Conversion of *trans*-Cinnamic Acid to L-Phenylalanine by Phenylalanine Ammonia Lyase

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The conversion of *trans*-cinnamic acid to phenylalanine using phenylalanine ammonia lyase (PAL) was examined. The optimum concentration of *trans*-cinnamic acid for the reaction was observed at 100 mM in cells and at 20 mM in cell free extracts, respectively. The production of L-phenylalanine was increased in both experiments as the concentration of ammonia was increased up to 10 M. The optimal pHs for the maximal conversion of *trans*-cinnamic acid to L-phenylalanine were 9.5 and 9.0 in experiments carried out with cells and with cell free extracts, respectively.

Introduction

L-Phenylalanine is an essential amino acid for human and animal nutrition and is a raw material for the production of aspartame (L-aspartidylphenylalanine methyl ester), an artificial sweetener which becomes an important commercial product. Economic production of L-phenylalanine has been the target for many research laboratories.

Phenylalanine Ammonia Lyase (PAL, E.C. 4.3.1.5) is an enzyme catalyzing deamination of L-phenylalanine to give *trans*-cinnamic acid and ammonia. It is distributed in many plants[1-5], in some fungi[6-11] and in *Streptomyces* strains.[12,13] In plants, PAL is known to catalyze the initial step of the biosynthetic pathways for phenylpropanoids, lignin and flavonoids.[3] PAL is reported to be induced in a variety of plants by illumination.[4,14] Increase of PAL activity is thought to be due to *de novo* synthesis.[14] However, Attridge *et al.*[4,15] mentioned that the PAL enzyme is regulated by inactivating enzymes and inactivators.

PAL in yeasts catalyzes the initial step in the catabolism of L-phenylalanine, which can serve as a sole carbon, a nitrogen and an energy sources.[16] Although it removes the amino group from L-phenylalanine, PAL does not contain pyridoxal 5'-phosphate.[17] Dehydroalanine was reported to be existing at the active site of the enzyme.[18] PAL isolated from yeast was reported to be inactivated by the reagents that are known to react with the carboxylic acid and the sulfhydryl groups.[17,19] Some halides also inhibit PAL.[19] Hodgins[17] reported that *trans*-cinnamic acid and its ring-hydroxyl derivatives strongly inhibited PAL as competitive inhibitors. Sato *et al.*[20,21] reported the relative inhibition(%) of PAL by cinnamic acid, its structural analogs and flavonoids. The kinetic constants of various new PAL inhibitors including phenylglycine were reported by us.[22]

We have been interested in the development of new biochemical methods for the production of L-phenylalanine. We examined various microorganisms[23] to find out the richest source of PAL. We found that *Rhodosporium trouloides*, an yeast was the best source for PAL. We examined inhibition of PAL by cinnamic acid and other inhibitors.[24] In the present report, we want to report its characteristics on the conversion of *trans*-cinnamic acid to L-phenylalanine.

Experimental

Materials. Bacto-yeast extract, Bacto-malt extract, Bacto-peptone and glucose were purchased from Difco Laboratories. L-Phenylalanine and *trans*-cinnamic acid were from Aldrich Chemical Co. Tris (hydroxymethyl)-aminomethane (Tris) was purchased from Sigma Chemical Co. Other chemicals and solvents were the first grade and were used without further purification. UV spectrophotometry used was LKB Biochrom Ultrospec 4050 equipped with temperature controller 4070. Autoclave was done at 15 lb/121℃ for 15 min. Yeast cells were cultured on a rotary shaker (180 rpm) at 28℃ or in a fermentor aerated at the rate of 5 L/min and stirred at 200 rpm at 28℃. Centrifugation was done by using Beckman J-21 Centrifuge and MSE Europa 65 Ultracentrifuge. Sonication was done with MSE-MK2 Sonicator. pH was measured by using the digital pH meter 202 of Nova Scientific Co.

Culture of Microorganisms. *Rhodosporium toruloides* (IFO 0559) was from the Institute for Fermentation, Osaka, Japan and maintained on malt extract agar slants which contained 2% malt extract, 0.1% peptone, 2% glucose and 3% agar in distilled water. For a seed culture, a medium (100 ml) composed of 1% malt extract and 0.1% yeast extract in distilled water was inoculated with a loopful inoculum of *Rhodosporium trouloides* and incubated at 28℃ on a rotary shaker (180 rpm) for 24 h. The seed culture was transferred to the growth medium containing 1% malt exract, 0.1% yeast extract and 0.1% L-phenylalanine in distilled water to give 5% v/v. The broth was incubated at 28℃ on a rotary shaker for 16 to 18 hours. Cells were harvested by centrifugation at 8,500 rpm for 15 min and washed twice with 0.9% saline. The weighed cells were either frozen and maintained at −20 ℃ or immediately disrupted by sonic oscillation.

Preparation of Cell free Extracts. The wet or frozen cells were resuspended in 2 ml of 50 mM Tris-HCl (pH 8.5) per g (wet weight). The suspension was disrupted for 10 min at maximum power in an ice-salt bath (sonication for 10 sec followed by 5 sec's break). This and all subsequent were carried out at 0℃. The supernatant obtained by centrifugation of the sonicated mixture at 16,000×g for 20 min was used as a cell free extract of *Rhodosporium toruloides*.

Enzyme assay. PAL was assayed by the method of Hodgins.[18] The standard assay mixture contained 2.8 m*l* of 0.833 mM L-phenylalanine in 0.1 M Tris-HCl (pH 8.5) and 0.2 m*l* of the cell free extract. The reaction was started by the addition of the cell free extract to the assay mixture and the accumulation of *trans*-cinnamic acid was monitored at 290 nm at 30℃.[25] The change in UV absorption was recorded for 2 min at 20 sec's interval. The PAL activity of the cell free extract was 0.333 unit/g of protein. The enzyme activity was defined as a 1 unit for the production of 1 μM of *trans*-cinnamic acid per seconds at 30℃. The amount of the protein in the cell free extract was determined by Lowry Method.[26]

Conversion of *trans*-cinnamic acid to L-phenylalanine with whole cells. The cells (2 g, wet weight) harvested and stored at −20℃ were suspended in 2 m*l* distilled water. The cell suspension (0.3 m*l*) was mixed with 1.2 m*l* of a reaction mixture and incubated in a 30℃ water bath at 120 strokes per min. for 1 hour. The incubated solution was centrifuged and the supernatant was analyzed for the content of L-phenylalanie by an automatic amino acid analyzer. The reaction mixture contained different amounts of *trans*-cinnamic acid with a fixed amount of ammonia (6.6 M). The pH of the reaction mixture was adjusted to pH 10.0 with concentrated HCl. For the study of the effect of the concentration of ammonia on the reaction, the reaction mixture was prepared with different amounts of ammonia with a fixed amount of *trans*-cinnamic acid (50 mM). The pH was also adjusted to pH 10.0 with concentrated HCl. For the study of the effect of pH, the reaction mixture was prepared by dissolving *trans*-cinnamic acid (50 mM) and ammonia (6.6 M). The pH of the solution was adjusted to the desired value by adding concentrated HCl.

Conversion of *trans*-cinnamic acid to L-phenylalanine with a cell free extracts. The cells (2 g, wet weight) harvested and stored at −20℃ were suspended in 2 m*l* of Tris-HCl buffer (50 mM, pH 8.5) and sonicated in an ice-bath for 10 min by repeating 10 sec's sonication with 5 min's interrupt for 15 min. The sonicated solution was centrifuged at 16,000 g for 20 min at 4℃. The cell free extract (0.2 m*l*) was added to the reaction mixture (0.8 m*l*) and incubated in a 30℃ water bath for 10 min at 120 strokes per min. The reaction was stopped by adding 100% trichloroacetic acid (0.11 m*l*). The mixture was kept in an ice bath for more than 30 min and centrifuged at 10,000×g for 10 min. The supernatant was analyzed for the content of L-phenylalanine by an automatic amino acid analyzer.

Results and Discussion

Conversion of *trans*-cinnamic acid to L-phenylalanine was carried out with the whole cells, or with the cell free extracts of *Rhodosporium toluloides* (IFO 0559). The yeast was cultured in the malt extract medium containing L-phenylalanine as the inducer of PAL. The harvested cells frozen or prepared freshly which were suspended in distilled water (2 g/m*l*) were added to the PAL reaction mixture containing *trans*-cinnamic acid and ammonia. The pH of the reaction mixture was adjusted to pH 10.0. The mixture was incubated for 1 hour. After the cells were removed by centrifugation, the concentration of L-phenylalanine in the reaction mixture was analyzed by an automatic amino acid analyzer. When the concentration of *trans*-cinnamic acid added to the reaction mixture was varied from 0 mM to 200 mM, maximal conversion of *trans*-cinnamic acid to L-phenylalanine (1.22 mM) was observed at 100 mM (Figure 1a). When the same experiment was carried out with the cell free extract, maximal production of L-phenylalanine (0.54 mM) was at 20 mM of *trans*-cinnamic acid at the same pH (Figure 1b). The cell free extract was prepared with cells (2 g/m*l*) suspended in Tris-HCl buffer by sonication followed by centrifugation. The cell free extract contained 0.333 unit of PAL per gram of proteins. The cell free extract (0.2 m*l*) was mixed with 8 m*l* of the PAL reaction mixture and the reaction was carried out for 10 min. However, 0.3 m*l* of the cell-suspended solution was added to 1.2 m*l* of PAL reaction mixture and the reaction was carried out for 1 hour. Under these conditions, about 2 times of L-phenylalanine was produced in the whole-cell incubation mixture than in the cell free extract incubation mixture. The existence of optimal concentration of *trans*-cinnamic acid was probably due to inhibition of PAL by *trans*-cinnamic acid.

Examination of the effect of the concentration of ammonia on the conversion of *trans*-cinnamic acid to L-phenylalanine with whole cells showed increase of the production of L-phe-

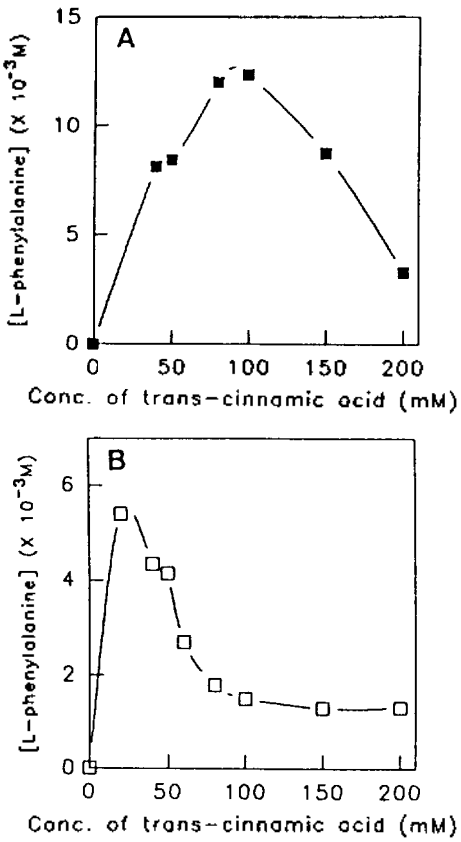

Figure 1. Effect of the concentration of *trans*-cinnamic acid on the conversion of *trans*-cinnamic acid to L-phenylalanine by the whole cells (A) and by the cell free extract (B). The concentration of L-phenylalanine in the incubated solution was obtained by an automatic amino acid analyzer. Whole cells and cell free extracts were incubated in the reaction mixture (pH 10.0, 6.6 M ammonia) as described in the experimental section.

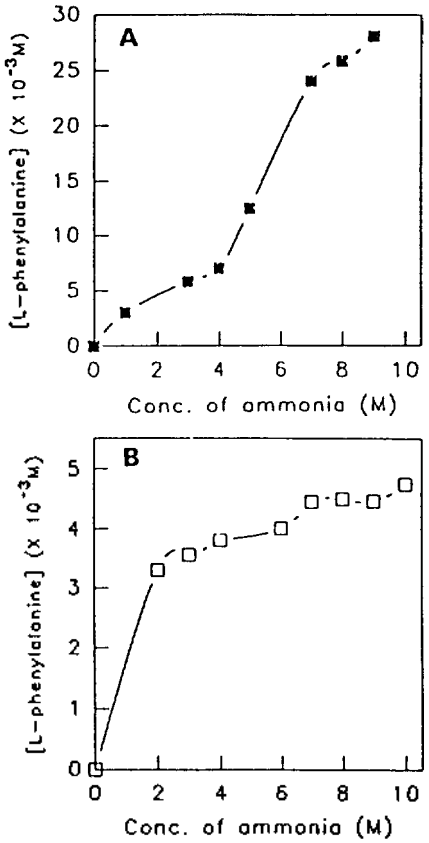

Figure 2. Effect of the concentration of ammonia on the conversion of *trans*-cinnamic acid to L-phenylalanine by the whole cells (A) and by the cell free extract (B). The cell suspension (0.3 m*l*) or the cell free extract (0.2 m*l*) were mixed with the substrate solution (1.2 m*l* or 0.8 m*l*), respectively; 50 mM of *trans*-cinnamic acid; pH 10.0) were mixed and incubated according to the method described in the experimental section. The content of L-phenylalanine was anlyzed by an automatic amino acid analyzer.

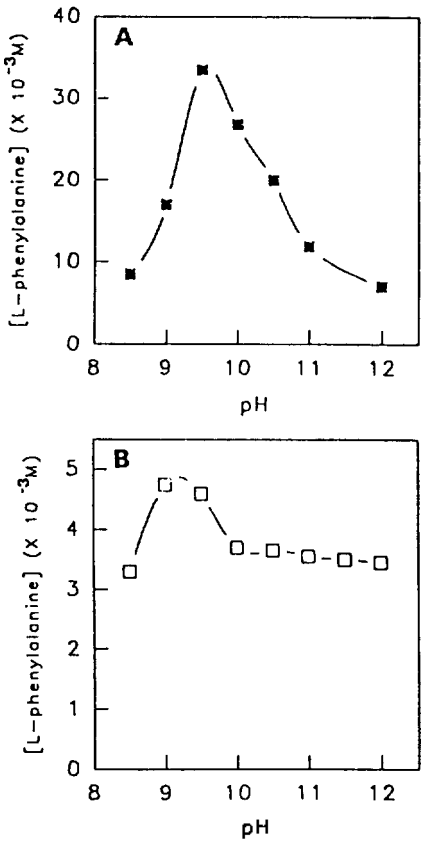

Figure 3. Effect of pH on the conversion of *trans*-cinnamic acid to L-phenylalanine by the whole cells (A) and by the cell free extract (B). Reactions were carried out at 30℃ for 1 hour with mixtures containing 0.3 m*l* of the cell suspension or 0.2 m*l* of the cell free extract with 1.2 m*l* or 0.8 m*l* of the substrate solution (50 mM of *trans*-cinnamic acid; 6.6 M of ammonia), respectively. The pH of the substrate solution and the cell free extract was adjusted with concentrated HCl or 1 M KOH.

nylalanine as the increase of the concentration of ammonia. Abrupt increase was observed as the concentration of ammonia was increased from 4 M to 8 M. However, the cell free extract showed little increase from 2 to 10 M of the ammonia concentration. Other differences between the results obtained with the whole cells and with the cell free extracts are due to the permeability problems.

The pH-rate profile shows a maximum rate for the ammonia lyase reaction by PAL at pH 8.5. But, when we examined the conversion of *trans*-cinnamic acid to L-phenylalanine, the whole cells and the cell free extract showed maximum production of L-phenylalanine at pH 9.5 and pH 9.0, respectively. The difference of optimal pH on production of L-phenylalanine was probably due to the differences of the pHs between outside and inside of the cells.

In conclusion we investigated the optimal conditions for the production of L-phenylalanine with the whole yeast cells in which PAL was induced with L-phenylalanine and with the cell free extracts containing PAL. In using the whole cell, L-phenylalanine was maximally produced in the incubating mixture containing 100 mM *trans*-cinnamic acid at pH 9.5. We could not increase the concentration of ammonia until it inhibit the conversion of *trans*-cinnamic acid and the more we increase the concentration of ammonia the more phenylalanine was produced up to as much as 10 M. In using the cell free extract system, the optimal concentration for the maximum production of L-phenylalanine was 20 mM for *trans*-cinnamic acid, and also 10 M for ammonia at pH 9.0. Industrial production of L-phenylalanine should be possible by developing new PAL having little inhibitory effect on the enzyme by *trans*-cinnamic acid. Furthermore development of new stable PAL system might be necessary.

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Formation of Soluble Intermediate During the Electrochemical Crystallization of Lead Dioxide

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Details on the electrochemical formation of lead dioxide from aqueous plumbous ion are studied by measuring current-time behavior with potential step method at a rotating platinum electrode. A cubic law without induction period can be applied to the crystallization of lead dioxide in both acetate and nitrate media. In the course of the mechanistic study, the presence of a soluble intermediate during the nucleation step is clearly observed with a rotating ring-disk electrode. Decrease in the anodic ring current due to the reduction of soluble species formed during the anodic crystallization of lead dioxide at disk is detected.

Introduction

Lead dioxide has been used as the positive plate in lead-acid battery and as an inert electrode for various anodic processes. There are numerous monographs and papers including several reviews[1-7] concerning the behavior of this electrode.

Electrochemical kinetics for the formation of lead dioxide have been studied in terms of nucleation and crystal growth[8-15]. Fleischmann and Thirsk have reported that the current-time relationship on the formation and growth of lead dioxide by the oxidation of $PbSO_4$ in potentiostatic condition obeys a cubic law as[9]

$$i = ABN_o t^3/3$$

where A is the potential dependent nucleation rate constant, B is the potential dependent rate constant of crystal growth, N_o is the maximum number of nuclei, and t is time. Derivation of the cubic growth law can be made by assuming that the crystal grows three-dimensionally and that there is a uniform probability for the formation of nuclei[8]. These assumptions are proven to be acceptable by morphological studies with scanning electron microscope (SEM)[13-16].

Fleischmann and Liler[8] investigated the kinetics of deposition of α-PbO_2 from acetate solution on a platinum substrate. The cubic growth law could also be applied in this case, however, an induction period, t_o, before nucleation had to be introduced as following to fit the experimental data,

$$i = ABN_o(t-t_o)^3/3$$

It was also shown that the current varied as the square of time

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