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Reverse Reaction of L-Phenylalanine Ammonia-Lyase derived from *Rhodotorula glutinis* for the Production of L-Phenylalanine

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Rhodotorula glutinis 의 L-Phenylalanine Ammonia-Lyase 의 역반응을 이용한 L-Phenylalanine 생성

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During the enzymatic production of L-phenylalanine exploiting L-phenylalanine ammonia lyase (E.C 4.3.1.5) and *trans*-cinnamic acid, the conversion yield of L-phenylalanine and the stability of L-phenylalanine ammonia-lyase *per se* or induced *Rhodotorula glutinis* IFO 0559 were investigated. And the glycerol added to the conversion reaction as stabilizer had effect only on L-phenylalanine and made it possible to obtain the 80% conversion yield from *trans*-cinnamic acid. In addition, the more rapid and reliable method than the thin layer chromatography for determining the conversion yield will be disscused.

L-Phenylalanine ammonia-lyase (PAL: EC 4,3, 1,5), an enzyme found in a variety of plants and yeasts catalyzes the deamination of L-phenylalanine to produce *trans*-cinnamic acid (1-7). Also, the reversibility of the enzyme reaction for the production of L-phenylalanine from *trans*-cinnamic acid has been suggested by some workers (7). In this report, the enzymatic and/or cellular stability and conversion yield during the time course reverse reaction were determined, and glycerol effect on the stability of not cell but enzyme led to the 80% conversion yield.

Materials and Methods

Culture of microorganisms

Optimal culture medium for the full induction of PAL in *Rhodotorula glutinis* IFO 0559 was composed of 1% peptone, 0.5% NaCl, and 1% L-phenylalanine (pH 6.2).

Fully induced cells were harvested by the centrifugation at 8,000g for 10 minutes after the 24 hr growth at 30°C, 150 rpm from the initial start A 660-0.15.

Assay of PAL activity

Activity of PAL was assayed by a modification of the method of Yamada *et al.*(7). The standard assay buffer contained 25 mM Tris. HCl (pH 8.8), and 0.005% cetylpyridinium chloride. 1 ml samples of the culture broth at appropriate times were harvested by centrifugation, washed once by suspending 3 ml of 0.9% saline, and resuspended in 3 ml of the standard assay buffer on ice. And immediately the reaction was carried out at 30°C for 10 min. *trans*-Cinnamic acid formation was determined by the spectrophotometry at 278 nm or 290 nm. For PAL activity assay of cells in conversion reaction mixture, the equivalent amounts of 1 ml culture broth were harvested and washed five times with 0.9% saline to completely wash out the inherent *trans*-cinnamic acid. PAL standard enzyme was purchased from the Sigma Chemical Co. (Sigma P 1016).

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The salts and glycerol in the commercial enzyme preparation were washed out at 4 °C by the ultrafiltration with Centricon microconcentrator (Amicon Corporation, Danvers, Mas.). And the PAL enzyme recovery from the conversion reaction mixture was also carried out with the microconcentrator.

Reverse reaction of PAL

For the preparation of conversion reaction substrate, 740 mg of *trans*-cinnamic acid was dissolved in 45 ml of 28% ammonium hydroxide, adjusted to pH 10.3 with distilled water in a final volume of 80 ml (final concentrations: 62.5 mM *trans*-cinnamic acid and 4.5 M NH_4OH).
Fully induced cell pellet was resuspended in 5 ml of the conversion reaction substrate and incubated at 30 °C for an appropriate time. In the other case of conversion reaction appropriate units of enzyme solution were mixed with the concentrated conversion reaction substrate to the final concentrations of 62.5 mM *trans*-cinnamic acid and 4.5 M NH_4OH.

Determination of conversion yield

For the reliable determination of conversion yield, three different methods were used. First, L-phenylalanine produced by the reaction was quantitized by thin layer chromatography using the ascending technique on a Merck 60 F 254 plate (solvent, 1-butanol: acetic acid: water, 4:1:1, vol/vol). The plates were developed for 4 hr and air-dried for 30 min. The chromatograms were sprayed with ninhydrin and baked at 100 °C for 5 min. In case of detection of *trans*-cinnamic acid, the thin layer chromatography was developed with a solvent (isopropanol: ammonium hydroxide: water, 20:1:2, vol/vol) and after removing the solvent by heating, the chromatograms were assayed upon the short-wave U.V. transilluminator in comparison with L-phenylalanine standards and *trans*-cinnamic acid standards. As the second approach high performance liquid chromatography in which mobile phase were composed of 30% acetonitrile and 30 mM sodium phosphate buffer (pH 6.5) was worked out for quantitation of L-phenylalanine by the U.V. detection at 254 nm. Finally, *trans*-cinnamic acid was detected by spectrophotometry without L-phenylalanine interference at some range of appropriate wavelength.

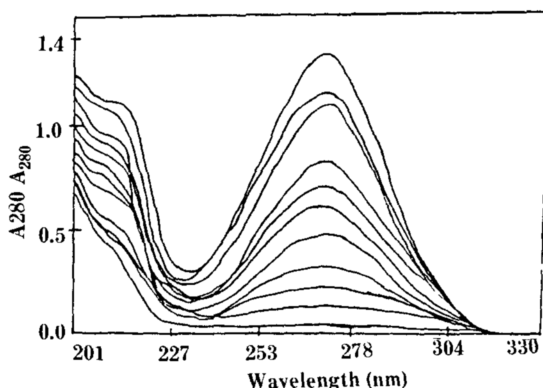

Fig. 1. The wavelength scanning of simulated conversion mixture.
Total concentration of L-phenylalanine plus *trans*-cinnamic acid was 62.5 μM, and the percentage values represent the ratio of L-phenylalanine to *trans*-cinnamic acid. Peak height was gradually decreased as the function of percentage values. Wavelength scanning procedure was guided by the operating instructions for Ultrospec (LKB) + Apple II computer.

Results and Discussion

Comparison of assay methods for the determination of conversion yield

The main point in common with all three assay methods is to exploit the U.V. absorbance of L-phenylalanine and/or *trans*-cinnamic acid. Both

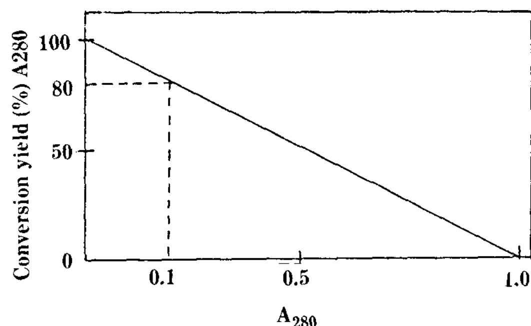

Fig. 2. Calibration standard curve for the determination of conversion yield.
For the use of this curve, the sample of the conversion mixture was 10 fold diluted and checked of absorbance at wavelength 280 nm.

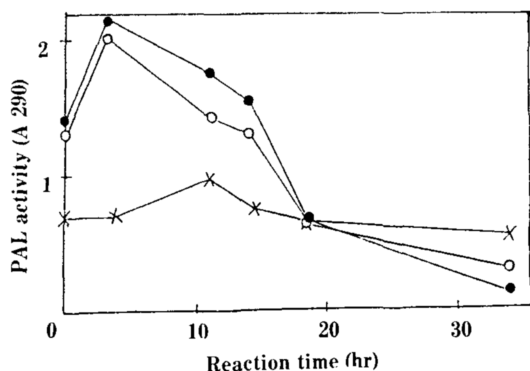

Fig. 3. The stability patterns of cellular PAL:
●, 0.3g of fully-induced cells in 1.5ml of standard conversion reaction substrate: ○, the same as ● except the addition of glycerol to a final concentration of 60%: ×, the same as ● except using of 0.15g of fully-induced cells.

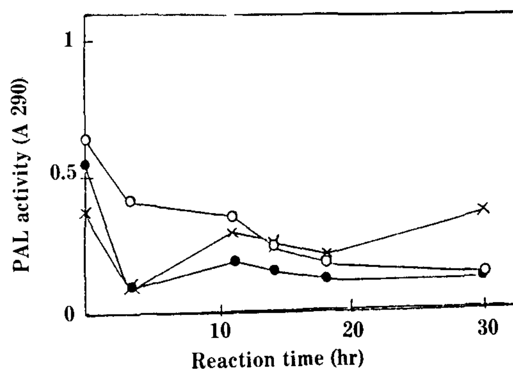

Fig. 4. The stability patterns of PAL standard:
●, 0.5 unit of PAL (Sigma Chemical Co.) were mixed with 1.5 × concentrated standard conversion substrate to a final volume of 1.5 ml: ○, the same as ● except the addition of 60% glycerol: ×, the same as ●, but 0.25 unit of enzyme was used instead of 0.5 unit.

HPLC system and spectrophotometry were rapid methods. Discernment of color or fluorescence of thin-layer chromatography is ocularly rough method. And HPLC operation of many samples included the laborous washing and loading of each ones. Furthermore, poor resolution of *trans*-cinnamic acid and L-phenylalanine with the mobile phase described in "Materials and Methods" and the wide difference between the extinction coefficient of L-phenylalanine and that of *trans*-cinnamic acid made it difficult to reliably quantitate the conversion yield. Fig. 1 shows the wavelength scanning profiles of simulated conversion reaction products and gradual decrease in peak height of *trans*-cinnamic acid in according as the conversion reaction occurs. Taking advantage of these profiles, we selected the optimal U.V. wavelength in which L-phenylalanine interference should not exist and the spectral sensitivity of *trans*-cinnamic acid should be in traceable scale.

At last, the calibration standard curve between the value of A 280 and conversion yield was plotted (Fig. 2).

Stability of PAL during the conversion reaction

The stability patterns of cells during the extreme reaction condition containing the high pH and concentration of ammonium ion are shown in Fig. 3 (closed circle). Interestingly, PAL activity of cells increased for some period of time. This phenomenon was true to the case when the concentration of cells in conversion reaction was half diluted, but appeared at a later time period.

The stability patterns of enzyme, free of cell were plotted in Fig. 4 (closed circle). It shows the gradual decrease of PAL activity during the reaction.

To know whether the cells in reaction mixture undergo lysis or release the PAL enzymes into the reaction medium, we harvested the cells or, if any, released enzymes of the reaction samples in two

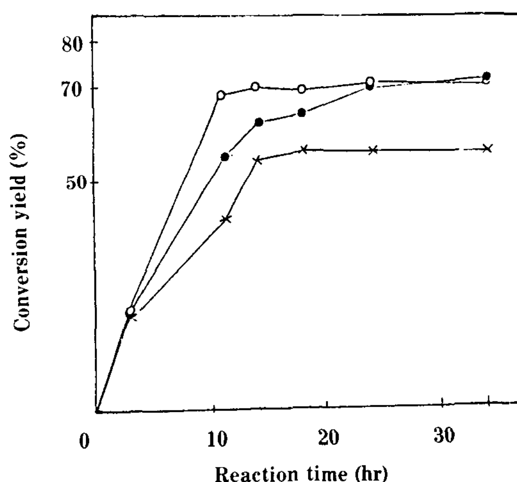

Fig. 5. Time courses of conversion yield using the fully induced cells:
●, ○, and × samples were taken out from the reaction mixture, described in Fig. 3.

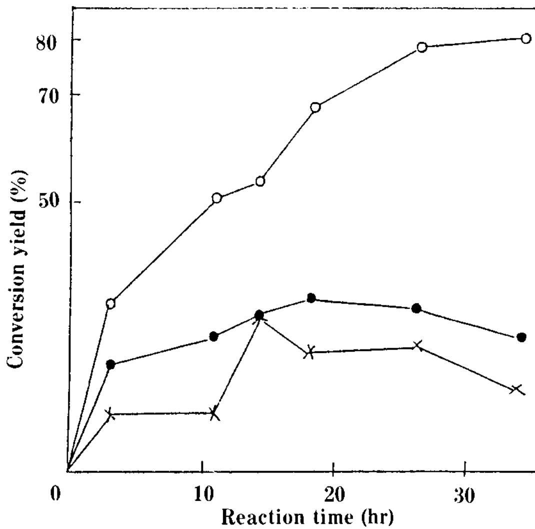

Fig. 6. Time courses of conversion yield using the PAL standard:

●, ○, and × samples were taken out from the reaction mixture, described in Fig. 4.

ways, that is, the centrifugation for only cells and the ultrafiltration with Centricon for cells and, if any, enzymes. There however, was no difference in PAL activity between two differently harvested reaction samples (data not shown). This indicates no release of PAL enzyme which was supposed to locate in periplasmic space of *R. glutinis.*

Parameters for the high conversion yield

We made the conversion reaction substrate at different concentrations of *trans*-cinnamic acid and ammonium ion. When the concentration of ammonium increased, the conversion yield was below the 70% (data not shown). It seems to be due to the unstability of PAL in a increased ammonium ion concentration.

To facilitate the maintenance of PAL stability, we used the glycerol as a stabilizer. The affirmative effect of glycerol worked on the stability of PAL enzyme, free of cells, but not on that of PAL embedded or entrapped in cells (Fig. 3, 4 open circles). And conversion yield in case of using the PAL enzyme with glycerol was as high as 80% (Fig. 6, open circles). When the concentration of cell or enzyme was reduced to half, somewhat greater stability of PAL activity but slightly less conversion yield than those at the original concentration were obtained (Fig. 3, 4, 5, cross). In the construction of conversion bioreactor using PAL system for the production of L-phenylalanine, we suggest that *R. glutinis* cells instead of PAL enzyme be used as a catalyst and be maintained at a low concentration. And because the maintenance of stability of PAL was verified to be critical point in conversion reaction, there should be some stabilizer, such as glycerol.

요 약

Rhodotorula glutinis IFO 0559에서 유래하는 L-phenylalanine ammonia lyase(E C 4. 3. 1. 5)를 이용하거나, 유도된 세포자체를 이용하여 *trans*-cinnamic acid로부터 L-phenylalanine을 생합성할 때 극단적인 반응액이 미치는 효소 및 세포의 안정성에 대하여 조사하였다. 그리고 안정제를 첨가한 60%의 glycerol은 효소의 안정화에 효과를 보였으며 *trans*-cinnamic acid에서 L-phenylalanine으로의 전이율은 80%까지 되었다. 이에 아울러 전이율을 보다 신속하고 정확히 측정할 수 있는 wavelength scanning 방법을 개발하였다.

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