Assay of L-DOPA Synthetic Activity

L-DOPA synthetic activity was measured with a reaction mixture containing 50 mM pyrocatechol, 50 mM sodium pyruvate, 0.65 M ammonium chloride (pH 8.5), 0.1 mM PLP, 0.1% sodium sulfite and a predetermined amount of crude-enzyme. The reaction solution was incubated at 37°C for 30 min, and mixed with an equal volume of 1 M HCl to stop the reaction. The protein aggregates were removed by centrifugation at 5,000×g for 20 min. The concentration of L-DOPA was determined by HPLC analysis. One unit of activity was defined as the amount of enzyme required to produce 1 μmole of L-DOPA per min under the specified conditions.

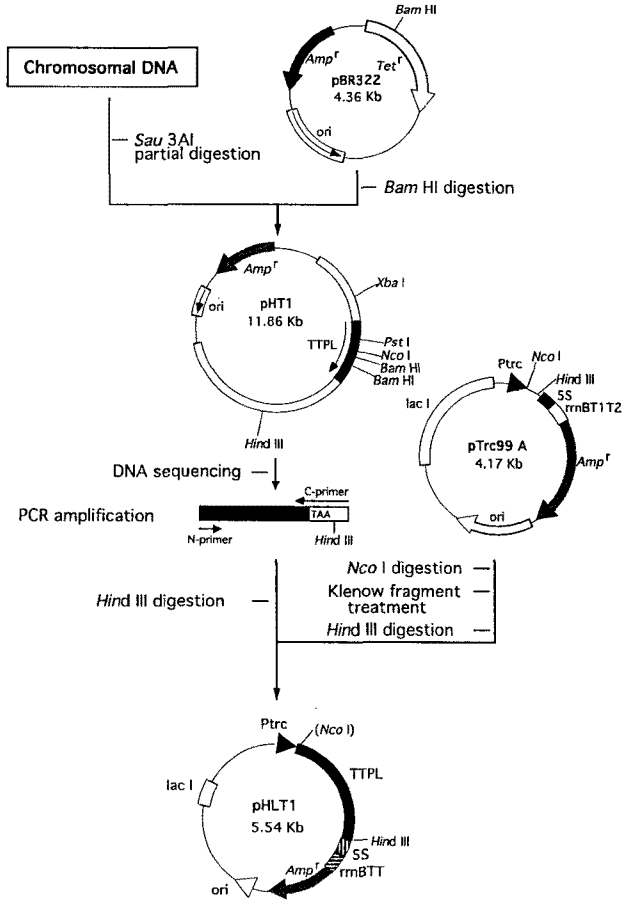

Fig. 1. Overall cloning and expression scheme of the TTPL gene from a thermophile isolated from nature.

Expression plasmid pHLT1 was constructed by inserting the TTPL gene into the *Hin*dIII and *Nco*I site of vector DNA (pTrc99A). The TTPL gene fragment was amplified by polymerase chain reaction (PCR), and digested with *Hin*dIII. The sequences of primers used for PCR were 5'-CAGCGACCCTGGGCGGAACC-3' (N-primer) and 5'-TGACTAAGTCAAGCTTATTAGCTGATCGGCTCGAAGCG-3' (C-primer including a *Hin*dIII site: underlined letters). The TTPL gene was ligated to pTrc99A serially treated with *Nco*I, Klenow fragment of DNA polymerase I, and *Hin*dIII.

Production of L-DOPA

The production of L-DOPA was performed with 10 ml of reaction mixture in a reactor equipped with a water-jacket. The reactor was tightly sealed with a rubber stopper and flushed with nitrogen gas to prevent the oxidation of pyrocatechol and L-DOPA. The reaction temperature was controlled at 18°C or 37°C by water circulation through the water-jacket. To avoid the depletion of the substrate, py-

rocatechol and sodium pyruvate were intermittently supplied to the reaction solution (up to 50 mM concentration) after the concentration of pyrocatechol in the reactor was determined by HPLC analysis.

Analysis

The concentrations of L-DOPA and pyrocatechol in the reaction solution were determined by HPLC (Younging, Korea). The column was μBondapak C18 (Waters, MA, USA). The eluent consisted of 5% (v/v) methanol, 2% (v/v) acetic acid, and 0.1 M sodium phosphate buffer. The flow rate of eluent was 1.0 ml per minute and materials eluted from the column were detected at UV 280 ηm. The protein concentration was measured with Coomassie brilliant blue G (Bio-rad, CA, USA) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Production of the TTPL from Recombinant *E. coli*

E. coli JM105 cells harboring plasmid pHLT1 (Fig. 1) were used for the preparation of the crude-enzyme solution following the methods described elsewhere. The proteins of the crude-enzyme solution were analyzed by 12% SDS-PAGE. As shown in Fig. 2, the TTPL corresponds to the protein band positioned at molecular weight 51.3 kDa of lane 2 and the enzyme content was 12-15% of the total *E. coli* proteins. The specific L-DOPA synthetic activity of the crude-extract of the recombinant *E. coli* cells was about 0.22 units/mg protein. When the crude-extract was partially purified by heat-treatment at 60°C, the specific activity increased up to 0.55 units/mg protein. The partially purified enzyme was used as the TTPL in this work.

Effect of Temperature

In order to investigate thermostability, the TTPL was incubated for 30 min at different temperatures in 20 mM potassium phosphate buffer (pH 8.0), and residual activity was measured. As shown in Fig. 3, the enzyme was stable up to 60°C, maintaining 95% of enzyme activity after the heat-treatment. Because stability of tyrosine phenol-lyase was a key factor determining the productivity of the L-DOPA synthetic reaction, the TTPL was thought to have significant potential in improving the enzymatic process.

Although the enzyme was thermostable up to 60°C, the optimal temperature for the L-DOPA synthetic reaction was found to be 37°C. At temperatures higher than the optimal, byproduct formation rapidly increased and the concentration of L-DOPA decreased in spite of the enzyme maintaining its stability.

Effect of pH

The L-DOPA synthetic activity of the TTPL was investigated under pH variations. Reaction solutions were titrated to different pHs with 6 N HCl and 5 M KOH, and used for the experiments. As shown in Fig. 4, the effect of pH on activity was quite large and maximum activity was observed at pH 8.3.

Effect of Ammonium Sources

Different ammonium salts were tested to measure the dependence of L-DOPA synthetic activity of the TTPL on ammonium sources. As shown in Fig. 5a, ammonium chloride was found to be the most effective ammonium source. The enzyme activity with ammonium acetate or ammonium sulfate was about 70% of that with ammonium chloride. Enzyme activity was highly dependent on the concentration of ammonium chloride and the optimal concentration of ammonium ion was determined to be 0.6 M (Fig. 5b).

Effect of Pyrocatechol Concentration

Thermostable enzymes are known to be stable in the

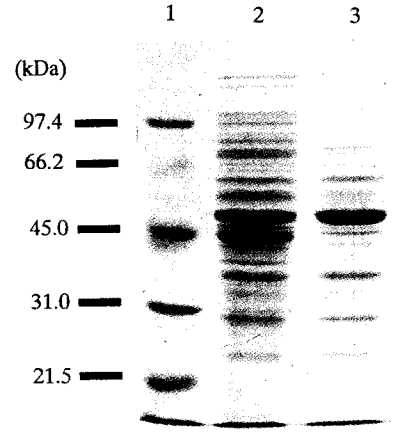

Fig. 2. Expression of recombinant tyrosine phenol-lyases of *E. coli* JM105 harboring plasmid pHLT1.
Lane 1, molecular weight standards; lane 2, *E. coli* JM105/pHLT1; lane 3, the TTPL purified partially by heat-treatment at 60°C.

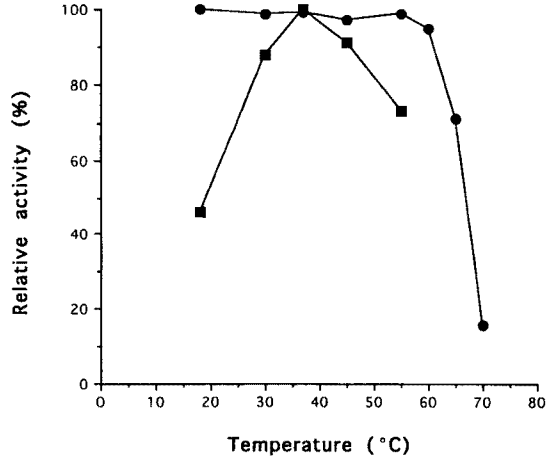

Fig. 3. Effect of temperature on the L-DOPA synthetic activity (■) and the stability (●) of the TTPL.

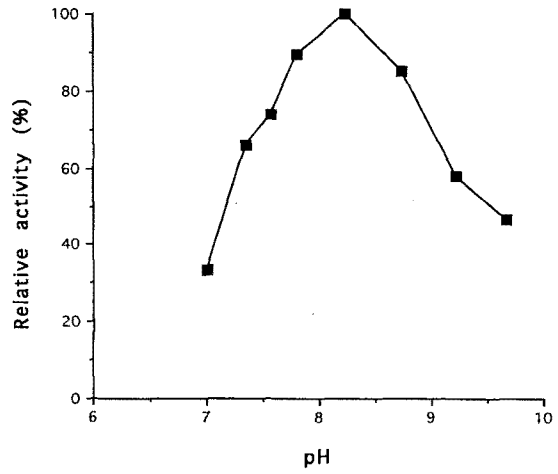

Fig. 4. Effect of pH on L-DOPA synthetic activity of the TTPL.

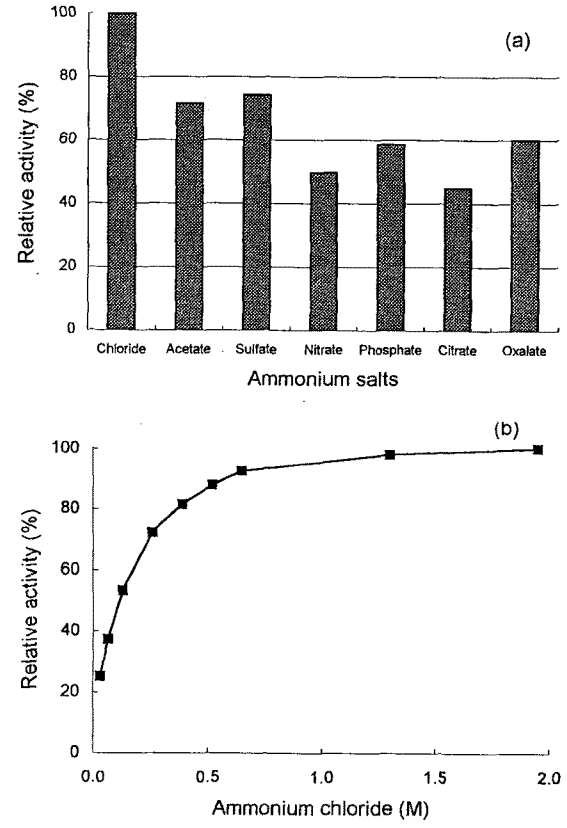

Fig. 5. Effect of (a) various ammonium salts and (b) concentrations of ammonium chloride on the L-DOPA synthetic activity of the TTPL.

presence of toxic chemicals, offering a significant potential in enzyme technology. In order to investigate the stability of the TTPL in the presence of pyrocatechol, the enzyme was incubated for 2 h at various pyrocatechol

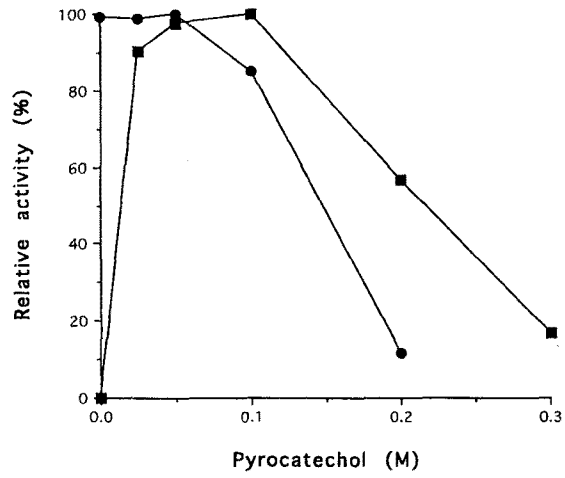

Fig. 6. Effect of pyrocatechol concentration on L-DOPA synthetic activity (■) and the stability (●).

concentrations and residual activity was measured. As shown in Fig. 6, the TTPL maintained its stability up to 50 mM pyrocatechol but lost 17% of its original activity in 100 mM pyrocatechol. When the enzyme was assayed with different pyrocatechol concentrations, enzyme activity increased moderately until 100 mM, and at higher concentrations, a substrate inhibition was observed. The tyrosine phenol-lyase of *Citrobacter freundii* KCTC 2006 showed a substrate inhibition for pyrocatechol at concentrations above 20 mM (8). From the above results, the TTPL was judged to be stable against inactivation by heat or pyrocatechol.

Effect of Ethanol

Ethanol was found to have the effect of increasing the L-DOPA synthetic activity of tyrosine phenol-lyase (8). In this work, the effect of ethanol was examined again with the TTPL, and the activity of the TTPL was observed to increase about 30% when 20% ethanol was included in the reaction solution (data not shown).

Production of L-DOPA by TTPL

L-DOPA production was carried out in a water-jacketed batch reactor containing 10 ml of reaction mixture. The reaction mixture consisted of 0.65 M ammonium chloride (pH 8.5), 50 mM sodium pyruvate, 50 mM pyrocatechol, 0.1 mM pyridoxal-5'-phosphate, 0.1% sodium sulfite, 5% ethanol and 1.5 units of the TTPL.

The time-courses of the L-DOPA synthetic reaction at 37°C and 18°C are shown in Fig. 7. When the reaction was conducted at 37°C, L-DOPA concentration increased rapidly for several hours, and reached 29.8 g/l in 6 h. However, L-DOPA concentrations didn't increase thereafter. By analyzing the reaction mixture with HPLC, this flattening-out of the profile of L-DOPA concentrations was found to be caused by the accumulation of byproducts. The byproduct was reported to be produced by a

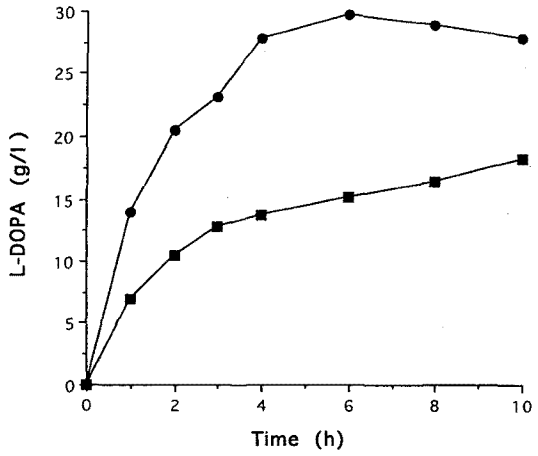

Fig. 7. Enzymatic production of L-DOPA by the TTPL at 18°C (■) and 37°C (●).

non-enzymatic reaction between the L-DOPA and excess pyruvate (5). The formation of byproducts increased with reaction temperature and consequently inhibited the synthesis of L-DOPA in this work.

Because the accumulation of byproduct was less serious at lower temperatures (5), L-DOPA synthesis was carried out at 18°C. However, as shown in Fig. 7, the enzymatic reaction was found to be inefficient at this low temperature, producing only 18 g/l of L-DOPA in ten hours. This low productivity was attributed to the fact that the enzyme showed only 46% activity at 18°C compared to its activity at 37°C.

Considering the low activity observed at 18°C, it appeared that the production of L-DOPA by the TTPL should be performed at 37°C in spite of the accumulation of byproducts. If the enzyme were immobilized on an insoluble matrix and used in a continuous reactor, the high productivity of the TTPL at 37°C could be maintained for long time because the accumulation of byproduct would be prevented by the replacement of the reaction medium.

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