Crystalline L-Aspartate β-Decarboxylase of Pseudomonas dacunhae

I. CRYSTALLIZATION AND SOME PHYSICOCHEMICAL PROPERTIES*

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SUMMARY

Crystalline L-aspartate β-decarboxylase of Pseudomonas dacunhae was prepared by the following steps: sonic disruption, ammonium sulfate fractionation, heat treatment, DEAE-Sephadex chromatography, Sephadex G-150 gel filtration, and hydroxylapatite chromatography, followed by crystallization. This procedure yields a pure enzyme with a 32% recovery of the activity in crude cell-free extracts. The specific activity (84 μmoles of CO₂ per min per mg) and the yield were significantly higher than those so far reported for the enzyme.

The purified enzyme appeared to be homogeneous in the ultracentrifuge (s₂₀,₅₀ = 20.7 S). Diffusion coefficient at zero protein concentration is 2.26 × 10⁻⁹ cm² per sec. The intrinsic viscosity is 0.054 dl per g. The partial specific volume, V, is 0.72 ml per g. The molecular weight computed from sedimentation-diffusion data (795,000) is in good agreement with the value obtained from the Archibald method (820,000). The frictional ratio, f/fmₐₓ, is 1.55. Electrolysis with carrier ampholytes revealed that this enzyme has an isoelectric point at around pH 4.2. The enzyme exhibits an absorption maxima at 278 nm and about 360 nm at pH 6.8. This enzyme contains approximately 16 moles of pyridoxal phosphate per mole of enzyme.

As reported in our previous paper (15), we found that Pseudomonas dacunhae produced markedly high activity of L-aspartate β-decarboxylase and used the enzyme for production of L-alanine from L-aspartic acid.

The present paper describes the preparation and some physicochemical properties of crystalline L-aspartate β-decarboxylase of P. dacunhae.

EXPERIMENTAL PROCEDURE

Materials-All chemicals unless otherwise specified were Katayama (Osaka) certified reagent grade. Pyridoxal phosphate, α-ketoglutarate (Nakai, Kyoto), and bovine serum albumin (Armour) were purchased from the sources designated. DEAE-Sephadex and Sephadex G-150 were purchased from Pharmacia. Hydroxylapatite was prepared by the method of Levin (16).

Culture of Microorganism—P. dacunhae IAM¹ 1152 was cultured under aerobic conditions at 30° for 20 hours in 20 liters of the medium containing 0.5% ammonium fumarate, 1% sodium fumarate, 0.9% peptone, 0.2% casein hydrolyzate, 0.05% KH₂PO₄, and 0.01% MgSO₄·7H₂O, pH 5.5, at an oxygen absorption rate of 0.82 mmole per liter per min. The cells were harvested by centrifugation. Approximately 4.5 g of cells (dry weight) were obtained per liter of medium.

Enzyme Assay—The assay of decarboxylase activity was carried out by manometric measurements of CO₂ liberated from L-aspartic acid under the following conditions. The main compartment of a Warburg vessel contained pyridoxal phosphate, 4 μmoles; α-ketoglutarate, 5 μmoles; sodium acetate buffer, pH 5.3, 400 μmoles; bovine serum albumin, 2 mg; and the enzyme in a final volume of 2.6 ml. The side arm contained sodium L-aspartate, 100 μmoles, and sodium acetate buffer, pH 5.3, 200 μmoles in 0.6 ml. After equilibration at 30°, the contents of the vessel were mixed and readings were made at 5-min intervals.

Protein Determination—Protein determination was carried out by the procedure of Lowry et al. (17). Crystalline serum albumin was used as a standard. After the purified proteins

* A preliminary note of some of this work has been published (1).

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Potassium phosphate buffer, pH 6.8, was dialyzed against the same Neurath type cell. Aspartate P-decarboxylase in 0.1 M potassium phosphate buffer, pH 6.8, in a water bath at 25°. The active fraction was concentrated with ammonium sulfate, dissolved in a small volume of buffer, and dialyzed overnight against distilled water. This solution was then adjusted to the concentration of 0.04 M potassium phosphate buffer, pH 6.8, containing 0.1 M potassium chloride. The yield in this step was generally 80%, and the purification achieved was usually 5-fold.

Isoelectric Point—The carrier ampholytes (LKB-Produkter AB) used in this investigation was selected to give a pH gradient between pH 3 and 6. Focusing and separation of the proteins were carried out at 4° in a special vertical electrolysis column of 110 ml of capacity, equipped with a cooling jacket (LKB-Produkter AB). Stabilization against convection was achieved by using a density gradient prepared from one dense and one less dense solution (21). Measurements of pH were made by a Hitachi model M-5 pH meter.

Partial Specific Volume—The partial specific volume was determined in 0.1 M sodium acetate buffer, pH 6.8, at 20° with a parallel stem pycnometer of approximately 3-ml capacity (22).

Coenzyme Content—Pyridoxal phosphate was determined spectrophotometrically on supernatant solutions obtained after precipitating the enzyme with metaphosphoric acid, with the molar absorbance index ε as 6770 at 295 mp (23) and by the phenylhydrazine method of Wads and Snell (24).

RESULTS

Purification Procedure

An outline of the purification procedure and the results of a representative preparation are given in Table I. All of the steps were carried out at 0–5°. Preparation of crude extract (Step 1), ammonium sulfate fractionation (Step 2), and heat treatment (Step 3) were described previously (1).

Step 4: Chromatography on DEAE-Sephadex—A column, 2.5 cm in diameter and 37.2 cm long, was filled with a suspension of DEAE-Sephadex in 0.14 M potassium phosphate buffer, pH 6.8. The DEAE-Sephadex was washed and equilibrated with the same buffer. The dialyzed material was used for the diffusion run together with the same buffer at 4°.

<table>
<thead>
<tr>
<th>Step and material</th>
<th>Volume</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Total activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>units/mg</td>
<td>units × 10⁶</td>
<td>%</td>
</tr>
<tr>
<td>1. Crude extract</td>
<td>470</td>
<td>18,400</td>
<td>1.24</td>
<td>228</td>
<td>100.0</td>
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<tr>
<td>2. Ammonium sulfate (30 to 50%)</td>
<td>117</td>
<td>8,990</td>
<td>2.37</td>
<td>213</td>
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<td>3. Heated supernatant</td>
<td>81.5</td>
<td>1,810</td>
<td>8.78</td>
<td>159</td>
<td>69.7</td>
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<td>4. DEAE-Sephadex</td>
<td>200</td>
<td>280</td>
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<td>124</td>
<td>51.4</td>
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<td>5. Sephadex G-150</td>
<td>74.5</td>
<td>181</td>
<td>64.1</td>
<td>116</td>
<td>50.9</td>
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<td>6. Hydroxylapatite</td>
<td>210</td>
<td>124</td>
<td>84.1</td>
<td>104</td>
<td>45.6</td>
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<td>7. Crystals</td>
<td></td>
<td>87.1</td>
<td>84.1</td>
<td>73.3</td>
<td>32.1</td>
</tr>
</tbody>
</table>

**TABLE I**

Summary of purification procedure

From 40 g of dry cells (see the text for details of procedure).

**Fig. 1.** Chromatography of aspartate β-decarboxylase on DEAE-Sephadex. Conditions are given in the text.
**Step 5: Sephadex G-150 Gel Filtration**—A column, 3 cm in diameter and 51.3 cm long, was filled with a suspension of Sephadex G-150 equilibrated with 0.04 M potassium phosphate buffer, pH 6.8, containing 0.1 M potassium chloride. The enzyme solution obtained in the former step was introduced into the column and eluted with the same buffer and the active fraction was collected (Fig. 2). The flow rate was approximately 60 ml per hour. A 1.5-fold purification was generally obtained by this step, with the yield of enzyme about 95%.

**Step 6: Hydroxylapatite Chromatography**—The active fraction in Step 5 was applied to a calcium hydroxylapatite column (3 x 24.5 cm) previously equilibrated with 0.05 M potassium phosphate buffer, pH 6.8. The enzyme was eluted with a linear gradient of potassium phosphate buffer, pH 6.8, from 0.05 M to 0.4 M. The flow rate was approximately 24 ml per hour. Fig. 3 shows the elution pattern obtained.

The fractions with constant specific activity were collected and concentrated by ammonium sulfate precipitation. The precipitate was collected, suspended in the buffer, and dialyzed overnight against distilled water. The clarified supernatant fraction generally contained the enzyme purified 1.3-fold relative to the preceding step, with a recovery of 90% of the enzyme activity. Enzymes were prepared in a partially resolved form; the degree of resolution varied 40 to 60% in different preparations. When enzyme preparations were incubated with pyridoxal phosphate, the absorbance in the 360 μm region markedly increased. Dialysis of the enzyme incubated with pyridoxal phosphate did not lead to significant alteration of the spectrum, and further addition of pyridoxal phosphate did not increase the 360 μm absorbance. Such dialyzed enzyme preparations gave maximal activity upon addition of α-ketoglutarate alone, and they are considered to represent the holoenzyme.

**Step 7: Crystallization**—Ammonium sulfate was added to a 15 mg per ml solution of the enzyme obtained in the former step until the solution became slightly turbid and insoluble matter was discarded by centrifugation. The enzyme crystallized from the supernatant solution by standing overnight at 5°C. This procedure resulted in a 68-fold purification with a overall yield of 32%. The crystalline enzyme possessed the highest activity ever reported (84 units per mg of protein). The enzyme has been repeatedly recrystallized without marked change in activity or in the appearance of the crystals.

The extinction of the enzyme at 280 μm was determined by weighing a desiccated portion of the purified enzyme on a Mettler microbalance. Based on these analyses, the extinction coefficient of the enzyme is $\varepsilon_{280} = 10.0$ at pH 6.8.

**Absorption Spectra**

Spectra of the crystalline enzyme, when recorded in a series of buffers in the pH range from 5.3 to 8.5, were found to be identical within the experimental limit. The complete absorption spectrum of the enzyme is shown in Fig. 4. In addition to the characteristic maximum at 278 μm, contributed by aromatic amino acid residues, an absorption peak at about 360 μm was observed.

**Coenzyme Content**

Spectrophotometric examination of the *Pseudomonas* enzyme showed an identical spectrum with those obtained with *Achromobacter* enzyme (10) and *Alcaligenes* (13). The absorption maximum at 360 μm was shown to be due to the presence of
The quantity of pyridoxal phosphate was computed to be 0.02 pmole per mg of protein. This corresponds to 16 moles of pyridoxal phosphate per 800,000 g of protein.

Pyridoxal phosphate was also determined by the phenyl-hydrazine method of Wada and Snell (24). With this procedure it was found that 3.02 mg of enzyme contained 0.061 pmole of pyridoxal phosphate, or 800,000 g of enzyme contained 16 moles of pyridoxal phosphate.

**Physicochemical Properties of Enzyme**

**Ultracentrifugal Analysis**—Fig. 5 shows the sedimentation pattern of crystalline enzyme. The crystalline aspartate β-decarboxylase appeared to be homogeneous in this pattern. The sedimentation coefficient was calculated by ultracentrifugation at 30,800 rpm. In order to determine the dependence of the sedimentation coefficient on concentration, velocity studies were made at protein concentrations of 0.8, 0.4, 0.2, 0.15, and 0.1 g/100 ml. Appropriate corrections were made for the temperature during ultracentrifugation and for the viscosity of the buffer in calculating the $s_{0.0}$ value. Extrapolation of the sedimentation data to zero concentration leads to a value of 20.7 S for $s_{0.0}$ (Fig. 6).

**Diffusion Coefficient**—Diffusion coefficients were calculated by the statistical or moment method (25). The values calculated were corrected to the diffusion coefficients corresponding to water at 20° ($D_{20, w}$). The values of $D_{20, w}$ are plotted against the concentration of enzyme. Extrapolation of zero concentration gave a value of $2.26 \times 10^{-7}$ cm$^2$ sec$^{-1}$ (Fig. 7).

**Partial Specific Volume**—The apparent specific volume was calculated from measurement of the density of aspartate β-decarboxylase solutions in 0.1 M sodium acetate buffer, pH 6.8, at three different concentrations. An average value of 0.72 ml per g was obtained.

**Frictional Coefficient Ratio**—The frictional ratio, $f/f_{min}$, was calculated from the sedimentation and diffusion coefficients with the following relation (26).

$$f/f_{min} = 1.00 \times 10^{-4} (1 - \rho V_{20}/D_{20, w} - s_{20, w} V_{20})^{1/2}$$

A value of 1.55 was obtained for the frictional ratio.

**Viscosity**—Aspartate β-decarboxylase solution in 0.1 M potassium phosphate buffer, pH 6.8, was made at five different concentrations at 25°. A value for the intrinsic viscosity, $[\eta]$, of 0.054 dl per g was obtained, as shown in Fig. 8.

**Molecular Weight Determination**—The molecular weight ($M$)
of crystalline aspartate β-decarboxylase, as determined from sedimentation and diffusion measurements, was calculated from the equation (26),

$$M = \frac{RT_0}{(1 - \bar{V}_p)D}$$

where $T$ is the absolute temperature, $R$ is the gas constant, and $\rho$ is the density of the solvent. A molecular weight of 795,000 was calculated for the holoenzyme with the use of the measured value for $S$ of $20.7 \times 10^{-13}$, $D$ of $2.26 \times 10^{-3}$, and $\bar{V}$ of 0.72.

The second method used for the determination of the molecular weight was the Archibald method with ultracentrifugation. A solution of 0.59% sample dialyzed against 0.1 M sodium acetate buffer, pH 6.8, was subjected to ultracentrifugation at 5,900 rpm. Six photographs were used. The protein concentration of the sample was determined by a separate run of ultracentrifugation with a synthetic boundary cell. The molecular weight computed for the individual patterns at different time intervals were found to decrease slightly with time. Extrapolation to zero time gave a value of $M = 820,000$. From these values, the molecular weight of the crystalline aspartate β-decarboxylase of *P. dacunhae* was estimated as approximately 800,000.

**Isoelectric Point**—With 1 ml of enzyme solution, containing 4 mg of protein, an experiment of isoelectric focusing was performed. After focusing for 48 hours with a constant potential of 800 volts and an initial current of 1.75 mA, the contents of the column were cut into 2-ml fractions. The amounts of protein in the fraction were estimated by measurements of absorbance at 280 μm. On the basis of isoelectric focusing aspartate β-decarboxylase appeared to be a homogeneous substance having a pI at pH 4.2 (Fig. 9).

**DISCUSSION**

Aspartate β-decarboxylase has been isolated in apparently homogeneous form from *Achromobacter d-15* (11) and *A. faecalis* (13). As shown in Table II, both are very similar to the present *Pseudomonas* enzyme in molecular weight and pyridoxal phosphate content. Although the crystalline form of the enzyme from *Achromobacter* is regular hexagonal plates, that from *Pseudomonas* is regular lozenge plates.

In contrast to several other previously purified vitamin B6 enzymes, aspartate β-decarboxylase does not exhibit peak absorbance in the range of 400 to 430 μm, nor does its spectrum change on alteration of pH.

*Pseudomonas* aspartate β-decarboxylase is markedly activated by both pyridoxal phosphate and a variety of α-keto acids. Since α-ketoglutarate activates this enzyme most markedly, it was used for the assay of enzymatic activity.

From 1 kg of lyophilized cells, approximately 2 g of crystalline aspartate β-decarboxylase were obtained by this procedure. The specific activity of the crystalline enzyme was slightly higher than any previously reported (Table II). This preparation of enzyme catalyzed the decarboxylation of 5,045 pmol of L-aspartate per hour per mg of protein at pH 5.3; hence its activity was 67,262 moles of aspartate decarboxylated per min per mole of enzyme and its catalytic activity was 4,204 moles of L-aspartate decarboxylated per min per mole of pyridoxal phosphate.*

**Table II**

*Comparison of various aspartate β-decarboxylase preparations*

<table>
<thead>
<tr>
<th>Sources of enzyme</th>
<th>$S_{avw}$</th>
<th>Molecular weight</th>
<th>Pyridoxal phosphate content</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Achromobacter d-15</em> (10, 11)</td>
<td>19.8</td>
<td>760,000</td>
<td>13</td>
<td>73.4</td>
</tr>
<tr>
<td><em>Alcaligenes faecalis</em> (13)</td>
<td>18-22</td>
<td>800,000</td>
<td>15.5</td>
<td>57.2</td>
</tr>
<tr>
<td><em>Pseudomonas dacunhae</em></td>
<td>20.7°</td>
<td>800,000</td>
<td>16</td>
<td>84.1</td>
</tr>
</tbody>
</table>

* This study.

° Extrapolation to zero concentration.
phosphate. The enzymatic properties will be published in a subsequent report.

In Table III the physicochemical properties of crystalline aspartate P-decarboxylase are summarized.

As described above, a value of 1.55 was obtained for the frictional ratio of aspartate P-decarboxylase. This value is very high in comparison with the data for globular proteins.

Preliminary studies indicate that the enzyme may be dissociated by high concentration of guanidine. Detailed studies of the dissociation of the enzyme will be published elsewhere.

Acknowledgments—The authors wish to express their thanks to Professor T. Suzuki of Osaka University, to Mr. T. Takayanagi and Dr. K. Fujii for their encouragement in this study, and to Mr. F. Murakami and Miss T. Nakagawa for technical assistance. We are also indebted to Dr. K. Kakiuchi of Institute for Protein Research, Osaka University, for performing the ultracentrifugation experiments.

REFERENCES


Table III

<table>
<thead>
<tr>
<th>Physicochemical properties of aspartate P-decarboxylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedimentation coefficient</td>
</tr>
<tr>
<td>Diffusion coefficient</td>
</tr>
<tr>
<td>Partial specific volume</td>
</tr>
<tr>
<td>Intrinsic viscosity</td>
</tr>
<tr>
<td>Extinction coefficient</td>
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<tr>
<td>Isoelectric point</td>
</tr>
<tr>
<td>Frictional ratio</td>
</tr>
<tr>
<td>Molecular weight</td>
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</table>

$^a$ From Archibald method (19).
$^b$ From sedimentation coefficient and diffusion coefficient.

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