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SHORT COMMUNICATION

PHENYLALANINE AND TYROSINE AMMONIA-LYASE ACTIVITY IN *SPOROBOLOMYCES PARAROSEUS*

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Abstract—Phenylalanine ammonia-lyase from *Sporobolomyces pararoseus* was purified more than 450-fold. Polyacrylamide disc gel electrophoresis of this purified enzyme gave a single major protein band. Tyrosine ammonia-lyase activity was monitored during the purification of phenylalanine ammonia-lyase. Deaminating activities for phenylalanine and tyrosine were not separated during the purification process. The existence of one ammonia-lyase with bisubstrate activity is postulated.

INTRODUCTION

PHENYLALANINE ammonia-lyase (PAL) (E.C. 4.3.1.5) has been studied in a variety of plants and fungi.[1-14] This enzyme catalyzes the deamination of L-phenylalanine to *trans*-cinnamic acid and ammonia. In the yeast strains studied thus far, purified PAL has been found to deaminate both phenylalanine and tyrosine. This was demonstrated in the yeast *Rhodotorula glutinis* by Ogata *et al.*[6] Recently Camm and Towers[12] postulated that in *S. roseus* (Sporobolomycetaceae) there were separate ammonia-lyases for phenylalanine and for tyrosine. The object of this study was to investigate the nature of the phenylalanine and tyrosine ammonia-lyases (PAL and TAL) present in *S. pararoseus* (ATCC-11386).

RESULTS

TAL Activity in S. pararoseus

An experiment was performed in which all but the last step in the purification procedure outlined for PAL was carried out. During the purification the activity of PAL and TAL was monitored spectrophotometrically. The ratio of enzyme activities was recorded for each of these steps (Table 1). As shown in this Table, there is a large transient increase of PAL and TAL activity following protamine sulfate treatment. This increased activity, which invariably occurs during this step is lost during the subsequent purification. TAL

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TABLE 1. PURIFICATION OF PAL AND TAL FROM *S. pararoseus*

Step	Total enzyme units PAL	Total enzyme units TAL	Ratio units PAL/TAL
1. Crude enzyme sonicate	12·4	2·95	4·20
2. Protamine sulfate (1 ml 5%/200 mg protein)	16·95	8·11	2·09
3. Ammonium sulfate fractionation			
22% saturation	10·70	2·10	5·10
26% saturation	1·48	0·298	4·97
4. Sodium citrate fractionation			
15·0% saturation	0·594	0·139	4·27
18·7% saturation	5·98	1·29	4·64
5. G-200 Sephadex chromatography			
Tube 35	0·0768	0·0120	6·40
40	0·1788	0·0300	5·96
45	0·2652	0·0432	6·19
46	0·2760	0·0444	6·21
47	0·2700	0 0408	6 61
50	0·2136	0·0336	6·36
55	0·0876	0·0144	6 08
Total combined above fractions	3·99	0·632	6·31

activity appears to be increased more than the PAL activity resulting in a decrease in the PAL to TAL ratio. Both enzyme activities, however, precipitated in the same fractions during ammonium sulfate and sodium citrate fractionations. The enzyme unit ratios of TAL to PAL were approximately the same for the major and minor fractions. This indicates that both enzymes precipitate at the same point during these salt fractionations.

After chromatography on G-200 Sephadex, the deaminating activity peaks for TAL and PAL coincided, 66·7% of the PAL activity and 49·0% of the TAL activity being recovered. The specific enzyme activity for PAL after the G-200 Sephadex fractions were pooled was 0·934 units/mg protein. The ratio of TAL to PAL units again was of a magnitude which indicated that there was little if any separation of these activities. To further ascertain whether TAL activity could be separated from PAL activity the purified enzyme was subjected to disc gel electrophoresis. Assay of both enzymes revealed only one activity peak for each enzyme and these peaks were again superimposable. The stained gel revealed one major protein band which corresponded in migration to the enzyme activities. Several media were substituted for the one described earlier, in an attempt to induce TAL formation. In two cases 1% L-tyrosine replaced 1% D,L-phenylalanine and in one of these salts[15] replaced malt and yeast extract. When tyrosine and salts were used as the growth medium the resultant enzyme, purified as before, gave a ratio of PAL to TAL units of 6·2. Under no conditions could a tyrosine specific ammonia-lyase be detected in this yeast.

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DISCUSSION

PAL was purified 450-fold using the described procedure which resulted in a specific enzyme activity of 1·31 units/mg protein. After purification the enzymes showed one major protein band on disc gel electrophoresis, a minor slower moving band and trace impurities. When a large amount of enzyme (100 μg) was used for electrophoresis, deaminating activity was found to correspond to the light slower moving band, representing only a small percentage of the total PAL activity. Due to the consistent appearance of this band and the fact that it apparently has some PAL activity, it is possible that it is an aggregate form of PAL with enzyme activity.

Phenylalanine and tyrosine ammonia-lyase activities in *S. pararoseus* were not separable at any stage in the purification. Because of the inseparability of these activities it is very likely that a single enzyme from *S. pararoseus* has bisubstrate activity for tyrosine and phenylalanine. Whether phenylalanine or tyrosine was used during yeast culture, essentially the same deamination activities were observed. There appears to be no independent PAL or TAL in this yeast when cultured under our conditions.

Camm and Towers[12] postulated, on the basis of experiments with a crude enzyme preparation, that the enzymes responsible for the deamination of tyrosine and phenylalanine in *S. roseus* were different proteins. Our data indicate a contrasting situation with *S. pararoseus* in that our purest enzyme deaminates both substrates. It is possible, however, that a minor species catalyzing solely tyrosine deamination could have been deactivated during purification.

EXPERIMENTAL

Enzyme Assay

The deamination of phenylalanine was measured by following the increase in absorbance at 290 nm.[16] The standard assay mixture contained 5 mM L-phenylalanine, 0·1 M Tris-HCl, pH 8·5, and enzyme at 30°. Tyrosine deamination was measured similarly, except that a wavelength of 310 nm was used (a molar extinction coefficient of 12,400 was utilized in activity calculations). In this latter assay L-tyrosine replaced L-phenylalanine in the standard system. An enzyme unit is defined as that amount of protein catalyzing the appearance of 1 μmole of product per min at 30°. For purified enzyme protein concentrations were measured by the modified Lowry method of Zak and Cohen[17] For crude protein determinations the Warburg–Christian spectrophotometric method was employed.[18]

Yeast culture. PAL was obtained from *S. pararoseus* grown in batch culture at 30° with a media of 0·7% Difco malt extract, 0·1% Difco yeast extract, and 0·1% D,L-phenylalanine. The organism was initially inoculated in 125 ml flasks containing approximately 40–50 ml of media. After 18 hr of growth on a rotary shaker, the culture was transferred to a 1-l. flask having 500 ml of the above media. Approximately 18 hr later this flask was used to inoculate a 20 l. carboy containing 15 l. of media with Antifoam A to prevent excess foaming. The carboys were aerated with compressed air. Under these conditions maximum PAL activity occurred during the late log phase which corresponded to 29 hr of cellular growth. The yeast cells (336 g from 5 carboys) were collected with a Sharples continuous flow centrifuge, washed with 1·5 l. of 0·5% NaCl–0·5% KCl and frozen.

Purification of PAL

Step 1. *Crude enzyme.* Table 2 summarizes a typical enzyme purification. All operations were performed at 0–5° except where otherwise stipulated. The washed cells were suspended with 600 ml of 50 mM Tris-HCl, pH 8·5. This mixture was divided into two 450-ml aliquots and each was sonicated with a Branson model W-185-w sonifier for 1·5 hr at maximum power, generating a temperature of 12°. The solutions were then centrifuged at 19,600 *g* for 10 min, and the supernatants (900 ml) retained.

Step 2. *Treatment with protamine sulfate.* One ml of 5% protamine sulfate suspension (Caibiochem) pH 8·5 (adjusted with NH_4OH) was added to the Step 1 supernatant for every 200 mg of protein present as measured spectrophotometrically.[17] The protein-protamine sulfate mixture was stirred for 1 hr and then centrifuged at 19,600 *g* for 10 min. The pellet was discarded.

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TABLE 2. PURIFICATION PROCEDURE

Purification step	Volume (ml)	Total mg protein	Total enzyme units	Specific activity (enzyme units/ mg protein)
1. Crude enzyme sonicate	900	46,700	126	0·003
2. Protamine sulfate supernatant	920	17,450	227	0 013
3. Ammonium sulfate fractionation	45	2923	134	0·046
4. Sodium citrate fractionation	20	892	92	0·103
5. 30% ammonium sulfate saturation	8	328	70	0·213
6. G-200 Sephadex	80	61	72	1·180
7. Third ammonium sulfate fractionation	10	45	59	1·310

Step 3. *Ammonium sulfate fractionation.* 46g $(NH_4)_2SO_4$ (7% saturation) was added to the protein solution (920 ml) and stirred for 20 min. The mixture was then centrifuged at 19,600 *g* for 10 min. The precipitate was dissolved in a minimum amount of 50 mM Tris-HCl, pH 8·5. The supernatant was brought to 14% saturation with 46 g $(NH_4)_2SO_4$. Similar $(NH_4)_2SO_4$ additions were continued until all the enzyme activity was present in the redissolved precipitates PAL precipitated between 21 and 28% $(NH_4)_2SO_4$ saturation. The most active fractions were pooled (45 ml).

Step 4. *Sodium citrate fractionation.* The same procedure was employed for sodium citrate fractionation as described for the $(NH_4)_2SO_4$ fractionation. PAL precipitated when 25–35 g of salt per 100 ml of solution had been added.

Step 5. *Second ammonium sulfate precipitate.* A second ammonium sulfate fractionation at 30% saturation (35 g salt/100 ml solution) served to concentrate PAL prior to G-200 Sephadex chromatography.

Step 6. *G-200 sephadex chromatography.* The active PAL (8·0 ml) was applied to a column (3 × 100 cm) of G-200 Sephadex which was equilibrated with 50 mM Tris-HCl, pH 8·5. Fractions (8 ml) were collected every 16 min. The enzyme was associated with the first major protein peak to be eluted. Those fractions which contained a specific activity greater than 0·5 units/mg were combined (80 ml).

Step 7. *Third ammonium sulfate precipitation.* PAL precipitated between 18 and 22% saturation with $(NH_4)_2SO_4$ during a third $(NH_4)_2SO_4$ fractionation. The most active fractions were dialyzed against 50 mM Tris-HCl, pH 8·5 and stored at −15°.

Polyacrylamide Gel Electrophoresis

Gel electrophoresis was performed as described by Davis.[19] Samples were applied in 50% sucrose and the polyacrylamide gels were subjected to 3·0 mamp/tube until the bromphenol blue marker reached the end of the gel (3 hr at 4°). The gels were run in duplicate, one gel being sliced and eluted in 0·5 ml of 50 mM Tris-HCl, pH 8·5 buffer and the other stained with 1% Amido-Schwartz. PAL activity corresponded with an intense band of low mobility and with a smaller band of even lower mobility.

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