Crystallization and Properties of L-Phenylalanine Ammonia-lyase from Rhodospiridium toruloides

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L-Phenylalanine ammonia-lyase was crystallized for the first time from a cell-free extract of Rhodospiridium toruloides IFO 0559. Heat treatment at 50°C for 5 min was a smart step for enzyme purification. Column chromatographies with DEAE-cellulose and hydroxyapatite, and gel filtration on a Sephadex G-200 column were used in the subsequent purification. The enzyme was purified to a homogeneous state and crystallized as fine needles with ammonium sulfate. The crystalline enzyme was pure by both analytical ultracentrifugation and polyacrylamide gel electrophoresis. The enzyme had a 8.2 s sedimentation velocity. The molecular weight of the enzyme was 165,000 by the dual methods of sedimentation equilibrium and gel filtration. The enzyme was composed of two identical subunits with a molecular weight of 80,000.

Materials and Methods

Chemicals. All of the chemicals used in this study were reagents of guaranteed grade, commercially available. Hydroxyapatite was prepared by the method of Tiselius et al.7) Microorganism and culture. Rhodospiridium toruloides IFO 0559 was purchased from the Institute for Fermentation, Osaka, and maintained on a malt slant. Composition of the culture medium was devised on the basis of the medium used by Ogata et al.8) The culture medium finally developed contained 2% L-phenylalanine, 0.25% K2HPO4, 0.1% KH2PO4, 0.01% MgSO4·7H2O, 0.05% yeast extract (Oriental Yeast Co.), and 2.0% sucrose. R. toruloides was first grown in 3 l of 5% malt aerobically at 30°C for 30 hr on a rotary shaker. The culture was then transferred to 30 l of the medium mentioned above in a 50-l jar fermentor. The yeast was grown at 30°C under vigorous aeration for 24 hr.

Enzyme assay. The activity of PAL was measured spectrophotometrically by monitoring the formation of cinnamic acid. The procedure was essentially the same as that used by Ogata et al.5) The reaction mixture contained 10 μmol L-phenylalanine, 200 μmol of Tris–HCl, pH 8.5,
and enzyme solution in a total volume of 3.0 ml. The reaction was started by the addition of enzyme solution and the increase in absorbance at 290 nm was monitored by a recorder at 25°C. One unit of the enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of trans-cinnamic acid per min. Specific activity was defined as units per mg protein, which was measured by the method of Lowry et al. with bovine serum albumin as a standard.

**Electrophoresis.** For the estimation of purity of the enzyme preparations, disc gel electrophoresis was done under the conditions described by Davis using 7.5% polyacrylamide gel and Tris-glycine buffer, pH 8.3. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was done in 10% polyacrylamide gel as described by Laemmli. Samples were treated with 3% SDS at 30°C for about 30 min before electrophoresis.

**Analytical ultracentrifugation.** Examination of purity of the crystalline enzyme was done with a Hitachi ultracentrifuge (SCP85H) at 60,000 rpm with schlieren optics attached. Estimation of the sedimentation coefficient and molecular weight measurement of the enzyme were done by the methods of sedimentation rate and sedimentation equilibrium, which were operated by a combination of a Hitachi UV scanner (ABS-7), an absorption scanner, and a UC processor (DA-7). Temperature was maintained at 20°C.

**Gel filtration.** The molecular weight of PAL was also measured with the crystalline preparation by gel filtration on a Sephadex G-200 column by the method of Andrews.

**Results**

**Purification of PAL**

Freshly harvested cells of *R. toruloides* (50 g wet wt.) were suspended in 10 mM Tris–HCl, pH 8.0, containing 10 mM L-phenylalanine. The cells were broken by two passages through a Dynomill or a French pressure cell press. The cell homogenate was centrifuged at 10,000 × g for 30 min at 4°C. The resulting supernatant (350 ml) was heated at 50°C for 5 min. The heat-denatured precipitate was removed by centrifugation at 10,000 × g for 20 min at 4°C. All purification steps below were done at 0–4°C unless otherwise stated. The supernatant solution from the preceding step was adsorbed onto a DEAE-cellulose column (3 × 30 cm), which had been equilibrated with 10 mM Tris-HCl, pH 8.0, containing 10 mM L-phenylalanine. After rinsing the column with the same buffer containing 0.05 M KCl, PAL was eluted from the column with an increasing concentration gradient of KCl to 0.3 M. The enzyme activity was eluted at about 0.2 M KCl. The active enzyme fractions were combined and precipitated by the addition of ammonium sulfate to 70% saturation. After the precipitate was collected by a conventional centrifugation, the resulting precipitate was dissolved in 5 mM potassium phosphate (KPB), pH 7.5, containing 10 mM L-phenylalanine and dialyzed against the same buffer overnight. The dialyzed enzyme solution was adsorbed onto a hydroxyapatite column (1 × 5 cm), which had been equilibrated with the same potassium phosphate. The hydroxyapatite column was rinsed with the same buffer until the optical density at 300 nm became less than 0.1. Then, PAL was eluted from the column with 50 mM potassium phosphate, pH 7.5, containing 10 mM L-phenylalanine. The enzyme solution was combined in a dialyzing tube and dialyzed against the same buffer in which ammonium sulfate was added to 80% saturation and the pH was maintained at 7.5 during the dialysis. Dialysis was done until the protein precipitated in the dialyzing tube. The precipitate was collected by centrifugation and dissolved in a minimal volume of 50 mM potassium phosphate, pH 7.5, containing 10 mM L-phenylalanine. After the insoluble materials were removed by centrifugation, the enzyme solution was put on a Sephadex G-200 column (1 × 115 cm), which had been equilibrated with 50 mM potassium phosphate, pH 7.5, containing 10 mM L-phenylalanine. PAL was eluted from the column as a sharp peak and some impurities followed. Since the fractions around the peak fractions appeared to be almost homogeneous, the corresponding fractions were combined and concentrated by dialysis as mentioned above. The precipitate of PAL was dissolved in a minimal volume of 50 mM potassium phosphate, pH 7.5, containing 10 mM L-phenylalanine. Insoluble materials were removed by a flush centrifugation. Solid ammonium sulfate was carefully added to the
Phenylalanine Ammonia-lyase of R. toruloides

Fig. 1. Crystals of L-Phenylalanine Ammonia-lyase from R. toruloides IFO 0559.

Table 1. Summary of Purification of L-Phenylalanine Ammonia-lyase from R. toruloides

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
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</thead>
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<tr>
<td>Cell-free extract</td>
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<td>79.0</td>
<td>0.016</td>
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<td>Heat treatment</td>
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<td>4.567</td>
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<td>Crystallization</td>
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<td>25.5</td>
<td>5.100</td>
<td>32</td>
</tr>
</tbody>
</table>

Fig. 2. Sedimentation Pattern of L-Phenylalanine Ammonia-lyase in Analytical Ultracentrifugation.

A crystalline preparation of PAL (about 10 mg/ml in 10 mM Tris-HCl, pH 8.0, containing 10 mM L-phenylalanine) was used and sedimentation patterns were taken at a wavelength of 310 nm. The photographs were taken as indicated after reaching 60,000 rpm.

Fig. 3. Polyacrylamide Gel Electrophoresis of L-Phenylalanine Ammonia-lyase.

A crystalline preparation of PAL (30 μg) was put on a gel column. Protein was stained with Coomassie brilliant blue.

Fig. 4. Molecular Weight Measurement of L-Phenylalanine Ammonia-lyase by Gel Filtration on Sephadex G-200 Column.

A crystalline preparation of PAL and the standard marker proteins (0.5 ml) were put on a Sephadex G-200 column (1 x 115 cm), equilibrated with 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM L-phenylalanine. Half-ml of fractions were collected. Standard marker proteins used were Lys, lysozyme (M₀ 14,000); BSA, bovine serum albumin (M₀ 69,000); LDH, NAD: lactate dehydrogenase from bovine heart (M₀ 150,000); and Cat, bacterial catalase (M₀ 240,000).

Physicochemical properties of PAL

The crystallized enzyme showed a single peak in analytical ultracentrifugation and the sedimentation velocity was calculated to be 8.2 s (Fig. 2). The same preparation also showed a single protein band in polyacrylamide gel
Fig. 5. SDS-Polyacrylamide Gel Electrophoresis of L-Phenylalanine Ammonia-lyase. 

A slab gel composed of 5% stacking gel and 10% separating gel were used. A mixture of standard marker proteins including phosphorylase b (Phos, M, 93,000), bovine serum albumin (BSA, 68,000), ovalbumin (Ova, 43,000), carbonic anhydrase (Anhy, 31,000), and lysozyme (Lys, 14,000) was used at the left lane. Various amount of crystalline preparation of PAL (5 µg, 10 µg and 15 µg) were put on the 2nd lane to 4th lane. Protein was stained with Coomassie brilliant blue.

electrophoresis as shown in Fig. 3. The absorption spectrum gave no characteristic features and the E\text{\%}^{1\%} at 280 nm was 13.6. In Fig. 4, the gel filtration profile for molecular weight measurement of PAL is shown and the apparent molecular weight of PAL was 162,000. It was evident that PAL was somewhat larger than NAD: lactate dehydrogenase (M, 150,000) and much smaller than catalase (M, 240,000). The molecular weight of PAL was also supported by an alternative measurement of molecular weight, by sedimentation equilibrium, yielding an apparent molecular weight of 165,000. On SDS-gel electrophoresis, one protein band having a molecular weight of 80,000 appeared indicating a possibility that PAL is composed of two identical subunits (Fig. 5).

Catalytic properties of PAL

When PAL was treated with various inhibitors, including sulfhydryl reagents, metal chelators, and carbonyl reagents, the enzyme was inhibited only with sulfhydryl reagents such as p-chloromercuribenzoate or mercuric chloride. PAL which had been treated with sodium borohydride showed no appreciable enzyme activity, as Hodgins reported.\textsuperscript{6} A group of carbonyl reagents were inert as inhibitors of PAL. The optimum pH of non-oxidative deamination of L-phenylalanine by PAL was observed at 8.5. Besides L-phenylalanine, L-tyrosine was also deaminated by the enzyme at a rate of about 50% for L-phenylalanine. Apparent Km values for L-phenylalanine and L-tyrosine were 0.29 mm and 0.18 mm, respectively. On the other hand, the enzyme was completely inert on D-phenylalanine and D-tyrosine. The enzyme activity was lost completely upon heating at 70°C for 10 min. However, almost of the enzyme activity was conserved on heating at 50°C for 10 min; this was the reason why heat treatment was used in a step of enzyme purification. The thermostability of PAL was enhanced by the presence of L-phenylalanine. The crystalline enzyme was stable and maintained its enzyme activity for at least one month when stored in a refrigerator at 4°C, though the cell-free extract was somewhat unstable during storage and lost its enzyme activity rapidly.

Discussion

Hodgins\textsuperscript{6} used Rhodotorula glutinis ATCC 15385 and highly purified PAL. We can see in the current issue of the American Type Culture Collection (ATCC) catalogue\textsuperscript{14} that this strain is reclassified as Rhodosporidium toruloides ATCC 10788 and also that the strain has been introduced from the Institute for Fermentation, Osaka (IFO). The strain is kept in IFO as Rhodosporidium toruloides IFO 0559 and is the same as Rhodotorula glutinis IFO 0559, which was used by Ogata et al.\textsuperscript{5,8} in early studies of PAL. With such a historical background for the yeast strain, we selected the strain for the purification of PAL. In this study, PAL has been purified and crystallized for the first time and compared some properties with the references.

Heat treatment at 50°C for 5 min allowed the enzyme purification with relative ease. The
heat treatment may allow the elimination of inactivating factors, probably proteases, from the enzyme solution in the presence of the substrate, L-phenylalanine. The presence of L-phenylalanine in the buffer solutions throughout the enzyme purification gave good results. PAL was finally purified to a homogenous state and a single protein band appeared on polyacrylamide gel electrophoresis. The relative mobility was almost 0.3 while the preparation by Hodgins gave 0.5, although his preparation that appeared elsewhere looks similar to ours. The difference may be due to the polymerizing state of the enzyme, since his preparation showed the molecular mass of 275,000 while our preparation gave it to be 165,000. The enzymes from potato tubers and maize seedlings also showed the higher molecular weights than our preparation, 330,000 and 306,000, respectively. Since our enzyme is a dimer composed of two identical subunits, the enzymes of Hodgins and from plants appear to be a tetramer. Such difference of the polymerizing state of the enzyme probably comes from the purification strategies used.

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References
16) E. A. Havir and K. K. Hanson, Biochemistry, 7, 1896 (1968).