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PURIFICATION OF PHENYLALANINE AMMONIA LYASE FROM *RHODOTORULA GLUTINIS*

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Key Word Index—*Rhodotorula glutinis*; yeast; phenylalanine ammonia lyase; enzyme; purification.

Abstract—A simple and rapid method employing four steps; sonication, salt precipitation, gel filtration on Sephacryl-S-400 followed by hydrophobic interaction chromatography on Phenyl Sepharose CL-4B for the purification of phenylalanine ammonia lyase from the yeast *Rhodotorula glutinis* is described. The preparation gave an overall yield of 33%, 195-fold purification and a high specific activity of 4.69 U mg^{-1} protein. The final preparation was homogeneous as judged from native polyacrylamide gel electrophoresis.

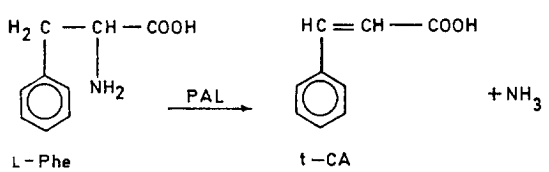
INTRODUCTION

Phenylalanine ammonia lyase (EC, 4.3.1.5, PAL) catalyses the spontaneous nonoxidative deamination of L-phenylalanine (L-Phe) to *trans*-cinnamic acid (t-CA) and ammonia [1].

The enzyme is widely distributed in higher plants [1–3], some fungi [4–5], yeasts [6–8] and in a single prokaryote, *Streptomyces* [9]. However, it is absent in true bacteria and animal tissues.

PAL is the first and key enzyme of the phenyl propanoid sequence, a secondary metabolic pathway in higher plants and is mainly involved in defence mechanisms [1, 10]. In microorganisms, it has a catabolic role, allowing them to utilize L-Phe as a sole source of carbon [7, 11].

PAL is one of the few nonhydrolytic enzymes that has commercial applications [12–20]. Despite its widespread distribution, PAL from *Rhodotorula* has been used exclusively for commercial purposes because of its high PAL levels and nonfastidious requirements for growth and PAL synthesis [8, 18]. Purified *Rhodotorula* PAL is effective in the treatment of certain mouse neoplastic tumors [12], is used for quantitative analysis of serum L-Phe in monitoring patients with phenylketonuria [13, 14] and also in the preparation of low Phe diets [15]. PAL-containing whole cells of *Rhodotorula* have been recently used to synthesize the essential amino acid, L-Phe [16–20] and its methyl ester [21] by the reversal of the physiological reaction. PAL has been purified from many sources [12, 22–25] including that from various strains of *Rhodotorula* [12, 23, 24] and has been characterized in detail. However, previous purification procedures of *Rhodotorula* PAL employed lengthy and time-consuming traditional methods of protein purification with resultant low specific activities and/or yields. There is therefore a need to develop a short and simple purification method for *Rhodotorula* PAL, the enzyme of commercial interest; the present work reports the results of such a study.



RESULTS AND DISCUSSION

Availability of a purified enzyme with a high specific activity and good yield is a prerequisite for a biocatalyst in commercial applications. In the present paper we describe a simple and rapid procedure for the purification of PAL from the yeast, *Rhodotorula glutinis*. A profile of a typical purification procedure is given in Table 1. Gel filtration of the salt-precipitated crude extract on Sephacryl-S-400 followed by hydrophobic interaction chromatography gave pure enzyme. It was observed that the addition of glycerol to the buffers throughout was necessary to retain enzyme activity during the purification. The final preparation gave 33% overall yield, 195-fold purification and a

Table 1. Purification of PAL from *Rhodotorula glutinis* NCYC 61*

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1} protein)	Yield (%)	Purification (fold)
Sonication	46	1940	0.024	100	1.0
30–65% $(NH_4)_2SO_4$ fractionation	37	624	0.060	80	2.5
Gel filtration on Sephacryl S-400	28	16	1.75	61	73
Phenyl Sepharose CL-4B hydrophobic interaction chromatography	15	3.2	4.69	33	195

*Data presented are for 40 g fresh weight of yeast derived from one litre of culture. Values given are means of three independent determinations.

PAL adsorbed on Phenyl Sepharose could be eluted successfully by employing a double gradient and the enzyme eluted with 10% glycerol and 0.5 M $(NH_4)_2SO_4$ (Fig. 1). The final preparation was homogeneous, as judged by the presence of a single protein band after native polyacrylamide gel electrophoresis (Fig. 2).

The PAL preparation from hydrophobic interaction chromatography step retained 80–85% of the initial activity when stored in 50 mM Tris-HCl buffer, pH 8.5 containing 20% glycerol at −20° for 2–3 months. Freezing the enzyme in the absence of glycerol resulted in inactivation and storage at 0–2° decreased the activity by 50% in 24 hr.

The present study extends and improves further the earlier work on the purification of *Rhodotorula* PAL [12, 23, 24]. Our method eliminates tedious, multistep procedures employed by previous workers and reduces the number of steps to just four (Table 2). The specific activity of the final preparation reported here (4.69 U mg^{-1} protein) is considerably higher than the values of 1.1 and 2.5 U mg^{-1} reported by Hodgins [24] and Fritz *et al.* [12] respectively and is also comparable to the specific activity of 5.1 U mg^{-1} reported by Adachi *et al.* [23] (Table 2). We are currently using the purified enzyme to quantify L-Phe in biological samples.

EXPERIMENTAL

Microorganism. The yeast strain *R. glutinis* NCYC 61, which was used for isolating PAL, was obtained from National Chemical Laboratories, Pune, India. The strain was maintained by a weekly transfer on agar slants containing 1% yeast extract, 1% peptone and 0.5% NaCl.

Chemicals. L-Phe and Tris were obtained from Sigma. Glycerol and $(NH_4)_2SO_4$ were products of

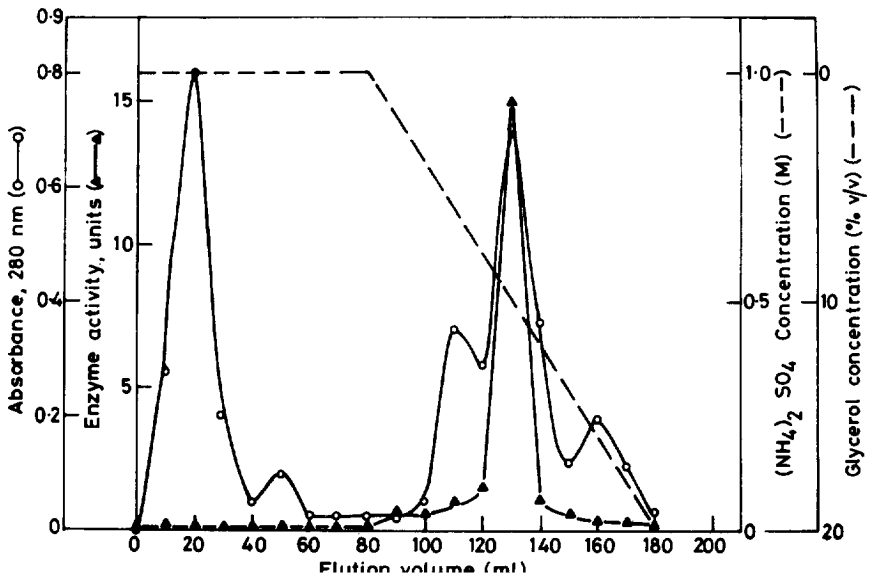


Fig. 2. Nondenaturing polyacrylamide gel electrophoresis of purified PAL. Direction of migration is from top to bottom.

Qualigens (India) Ltd. Sephacryl-S-400 and Phenyl Sepharose gels were procured from Pharmacia.

Culture conditions. R. glutinis cells were grown in a culture medium containing 1% yeast extract, 1% peptone and 0.5% NaCl on a bacterial shaker (160 rpm) at 30°. Cells were harvested in the late log-phase (24–27 hr) when maximal PAL activity was observed.

PAL assay. PAL activity was measured by following the formation of t-CA from L-Phe at A_{290} nm [16–19]. The reaction mixture (1 ml) containing 50 mM Tris-HCl buffer pH 8.5, 75 mM L-Phe and enough enzyme to give an absorbance change of 0.01–0.05 min^{-1} was incubated at 30° for 10 min. The reaction was then terminated by inactivating the enzyme with concd HCl and the A_{290} of the clear soln was measured. Reaction mixt. in which the substrate was added after termination of the reaction served as control. The enzyme activity is expressed as units; one unit of enzyme is defined as the amount required to convert one μmol of L-Phe per min at 30°.

Protein determination. This was done by a modified Bradford method [26] using bovine serum albumin.

Purification of R. glutinis PAL. All operations were carried out at 0–4° unless otherwise stated. All centrifugations were at 17 000 *g* for 20 min in Sorvall RC-5 refrigerated centrifuge.

(a) *Preparation of crude extract. R. glutinis* cells (35–40 g) obtained from 1 litre of culture were suspended in 100 ml 10 mM Tris-HCl buffer, pH 7 containing 25% glycerol. The cell suspension was sonicated (50 Hz sec^{-1}) in a Vibra Cell VC-300 sonicator for 10 min in ice. Then the suspension was centrifuged. After assaying the sediment and supernatant separately for PAL activity the sediment was sonicated exactly as before (×3). After four sonications, the residue containing negligible PAL activity was rejected and the four supernatants were pooled (400 ml). This prepn is referred to as the crude extract.

(b) *Ammonium sulphate fractionation.* The crude extract was brought to 30% $(NH_4)_2SO_4$ satn by slowly adding powdered $(NH_4)_2SO_4$ with stirring. The soln was allowed to stand for 1 hr and the ppt. formed was removed by centrifugation. The supernatant was then brought to 65% $(NH_4)_2SO_4$ satn and the ppt. which contained the enzyme activity was dissolved in 10 ml of 50 mM Tris-HCl buffer, pH 8.5 containing 25% glycerol (buffer A).

(c) *Sephacryl-S-400 gel filtration.* A 10-ml sample of partially purified PAL preparation from the previous step was loaded onto a Sephacryl-S-400 gel column (100 × 2.6 cm, bed vol.; 450 ml), prequilibrated with buffer A. Then the column was washed with 2 vol. of the same buffer at 28 ml hr^{-1} and 7 ml fractions were collected. The active fractions were pooled and the pooled enzyme was concd by precipitation with $(NH_4)_2SO_4$ (85% satn). The ppt obtained was redissolved in 100 ml of 50 mM Tris-HCl buffer pH 7.5 containing 1 M $(NH_4)_2SO_4$ (buffer B).

(d) *Hydrophobic interaction chromatography.* A 10-ml sample of enzyme from gel filtration step was applied onto a Phenyl Sepharose CL-4B column (20 × 0.8 cm, total bed vol. 40 ml) preequilibrated with buffer B. The column was washed with 80 ml buffer B at 20 ml hr^{-1}. Finally, PAL was eluted with a double gradient of decreasing $(NH_4)_2SO_4$ concn (1–0.0 M) and increasing glycerol concn (0–20%) in Tris-HCl buffer, pH 7.5, and 10 ml fractions were collected. The active fractions were pooled, concd by $(NH_4)_2SO_4$ and redissolved in Tris-HCl buffer, pH 7.5 containing 20% glycerol. The enzyme solns were stored at −20° for further use. Glycerol was removed from the samples on the day of use by dialysis.

Electrophoresis: Polyacryamide gel electrophoresis

Table 2. Comparison of *Rhodotorula* PAL purification by different workers

Organism	Number of steps employed	Purification (fold)	Yield (%)	Specific activity (U mg^{-1})	Reference
R. glutinis ATCC 15385	8	49	17	1.1	[24]
R. glutinis IFO 0559	8	250	23	2.5	[12]
R. glutinis IFO 0559	6	300	32	5.1	[23]
R. glutinis NCYC 61	4	195	33	4.69	Present study

of the native enzyme on 7.5% gel was performed at 2° according to the method of ref. [27].

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