Partial Purification and Some Properties of Tyrosine Phenol-Lyase from *Aeromonas phenologenes* ATCC 29063

G. M. CARMAN and R. E. LEVIN*

Department of Food Science and Nutrition, University of Massachusetts, Amherst, Massachusetts 01002

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Tyrosine phenol-lyase was purified 32-fold from *Aeromonas phenologenes* ATCC 29063, the organism that produces phenol in refrigerated haddock. The purification procedure included ammonium sulfate fractionation, protamine sulfate treatment, and column chromatography with Sephadex G-200, diethylaminoethyl-cellulose, and hydroxypapatite. The enzyme was found to be thermally inactivated at temperatures above 40°C. The optimum pH of the enzyme was found to be pH 8.5. The Michaelis constants for L-tyrosine and pyridoxal phosphate were 2.3 × 10⁻⁴ M and 3.2 × 10⁻⁴ M, respectively. The molecular weight of tyrosine phenol-lyase was found by gel filtration and electrophoresis to be approximately 380,000.

The major high-boiling volatile compound produced during the refrigerated storage of haddock has been shown to be phenol (4). An organism isolated from haddock, capable of producing phenol, has been found to be an aeromonad. An isolate of this organism has been deposited as *Aeromonas phenologenes* ATCC 29063 (5). Preliminary studies have shown that resting-cell suspensions of *A. phenologenes* produce phenol, pyruvate, and ammonia from L-tyrosine. The responsible enzyme is tyrosine phenol-lyase (TPL, deaminating, EC 4.1.99.2) (3, 9-11, 16).

This study describes the induction, partial purification, and certain properties of TPL from *A. phenologenes* ATCC 29063.

**MATERIALS AND METHODS**

Maintenance of stock cultures. Stock cultures of *A. phenologenes* were maintained on tryptic soy agar slants without dextrose (Difco Laboratories), incubated at 30°C, and transferred every 3rd week.

Preparation of resting cells. Baffled Erlenmeyer flasks (1 liter) containing 250 ml of tryptic soy broth (TSB) without dextrose (Difco Laboratories) were inoculated with 10 ml of 1-day-old cultures of *A. phenologenes*. The cultures were grown at 30°C with rotary agitation at 300 rpm for 20 h. Cells were harvested by centrifugation at 6,800 × g for 10 min at 2°C and washed twice in 0.02 M phosphate buffer (PB) at pH 7.0. The cell pellet was suspended in PB, and the dry cell weight was determined as previously described (5).

Growth of bacteria for purification of TPL. Cultures of *A. phenologenes* were grown at 30°C in 5 liters of TSB containing 0.1% L-tyrosine (Nutritional Biochemicals Corp.) in a 7-liter fermentor (Microferm, New Brunswick Scientific Co.). Air at a rate of 12 liters/min was passed through the culture, which was stirred at 700 rpm. Cultures were grown until they attained a dry-cell-weight density of 5.8 mg/ml. Cells were harvested by centrifugation for 10 min at 6,800 × g and the cell paste was frozen and stored at −20°C.

Enzyme assays. TPL activity was determined by measuring the formation of pyruvate from L-tyrosine at 30°C by following the decrease in absorbance at 340 nm in the presence of excess reduced nicotinamide adenine dinucleotide (NADH) and lactate dehydrogenase (LDH) for 10 min. Reaction mixtures contained 7.5 μmol of L-tyrosine, 0.3 μmol of pyridoxal 5′-phosphate (PLP, Sigma Chemical Co.), 1.5 μmol of NADH (Sigma Chemical Co.), 5 IU of LDH (Sigma Chemical Co.), 300 μmol of PB at pH 8.0, and enzyme, in a total volume of 3.0 ml. Alternatively, NADH and LDH were omitted from the reaction mixtures, and enzyme activity was determined by measuring the 2,4-dinitrophenylhydrazine derivative of pyruvate after a reaction period of 20 min at 440 nm, according to the method of Friedemann and Haugen (8). A unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of pyruvate per min under the assay conditions described. The specific activity is expressed as units per milligram of protein.

Protein determination. Protein was determined by the method of Lowry et al. (14). Crystalline bovine serum albumin (Nutritional Biochemicals Corp.) was used as the standard. Protein was monitored during purification by measuring the absorbance of fractions at 280 nm.

Phenol determination. Phenol was determined by gas chromatography as previously described (4).

(i) Preparation of crude cell-free extracts. Ali-
quotas (4 g of dry cell weight) of frozen cells were thawed, washed, and suspended in 20 ml of PB containing 5 μmol of 2-mercaptoethanol (ME) per ml at pH 7.0. The cells were disrupted by ultrasonic treatment for 10 min at 10°C using a chamber-type Bronwill Biosonic sonicator (Bronwill Scientific Co.). Cell debris was removed by centrifugation for 10 min at 12,000 × g, and the supernatant fraction was decanted and further treated as described below.

(ii) Fractionation of crude cell-free extracts with ammonium sulfate. The supernatant fraction from disrupted-cell suspensions was adjusted to a protein concentration of 10 mg/ml by the addition of PB + ME at pH 7.0 to a final volume of 100 ml. Enzyme-grade ammonium sulfate (Nutritional Biochemicals Corp.) was added to the crude enzyme to 0.45 saturation with stirring. After 30 min, the precipitate was removed by centrifugation at 12,000 × g for 30 min and discarded. The ammonium sulfate concentration was then increased to 0.60 saturation. After 60 min, the precipitate was collected by centrifugation at 12,000 × g for 30 min and dissolved in approximately 5 ml of PB + ME at pH 7.0. The percentage of saturation of ammonium sulfate was determined from a nomogram (7).

(iii) Protamine sulfate treatment. Protamine sulfate (2.0%, Nutritional Biochemicals Corp.) was added to the ammonium sulfate fraction with stirring (1.0 ml of 2.0% protamine sulfate per 140 mg of protein). After 20 min the precipitate was collected by centrifugation at 12,000 × g for 10 min and discarded.

(iv) Sephadex G-200 column chromatography. The enzyme solution (6.5 ml) from the previous step was applied to a Sephadex G-200 (Pharmacia Fine Chemicals Co.) gel bed (2.5 by 40 cm) equilibrated with 0.02 M PB + ME at pH 7.0. Buffer was allowed to flow through the column at a rate of 20 ml/h. Fractions (5.0 ml) were collected and assayed for TPL activity; protein was monitored by following the absorbance at 280 nm.

(v) Diethylaminoethyl (DEAE)-cellulose column chromatography. Sephadex G-200 fractions containing the highest specific activity were pooled to a total volume of 25 ml and subjected to chromatography on a DEAE-cellulose (Nutritional Biochemicals Corp.) column (2.5 by 33 cm). The column was equilibrated with 0.02 M PB + ME at pH 7.0. Elution of the enzyme was achieved by passing 500 ml of a linear PB gradient (pH 7.0, 0.02 to 0.4 M) containing ME through the column at a flow rate of 1 ml/min. Fractions (7.0 ml) were collected and assayed for TPL activity. Fractions with the highest specific activity were pooled and dialyzed for 16 h against 0.02 M PB + ME at pH 7.0.

(vi) Hydroxyapatite (HA) column chromatography. The dialyzed enzyme from the previous step (35 ml) was applied to a column of HA (2.5 by 15 cm) equilibrated with 0.02 M PB + ME at pH 7.0. Elution of the enzyme was achieved by passing 200 ml of a linear PB gradient (pH 7.0, 0.02 to 0.2 M) containing ME through the column at a flow rate of 30 ml/h. Fractions (5.0 ml) were collected and assayed for activity.

Analytical polyacrylamide electrophoresis. The system for polyacrylamide gel electrophoresis was that described by Davis (6) in a Shandon analytical polyacrylamide electrophoresis apparatus (Shandon Scientific Co.). Sambler columns (5 by 60 mm) by a procedure described by Broome (2). Electrophoresis was performed at pH 8.3 in 5.0% acrylamide (Eastman Kodak Co.) gels at 4 mA per gel tube. Gels were stained in 1% Buffalo black in 7% acetic acid and destained electrographically in 7% acetic acid.

Determination of molecular weight. (i) Gel filtration. The molecular weight of TPL was determined by elution through a column of Sephadex G-200 (2.5 by 40 cm) equilibrated with 0.02 M PB + ME at pH 7.0 according to the method of Andrews (1). Samples (10 mg of protein in 1.0 ml) of each reference protein (chymotrypsinogen A, molecular weight 25,000; ovalbumin, molecular weight 45,000; aldolase, molecular weight 158,000; catalase, molecular weight 244,000; and β-galactosidase, molecular weight 520,000) were injected onto the column, and 3.0-ml fractions were collected at a flow rate of 10 ml/h. Protein was monitored by the increase in absorbance at 280 nm. Blue dextran (Pharmacia Fine Chemicals Co.) was used to estimate the void volume.

(ii) Electrophoresis. Molecular-weight determination by electrophoresis was performed in an electrophoresis apparatus (Pharmacia GE-4). Samples (16 μg) of each reference protein (bovine serum albumin, molecular weight 67,000; ceruloplasmin, molecular weight 150,000; catalase, molecular weight 244,000; aperoferritin, molecular weight 460,000; and α-2-macroglobulin, molecular weight 820,000) were subjected to electrophoresis on a 4 to 30% gradient polyacrylamide gel slab purchased from Pharmacia Fine Chemicals Co. Electrophoresis was performed at pH 8.3 at 125 V for 15 h. Gel slabs were stained in 1% Buffalo black in 7% acetic acid and destained electrophoretically. A standard curve was generated by plotting the log of molecular weight of each reference protein versus its migration distance.

RESULTS

Induction of TPL. Resting-cell suspensions prepared form cultures grown in TSB supplemented with 0.1% L-tyrosine showed approximately a sixfold increase in the production of phenol as compared with cell suspensions from cultures grown in unsupplemented TSB (Fig. 1). Cultures of A. phenologenes were grown in TSB containing various concentrations of L-tyrosine. Crude cell-free extracts were prepared for each culture and the specific activity was determined. Figure 2 shows that an approximate sixfold increase in specific activity of TPL was again achieved by induction. No significant increase in TPL formation occurred with over 0.1% L-tyrosine in the culture medium.

Purification of the enzyme. A summary of the purification procedure is given in Table 1.
Crude TPL was precipitated between 0.45 to 0.60 saturation with ammonium sulfate and treated with protamine sulfate. The elution patterns for TPL and protein on Sephadex G-200 are shown in Fig. 3. When pooled fractions (19 to 23) from a Sephadex G-200 column were applied to a DEAE-cellulose column, TPL activity was eluted between 0.13 and 0.17 M PB (Fig. 4). When the most active DEAE-cellulose fractions (35 to 39) were pooled, dialyzed, and applied to a column of HA, TPL activity was eluted at a PB concentration of about 0.05 M (Fig. 5). The above purification procedure resulted in an increase in specific activity for TPL of approximately 32-fold with a 14% recovery.

Additional techniques were used in attempting to purify the enzyme further. These included affinity chromatography, carboxymethylcellulose chromatography, and preparative acrylamide gel electrophoresis. These procedures either failed to yield pure enzyme or resulted in a considerable loss of enzyme activity.

Homogeneity. An enzyme sample (80 μg) equivalent to that of step (vi) in Table 1 was applied to a 5% polyacrylamide gel and subjected to electrophoresis at pH 8.3. One major and two minor bands were evident (Fig. 6).

Stability of enzyme. The HA enzyme preparation lost about 50% of its activity when stored for approximately 1 week at 2°C in 0.02 M PB + ME at pH 7.0. The enzyme was not stable to freezing and thawing.

Effect of heating on enzyme activity. The enzyme was reasonably stable up to 40°C, with rapid inactivation above 50°C (Fig. 7). Almost all activity was lost on heating the enzyme for 25 min at 60°C (Fig. 8).

Optimum pH. The activity of TPL was assayed at various pH values. The pH optimum for the enzyme was found to be pH 8.5 (Fig. 9).

Determination of $K_m$ for L-tyrosine. The apparent $K_m$ for L-tyrosine was calculated to be $2.3 \times 10^{-4}$ M by the method of Lineweaver and Burk (13) (Fig. 10).

Determination of $K_m$ for PLP. Figure 11 is a Lineweaver-Burk plot for PLP. The $K_m$ was calculated to be $3.2 \times 10^{-4}$ M.

Apparent molecular weight of TPL. The molecular weight estimated by Sephadex G-200 column chromatography was found to be approximately 370,000. Electrophoresis yielded a molecular weight of approximately 390,000.

**DISCUSSION**

Maximum TPL formation from *A. phenologenes* was observed when cultures were grown in a medium containing 0.1% L-tyrosine. Kumagai et al. (9, 12) reported similar induction of TPL with *Escherichia intermedia* and *Erwinia herbicola*.

The specific activity (0.086) in crude cell-free extracts of TPL from *A. phenologenes* was higher than that found by Kumagai et al. (9,
TABLE 1. Purification procedure for TPL

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Vol (ml)</th>
<th>Total units</th>
<th>Total protein (mg)</th>
<th>Sp act</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Crude cell-free extract</td>
<td>100</td>
<td>105</td>
<td>1220</td>
<td>0.086</td>
<td>100</td>
</tr>
<tr>
<td>(ii) Ammonium sulfate (0.45 to 0.60 saturation)</td>
<td>5</td>
<td>66</td>
<td>238</td>
<td>0.277</td>
<td>62.8</td>
</tr>
<tr>
<td>(iii) Protamine sulfate</td>
<td>6.5</td>
<td>64</td>
<td>224</td>
<td>0.285</td>
<td>60.9</td>
</tr>
<tr>
<td>(iv) Sephadex G-200</td>
<td>25</td>
<td>25</td>
<td>62.5</td>
<td>0.40</td>
<td>23.8</td>
</tr>
<tr>
<td>(v) DEAE-cellulose</td>
<td>35</td>
<td>20</td>
<td>15.3</td>
<td>1.13</td>
<td>19.0</td>
</tr>
<tr>
<td>(vi) HA</td>
<td>15</td>
<td>15</td>
<td>5.35</td>
<td>2.80</td>
<td>14.2</td>
</tr>
</tbody>
</table>

Enzyme (80 μg) from an HA column failed to yield a homogeneous preparation when subjected to polyacrylamide gel electrophoresis. Extensive efforts to further purify the enzyme failed. Kumagai et al. (10, 12) reported a 107-fold purification of TPL from E. intermedia and a 22-fold purification from E. herbicola. Electrophoresis of crystalline preparations of TPL from E. intermedia and E. herbicola (30 and 27 μg, respectively) indicated that these enzyme preparations were homogeneous.

The $K_m$ and the effects of heating on TPL from A. phenologenes enzyme were similar to those reported for TPL isolated from E. intermedia (10) and E. herbicola (12). The pH optimum of 8.5 for TPL from A. phenologenes differs only slightly from that of 8.2 reported for TPL from E. intermedia (10) and E. herbicola (12). The Michaelis constant ($3.2 \times 10^{-5}$ M) for PLP with TPL from A. phenologenes differed from the value of $1.3 \times 10^{-6}$ M for PLP with TPL isolated from E. intermedia and E.
FIG. 6. Acrylamide gel electrophoresis of TPL. A 0.1-ml sample of the HA effluent containing 80 μg of protein was applied to the stacking gel in a Sephadex-sucrose mixture and subjected to electrophoresis at a current of 4 mA at pH 8.3. Location of two minor protein bands is indicated by arrows.

Fig. 7. Thermal stability of TPL. Aliquots (0.5 ml) of enzyme equivalent to that obtained from step (vi) in Table 1 were heated for 10 min at the indicated temperatures in a controlled-temperature water bath. After incubation, the enzyme was cooled in ice and assayed for activity at 30°C by measuring the decrease in absorbance of NADH at 340 nm in standard assay mixtures containing an excess of LDH (5 IU) and NADH (0.5 μmol per ml).

Fig. 8. Heat inactivation of TPL at 60°C. Aliquots (0.5 ml) of enzyme equivalent to that obtained from step (vi) in Table 1 were heated for 5-min intervals for 30 min in a controlled-temperature water bath at 60°C. The enzyme aliquots were then cooled in an ice bath. Enzyme activity was assayed as in Fig. 8.
A. PHENOLOGENES TYROSINE PHENOL-LYASE

Fig. 9. Effect of pH on TPL activity. The reaction was carried out in standard reaction mixtures containing 100 μg of the indicated buffers per ml. Enzyme activity was assayed as in Fig. 8.

Fig. 10. Effect of substrate concentration on TPL activity. An enzyme preparation equivalent to that obtained from step (vi) in Table 1 containing 0.055 U of activity was assayed with various concentrations of L-tyrosine at 30°C. Enzyme activity was assayed as in Fig. 8. Velocity (V) is expressed as units per milliliter.

Fig. 11. TPL activity as a function of PLP concentration. The enzyme was dialyzed extensively at 2°C against 0.02 M PB + ME at pH 7.0. TPL (0.02 U) was assayed with various concentrations of PLP. Enzyme activity was assayed as in Fig. 8. Velocity (V) is expressed as units per milliliter.

herbicola by Kumagai and co-workers (11, 12). This may be due to a reduced affinity of TPL for PLP after crystallization of the enzyme by these authors (11, 15) or to a fundamental difference in TPL from A. phenologenes.

The molecular weight of TPL from A. phenologenes was found to be approximately 380,000 ± 10,000. This value differs from the molecular weights determined for TPL isolated from E. intermedia of 170,000 (10) and E. herbicola of 259,000 (12).

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LITERATURE CITED


