Phenylalanine Ammonia-lyase

INDUCTION AND PURIFICATION FROM YEAST AND CLEARANCE IN MAMMALS*

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Phenylalanine ammonia-lyase (EC 4.3.1.5) catalyzes the deamination of L-phenylalanine to form trans-cinnamic acid and tyrosine to trans-coumaric acid. Maximal enzyme activity in Rhodotorula glutinis (2 units/g. wet weight, of yeast) was induced in late-log phase (12 to 14 hours) of growth in a culture medium containing 1.0% malt extract, 0.1% yeast extract, and 0.1% L-phenylalanine. A highly purified enzyme was obtained by fractionation with ammonium sulfate and sodium citrate followed by chromatography on DEAE-cellulose and Sephadex G-200. The active preparation yielded a major component on three different polyacrylamide gel electrophoretic systems. Antisera to phenylalanine ammonia-lyase was raised in rabbits and detected by double immunodiffusion. The antigen-antibody complex was enzymatically active in vitro. The biological half-life of the enzyme was approximately 21 hours in several mammalian species (mice without and with BW10232 adenocarcinoma and B16 melanoma, rats, and monkeys) after a single injection; however, upon repeated administration, phenylalanine ammonia-lyase had a much shorter biological half-life. The onset of rapid clearance occurred earlier in tumor-bearing than in nontumor-bearing mice indicating a direct or indirect influence by the tumor on the biological half-life of phenylalanine ammonia-lyase.

Experimental Procedure

Yeast Culture—Rhodotorula glutinis (5559 Institute for Fermentation, Osaka, Japan) for these experiments was either purchased from P-L Biochemicals, Inc., or cultured in a New Brunswick 14L fermentor in a medium containing malt extract (1.0%), yeast extract (0.10%), and L-phenylalanine (0.10%) in deionized water. Bacto-malt extract Difco certified and Bacto-Yeast extract Difco certified was purchased from Difco Laboratories, Detroit, Mich. Yeast from a 3% agar slant containing malt extract (0.1%) was used to initiate growth in 250 ml (500-ml flask) for 24 to 36 hours. This inoculum (250 ml) was then added to 12 liters of growth medium and incubated at 30° with 12 p.s.i. of air pressure and a mixing speed of 600 rpm. The cells were collected in a Cepa continuous flow centrifuge, and then either frozen and maintained at -5° or immediately disrupted by sonic oscillation.

Enzyme Assay—Phenylalanine ammonia-lyase was assayed as previously described with the following modification (10). The reaction was started by the addition of enzyme to 0.833 mm L-phenylalanine in Tris-HCl buffer (0.1 M, pH 8.5) and was monitored at 290 nm with a Gilford model 240 spectrophotometer at 30°. The concentration of enzyme was adjusted so that the change in optical density was in a range of 0.0075 to 0.018/min. The activity was determined from an optical density change over a time period of 6 to 10 min. One unit of enzyme was defined as that amount of protein that catalyzed the appearance of 1 micromole of cinnamic acid/min at 30°. Protein concentration was determined by the spectrophotometric method of Warburg and Christian (16). The activity of the enzyme using tyrosine as the substrate was also measured. The reaction mixture contained 0.833 mm L-tyrosine in Tris-HCl buffer (0.1 M, pH 8.5) at 30°. The initial rate of the reaction was monitored at 315 nm, and tyrosine ammonia-lyase activity was calculated using the extinction coefficient (10,000) for p-coumaric acid. The specific activity was expressed as micromoles of p-coumaric acid formed/min at 30°/mg of protein. Throughout this paper, tyrosine ammonia-lyase activity is shown as a percentage of phenylalanine ammonia-lyase activity.

Enzyme Purification—One kilogram of yeast grown under the conditions for induction of tyrosine ammonia-lyase was used to initiate growth in 250 ml of a medium containing malt extract (1.0%), yeast extract (0.10%), and L-phenylalanine (0.10%) in deionized water. The cells were collected in a Cepa continuous flow centrifuge, and then either frozen and maintained at -5° or immediately disrupted by sonic oscillation. The active preparation yielded a major component on three different polyacrylamide gel electrophoretic systems. Antisera to phenylalanine ammonia-lyase was raised in rabbits and detected by double immunodiffusion. The antigen-antibody complex was enzymically active in vitro. The biological half-life of the enzyme was approximately 21 hours in several mammalian species (mice without and with BW10232 adenocarcinoma and B16 melanoma, rats, and monkeys) after a single injection; however, upon repeated administration, phenylalanine ammonia-lyase had a much shorter biological half-life. The onset of rapid clearance occurred earlier in tumor-bearing than in nontumor-bearing mice indicating a direct or indirect influence by the tumor on the biological half-life of phenylalanine ammonia-lyase.
conditions described for optimal enzyme activity was suspended in 1 liter of Tris-HCl buffer (0.05 M, pH 8.5) and 200-ml aliquots were disrupted for 30 min with a Branson model W-185D sonifier with a W horn at maximum power. The suspension was kept cold by submerging the sonifier in an ice-water bath during this step. This and all subsequent operations were carried out at 0°C. The debris was removed by centrifugation (16,000 × g for 10 min) in a Sorvall model RC-2B centrifuge using a GSA rotor with stainless steel bottles, and the supernatant was submitted to the following ammonium sulfate fractionation.

Ammonium sulfate (65% saturation) was added and the mixture allowed to stand for a minimum of 20 min with stirring (4°C). The suspension was centrifuged (16,000 × g for 10 min) and the precipitate dissolved in 1 liter of Tris-HCl buffer (0.05 M, pH 8.5). The solubilized proteins were then fractionated by the stepwise addition of solid ammonium sulfate into five fractions: 0 to 19, 19 to 28, 28 to 37, 37 to 45, and 45 to 54% saturation. All precipitates from this fractionation were dissolved in approximately 300 ml of Tris-HCl buffer (0.05 M, pH 8.5) and assayed. The protein fraction precipitated by 28 to 37% ammonium sulfate saturation contained 85 to 90% of the phenylalanine ammonia-lyase activity. The addition of solid ammonium sulfate to a final saturation of 85% precipitated the enzyme for storage overnight at 4°C without loss of activity.

The stored suspension was centrifuged (16,000 × g for 10 min) and the precipitate dissolved in approximately 250 ml of Tris-HCl buffer (0.05 M, pH 8.5) and fractionated by the stepwise addition of solid ammonium sulfate into six fractions: 0 to 15, 15 to 23, 23 to 30, 30 to 37, 37 to 44, and 44 to 52% saturation. All precipitates from the six steps were dissolved in approximately 150 ml of Tris-HCl buffer (0.05 M, pH 8.5) and assayed. The fraction precipitated by 30 to 37% ammonium sulfate saturation contained 80 to 85% of the phenylalanine ammonia-lyase activity. This precipitate was dissolved in the minimum amount of Tris-HCl buffer (0.05 M, pH 8.5) and dialyzed against 6 liters of Tris-HCl buffer (0.005 M, pH 8.5) with one change for a total of 8 hours.

Approximately 4 g of protein containing the enzyme were submitted to chromatography on a DEAE-cellulose (DE70) column (2.5 × 100 cm) equilibrated with Tris-HCl buffer (0.005 M, pH 8.5). The column was washed with the same buffer and the enzyme activity was eluted with a gradient of 0 to 0.5 M NaCl in Tris-HCl buffer (0.005 M, pH 8.5) with a flow rate of 1 ml/min. Fractions of 10 ml were collected and assayed for enzyme activity. Those fractions that contained an activity of 1.0 units/mg of protein or greater were pooled, precipitated with 40% ammonium sulfate (w/v), and the precipitate was redissolved in 50 ml (765 mg of protein) of Tris-HCl buffer (0.05 M, pH 8.5).

The sample was then chromatographed on a Sephadex G-200 column (5 × 150 cm) which had been equilibrated with Tris-HCl buffer (0.005 M, pH 8.5). Fractions (10 ml) were collected and those that contained a specific activity of 2.0 units/mg of protein or greater were pooled, precipitated with 30% ammonium sulfate (w/v), and the precipitate was redissolved in 50 ml (765 mg of protein) of Tris-HCl buffer (0.05 M, pH 8.5).

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Yeast Culture—The rate of growth of Rhodotorula glutinis and the induction of phenylalanine ammonia-lyase is shown in Fig. 1. Phenylalanine ammonia-lyase activity is detectable after 7 hours of growth (mid-log) and reaches a maximum between the 12th and 14th hour (late-log) but declines rapidly when the yeast reaches stationary phase. Variation of initial extract concentrations (malt and/or yeast) gave similar curves except that the growth and induction peaks were much broader. When the malt extract concentration was decreased The sample was then chromatographed on a Sephadex G-200 column (5 × 150 cm) which had been equilibrated with Tris-HCl buffer (0.005 M, pH 8.5). Fractions (10 ml) were collected and those that contained a specific activity of 2.0 units/mg of protein or greater were pooled, precipitated with 40% ammonium sulfate (w/v), and the precipitate was redissolved in 50 ml (765 mg of protein) of Tris-HCl buffer (0.05 M, pH 8.5).

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Clearance Rates of Phenylalanine Ammonia-lyase—Purified phenylalanine ammonia-lyase, injected intraperitoneally into mice, was measured by assaying the enzymatic activity in the plasma. Several samples of 0.03 ml of blood, representing less than 2% of the blood volume of the mouse, were readily obtained from the tip of the tail. Using heparinized capillary tubes, samples were collected and centrifuged in an Adams Micro-Hematocrit centrifuge. After the measurement of the hematocrit, the plasma was removed and placed in a micro test culture plate for the analysis of phenylalanine ammonia-lyase activity. The plasma was also used in the double immunodiffusion method to detect phenylalanine ammonia-lyase protein and/or the presence of antibodies.

The following species have been used to measure the clearance rate of either single or multiple injections at two dose levels of phenylalanine ammonia-lyase: (a) C57BL/6 female mice with and without the tumors, BW10232 mammary adenocarcinoma or B16 melanoma; (b) RDF, male mice; (c) Sprague-Dawley male rats; and (d) squirrel monkeys.
(to 0.5% or less), the enzyme yield from yeast remained the same but the enzyme fractionated differently in ammonium sulfate and was unstable throughout purification.

**Enzyme Purification**—A typical purification scheme for phenylalanine ammonia-lyase is summarized in Table I. From 1 kg of yeast, approximately 460 units of highly purified enzyme were obtained using the ammonium sulfate and sodium citrate fractionations. DEAE-cellulose chromatography, and Sephadex G-200 chromatography. The overall yield was about 23% and the specific activity of highly purified enzyme was 2.5 units/mg of protein. Several modifications to the previously published purification procedure (10) should be emphasized. DEAE-cellulose chromatography was utilized to replace a protamine sulfate step. The treatment of the sonic extract with protamine sulfate resulted in an enzyme preparation that was slightly more soluble in solutions of ammonium sulfate and sodium citrate. Furthermore, such preparations contained a highly toxic contaminant that was difficult to remove subsequently. Also, the specific activity of the enzyme was about 2.5 times greater than that previously obtained in other laboratories (8, 10, 20). The reasons for this are unknown, but the conditions utilized for yeast growth reported here are more precisely defined. Throughout the purification procedure, the tyrosine ammonia-lyase activity remained constant and was approximately 28% of the phenylalanine ammonia-lyase activity. Upon reduction of phenylalanine ammonia-lyase activity with sodium borohydride (11), the tyrosine ammonia-lyase activity was proportional to the decrease in phenylalanine ammonia-lyase activity.

The preparations of phenylalanine ammonia-lyase submitted to standard polyacrylamide gel electrophoresis, sodium dodecyl sulfate gels, and to isoelectric focusing demonstrated a single major component (Fig. 2). The enzyme has been shown to have a molecular weight of approximately 330,000 (20), and it consists of four subunits which are either very similar or identical as demonstrated by sodium dodecyl sulfate gel electrophoresis (11). The isoelectric focusing of phenylalanine ammonia-lyase demonstrated that it has an isoelectric point of approximately 5.4.

**Measurement of Phenylalanine, Tyrosine, and Phenylalanine Ammonia-lyase in Plasma**—The concentration of phenylalanine and tyrosine in the peripheral blood of several rodents is 60 to 80 nmol/ml (21-23). In our studies, values of 47.5 ± 5 nmol/ml S.E. and 45.0 ± 10 nmol/ml S.E. of phenylalanine and tyrosine, respectively, were obtained from the peripheral blood of squirrel monkeys. After a single injection of phenylalanine ammonia-lyase (100 units/kg), however, the concentrations of these amino acids were reduced to almost undetectable levels within 4 to 8 hours and remained low for 24 hours. When 50 units/kg of phenylalanine ammonia-lyase were injected,
Phenylalanine Ammonia-lyase: Induction, Purification, Clearance

reductions in phenylalanine and tyrosine were correlated inversely with circulating levels of phenylalanine ammonia-lyase (Table II).

After intraperitoneal injection, the activity of phenylalanine ammonia-lyase reached a maximum in the circulation within 4 to 8 hours, but decreased subsequently at a constant rate. Table III compares the plasma half-life of phenylalanine ammonia-lyase after a single injection in mice of several strains, rats, and squirrel monkeys. The half-life was calculated to be 21 ± 2 hours, and although not shown here, was found to be independent of the dose administered over a range of 10 to 100 units/kg. Also, the half-life of phenylalanine ammonia-lyase was unaffected by the presence of tumors in mice after a single injection.

Following repeated injections of enzyme, however, a different pattern emerged (Fig. 3). Groups of six mice were administered phenylalanine ammonia-lyase intraperitoneally at a concentration of 100 units/kg every other day for 24 days on odd days. The circulating levels of enzyme were determined at 24 hours on even days. The open circles of Fig. 3 show the theoretical curve expected for circulating levels of phenylalanine ammonia-lyase based on the half-life obtained from a single injection. In the experimental control animals, it was found that the circulating levels of enzyme increased to values above that expected from the theoretical curve. Within 12 days or six injections of the enzyme, however, phenylalanine ammonia-lyase was cleared at an increasingly rapid rate. By the 16th day, circulating levels of phenylalanine ammonia-lyase could not be detected 24 hours after injection. A similar experiment performed in mice bearing the BW10232 mammary tumor or the B16 melanoma gave a still different pattern of clearance. The onset of increased clearance of the enzyme occurred about two times earlier than that observed in non-tumor-bearing mice. These results indicate that the tumor causes, either directly or indirectly, earlier clearance of the enzyme. In the B16 melanoma-bearing mice, some fluctuation in clearance occurred but the pattern was very similar to that observed in mice with the BW10232 mammary tumor.

The half-life of the enzyme in normal mice after seven injections of phenylalanine ammonia-lyase was 1.7 hours (Fig. 4). This increased rate of clearance was found regardless of whether the animals received injections of enzyme every other day or once a week. The clearance of the enzyme was also measured using the double immunodiffusion technique at each time point on the curve. The presence of phenylalanine ammonia-lyase determined qualitatively agreed with the levels of circulating phenylalanine ammonia-lyase determined enzymatically. The corresponding experiments for the detection of

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**Table II**

<table>
<thead>
<tr>
<th>Time after enzyme injection</th>
<th>Levels of phenylalanine and/or tyrosine</th>
<th>Circulating enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>hr</td>
<td>%</td>
<td>units ± S.D./ml plasma</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td>0.29 ± 0.08</td>
</tr>
<tr>
<td>8</td>
<td>7</td>
<td>0.35 ± 0.08</td>
</tr>
<tr>
<td>24</td>
<td>14</td>
<td>0.18 ± 0.04</td>
</tr>
<tr>
<td>48</td>
<td>26</td>
<td>0.08 ± 0.01</td>
</tr>
</tbody>
</table>

* Amino acid analysis on Beckman model 119 analyzer. Normal base-line levels: tyrosine, 45.0 ± 10 S.E. nmol/ml of plasma; phenylalanine, 47.5 ± 5 S.E. nmol/ml of plasma.

**Table III**

<table>
<thead>
<tr>
<th>Species*</th>
<th>Weight range</th>
<th>Plasma half-life after single injection 100 units/kg (hr ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDF, male mice</td>
<td>14-16</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>C57BL/6 female mice</td>
<td>14-16</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>C57BL/6 female mice (BW10232-11th day tumor)</td>
<td>14-16</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>C57BL/6 female mice (B16 melanoma-11th day tumor)</td>
<td>14-16</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>Sprague-Dawley male rats</td>
<td>200</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>Squirrel monkeys</td>
<td>500-600</td>
<td>17 ± 1</td>
</tr>
</tbody>
</table>

* The biological half-life of phenylalanine ammonia-lyase was determined in groups of 10 animals except in squirrel monkeys where 5 were used.

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**Fig. 3** Composite of the 24-hour circulating levels of phenylalanine ammonia-lyase in groups of six normal and six tumor-bearing C57BL/6 mice. Phenylalanine ammonia-lyase (100 units/kg, body weight) was injected on odd days and circulating activity assayed 24 hours later on even days. In tumor-bearing mice, the tumor inoculum was given on Day 0. A theoretical curve (O—O) was calculated based on a 21-hour biological half-life and assuming that the accumulation of enzyme was additive. Circulating levels of enzyme at 24 hours were obtained in normal mice (●—●), BW10232 tumor-bearing mice (×—×), and B16 tumor-bearing mice (Δ—Δ).

**Fig. 4** Half-life of circulating phenylalanine ammonia-lyase after a single injection (100 units/kg, ×—×) and after seven injections (100 units/kg on odd days, ●—●) in six normal C57BL/6 mice. Each point represents the average of six mice.
antibody to phenylalanine ammonia-lyase produced in mice was attempted; however, the results were negative by the Ouchterlony technique.

DISCUSSION

Phenylalanine ammonia-lyase has now been purified from yeast in large quantities by an improved procedure. The new developments involved precise control of the induction of the enzyme and an improved purification scheme. DEAE-cellulose chromatography was substituted for the protamine sulfate precipitation step. The enzyme obtained from such procedures show a single major component on polyacrylamide gels, and had no acute toxicity in mice when injected at a concentration of 1000 units/kg, a dose 3- to 10-fold greater than that used in therapeutic or clearance studies.

The enzyme was shown to be highly immunogenic when injected in rabbits, and the antibody obtained precipitates phenylalanine ammonia-lyase as demonstrated using the Ouchterlony double diffusion method. The phenylalanine ammonia-lyase-antiphenylalanine ammonia-lyase complex retained essentially all of the enzymatic activity as demonstrated in vitro.

Following a single injection of the enzyme in mice, rats, or monkeys, the half-life was approximately 21 hours. Maximum levels of circulating enzyme were detected after 4 to 8 hours, illustrating passage of the enzyme from the peritoneal cavity into the circulation. The half-life of the enzyme after a single injection was not influenced by the presence of at least two different types of tumors (adenocarcinoma BW10232 and B16 melanoma). In contrast, repeated injections of the enzyme brought about a very rapid clearance of phenylalanine ammonia-lyase (half-life of 1.7 hours). Interestingly, the tumor-bearing animals increased the onset of clearance of the enzyme by a time factor of approximately 2. Whether this is a direct or indirect effect has not yet been established. However, when plasma from animals that received repeated injections of phenylalanine ammonia-lyase in vivo were incubated with exogenous phenylalanine ammonia-lyase in vitro, enzyme activity was retained. Attempts to demonstrate that the increase in clearance rate was brought about by the immune system were unsuccessful using the Ouchterlony technique. Recent data using the sensitive, passive hemagglutination assay, however, demonstrated the presence of an antibody which accounts, at least in part, for the rapid clearance of the enzyme.

Asparaginase isolated from Escherichia coli and other microorganisms has been utilized for the therapeutic treatment of leukemias in mice and humans (24, 25). The effectiveness of the enzyme depends upon the Michaelis constant for the substrate, the clearance of the enzyme from the host, and the susceptibility of the tumor cells to deprivation of the amino acid, asparagine. In the case of phenylalanine ammonia-lyase, even though the Michaelis-Menten constant (10⁻⁴ M for phenylalanine) is relatively high (15), circulating levels of phenylalanine and tyrosine were markedly depleted after a single injection of enzyme.

The half-life of phenylalanine ammonia-lyase is decreased appreciably after repeated injections, especially in tumor-bearing mice. Consequently, therapeutic effectiveness will probably depend significantly on the rate of clearance of the enzyme. Modification of enzymes to render them nonimmunogenic or to increase their half-life have been successful with asparaginases (26, 27), and currently are being applied to phenylalanine ammonia-lyase.

Since an inverse correlation between the circulating levels of phenylalanine ammonia-lyase and phenylalanine (or tyrosine) was obtained, the ease of measurement of enzyme rather than amino acids in plasma may be extremely useful for the assessment of physiological effects on the host.

Acknowledgments—We thank Richard Eberle for excellent technical assistance. We also wish to thank Dr. Phil Poffenbarger for the amino acid analyses.

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