Isolation of Crystalline pH 6 Acetolactate-forming Enzyme from Aerobacter aerogenes

Fredrik C. Stormer
From the Department of Microbiology, Ullevål Hospital, Oslo, Norway

SUMMARY
The pH 6 acetolactate-forming enzyme has been isolated from Aerobacter aerogenes and crystallized in the presence of sodium pyruvate, cocarboxylase, and MgCl₂. Two different crystal forms, one plate-shaped and another needle-shaped, are described. The latter crystalline form, which is developed in the absence of (NH₄)₂SO₄, is very slightly soluble at neutral pH and has low specific activity when brought into solution at high or low pH. Evidence that the needle-shaped crystals are the enzyme protein, and not an artifact, is described. Specific activity does not increase significantly by recrystallization of the plate-shaped crystals, and the enzyme is homogenous on electrophoresis and ultracentrifugation.

The formation of α-acetolactate from pyruvate in biological systems was first postulated by Watt and Krampitz in 1947 (1); in 1952, Juni (2) showed that the formation of acetoin (acetyl-methylcarbinol) by extracts of Aerobacter aerogenes is catalyzed by two different enzyme systems.

The detection of acetoin, known as the Voges-Proskauer test, is used to distinguish between the colon and Aerobacter groups of bacteria.

This paper describes the isolation in crystalline form of the pH 6 acetolactate-forming enzyme from A. aerogenes.

EXPERIMENTAL PROCEDURE

Materials and Methods

Chemicals—Cocarboxylase and sodium pyruvate were obtained from Sigma. DEAE-Sephadex A-50 (bead form) was purchased from the Pharmacia Laboratories, and hydroxylapatite was obtained from the Bio-Rad Laboratories. Acetolactate ethylester was a gift from T. Stensrud of the University of Oslo.

The stock culture of A. aerogenes, strain 1033, was a gift from Professor H. E. Umbarger of the University of Purdue, Lafayette, Indiana. The cells were routinely maintained on nutrient agar slants.

Conditions for Growth of A. aerogenes—The bacteria were grown with maximal aeration at 37°C in the minimal medium of Davis and Mingioli (5) with the use of distilled water and supplemented with trace elements (6). The addition of trace elements was found necessary to give optimal growth conditions; 1% Bacto-dextrose was used as the source of carbon.

The cells, allowed to reach the stationary phase, were harvested when the pH in the culture had dropped to approximately 5.8, usually after 20 hours. The suspension was chilled to 0-15°C, and the cells were sedimented in a refrigerated SS-1 Servall centrifuge equipped with continuous flow system. The cells could be stored at -25°C for several months without detectable loss of the pH 6 acetolactate-forming enzyme activity.

Buffers—The standard buffer used for preparing solutions of enzyme preparation throughout this work contained 50 mM phosphate, 1 mM MgCl₂, 5 mM sodium pyruvate, and 0.2 mM cocarboxylase, pH 6.0.

Assay Procedures—The pH 6 acetolactate-forming enzyme activity was determined by measuring the amount of acetolactate formed in the assay. In a total volume of 1.0 ml, the complete incubation mixture contained 50 μmoles of acetate, pH 5.8; 40 μg of cocarboxylase; 40 μmoles of sodium pyruvate; and the enzyme. Acetolactate was detected as acetoin as described previously (7).

Specific activity is expressed as micromoles of acetolactate formed per mg of protein per hour.

Acetolactate decarboxylase activity was determined by incubating a mixture containing the enzyme extract; 100 μmoles of acetate, pH 5.8; and 40 μmoles of N-acetolactate in a final volume of 1.0 ml. After 20 min at 37°C, the reaction was stopped by adding 1.0 ml of 2.5 M NaOH. Of this solution, 0.1 ml was
withdrawn, added to 0.4 ml of 2.5 M NaOH, and tested for acetoin (7).

Protein was determined by the colorimetric method of Lowry et al. (8) with bovine serum albumin as a standard.

RESULTS

Purification of pH 6 Acetolactate-forming Enzyme from A. aerogenes—The following procedure for purification of the pH 6 acetolactate-forming enzyme resulted in a 120-fold purification with a 20 to 30% yield. Unless otherwise stated, all operations were performed at 0-5°C, and Table I summarizes the data for a typical preparation.

Preparation of Extract—Frozen cells (185 g) were suspended in 5 volumes of the standard buffer (see "Experimental Procedure"). Aliquots of 30 ml were subjected to ultrasound for 10 min with a MSE ultrasonic power unit, model 60 w. The temperature of the sonic extract increased up to 15°C during this operation.

The material was centrifuged for 10 min at 16,000 × g, and unbroken cells were resuspended in the buffer and treated by sonic disruption. The supernatant was centrifuged for 30 min at 40,000 × g, and the clear solution was collected to yield Fraction I.

Heat Treatment—The above solution was divided into two portions and heated to 55°C, with continuous stirring, by placing the containers in a water bath at 80°C. The solutions were kept at 55°C for 2 min and cooled to 0°C in an ice-water bath with continuous stirring. After 20 min the inactive precipitate was removed by centrifugation and discarded (Fraction II).

Streptomycin Precipitation—To the 990 ml of Fraction II were added, with stirring, 178 ml of a 10% streptomycin sulfate solution adjusted to pH 6 with phosphate. After standing for 15 min, the suspension was centrifuged and the supernatant collected (Fraction III).

First Ammonium Sulfate Fractionation—To Fraction III were added, with stirring, 450 g of solid (NH₄)₂SO₄ and the pH was adjusted to 6.0 by adding 1 M K₂HPO₄ by drops. The suspension was allowed to stand for 20 min after the salt had dissolved.

The precipitate was dissolved after centrifugation at 10,000 × g for 10 minutes in sufficient 0.05 M phosphate to provide a protein concentration of 20 to 30 mg per ml. This solution was then dialyzed against 2 liters of the same buffer overnight. A small precipitate, which appeared during dialysis, was removed by centrifugation at 10,000 × g and yielded a clear supernatant (Fraction IV).

DEAE-Sephadex A-50 Chromatography—Fraction IV was adjusted to pH 7 by adding 1 M K₂HPO₄ and distilled water to yield a final phosphate concentration of 0.05 M. This 350-ml solution was loaded on the column (11 × 5 cm), previously equilibrated with the same buffer, at a flow rate of 3.0 ml per min. The column was eluted with a linear gradient formed from 500 ml of 0.05 M and 500 ml of 0.5 M phosphate, pH 7.0 (total volume of 1 liter), was applied at a flow rate of 1.5 ml per min, and 10-ml fractions were collected. These fractions were assayed for the pH 6 acetolactate-forming enzyme activity, and their protein content was determined.

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![Image](http://www.jbc.org/)

**FIG. 1.** Chromatography of Fraction IV on DEAE-Sephadex A-50. A column, 11 x 5 cm, was prepared and loaded with 350 ml of Fraction IV at a flow rate of 3.0 ml per min. A constant linear gradient from 0.05 M to 0.5 M phosphate, pH 7.0 (total volume of 1 liter), was applied at a flow rate of 1.5 ml per min, and 10-ml fractions were collected. These fractions were assayed for the pH 6 acetolactate-forming enzyme activity. ---; and their protein content was determined, -.

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**FIG. 2.** Chromatography of Fraction V on hydroxylapatite. The column was prepared as described in the text and loaded with 125 mg of Fraction V at a flow rate of 2.5 ml per min. A stepwise elution was performed with 5 ml-portions of phosphate, pH 6.0: three times, 0.35 M; six times, 0.9 M. These fractions were collected and assayed for the pH 6 acetolactate-forming enzyme activity. ---; and their protein content was determined, ---.

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FIG. 3. Photograph of the crystalline pH 6 acetolactate-forming enzyme. A, soluble crystals, X 162; B, insoluble crystals, X 360.

which contained most of the enzyme activity was collected (Fig. 2). The gel was discarded, and the procedure was repeated with a new batch of protein. Gel, 4.3 g, dry weight, was used each time, and during this purification step all buffers contained 2 mM MgCl₂, 10 mM sodium pyruvate, and 0.4 mM cocarboxylase. The fractions were combined, volume 250 ml, and solid 97 g of (NH₄)₂SO₄ were added, and the pH adjusted as earlier. The active precipitate was dissolved in 0.05 M phosphate, pH 6.0, to yield a protein concentration of 17 mg per ml (Fraction VI). Although the enzyme is not completely separated from the protein that follows it, it is sufficiently pure for crystallization.

First Crystallization—The protein concentration from the above step should not be lower than 17 mg per ml. In about 8 hours, a "silky shimmer" was observed; after an additional 24 hours, a heavy, white precipitate was revealed, which consisted of thin plates (Fig. 3A). Occasionally, the time for the appearance of the crystals could be somewhat longer, and the enzyme was crystallized with greater consistency if seed crystals were added to the supernatant solution before storage.

The precipitate was spun down at 12,000 x g for 10 min and

Fig. 5. Enzyme activity as a function of storage time at 0°. Each tube contained in 1 ml: 0.5 ml of enzyme protein, specific activity, 3120; 50 FM phosphate, pH 6.0; and where indicated in the following concentrations: sodium pyruvate, 20 μM; cocarboxylase, 0.1 μM; and MgCl₂, 0.5 μM. Samples were withdrawn at the intervals indicated and tested for activity. ■, sodium pyruvate, cocarboxylase, and MgCl₂; ▼, sodium pyruvate and cocarboxylase or cocarboxylase and MgCl₂; +, sodium pyruvate and MgCl₂; ○, no additions.

precipitate was collected by centrifugation and dissolved in 100 ml of 0.05 M phosphate, pH 6.0 (Fraction V).

Hydroxyapatite Chromatography—An aliquot of Fraction V containing 125 mg of protein was loaded on the column prepared as previously described (9, 10) and washed with a few milliliters of 0.05 M phosphate, pH 6.0. The column was eluted three times with 5 ml of 0.35 M phosphate and three times with 5 ml of 0.9 M phosphate, pH 6.0. The 0.9 M eluant, Fractions 4 to 6,
dissolved, by gentle shaking at 37°, in a sufficient volume of standard buffer containing 0.2 M (NH₄)₂SO₄, pH 6.0, to yield a protein concentration of 7.5 mg per ml (Fraction VII). More than 85% of the enzyme from the last step was crystallized (based on specific activity) in this way.

Second Crystallization—Recrystallization was carried out as follows. Insoluble material in Fraction VII was removed by centrifugation, and to the clear supernatant, 1.0 ml of saturated (NH₄)₂SO₄ in the standard buffer was added dropwise until the solution became slightly turbid. Crystalline enzyme appeared in a few hours, with the same shape as in the first crystallization, and after standing overnight, the heavy crystalline precipitate was collected by centrifugation (Fraction VIII). No further increase in specific activity was observed when Fraction VIII was recrystallized by the same procedure as described above.

Third Crystallization—If the plate-shaped crystals were dissolved in the standard buffer, 50 mM acetate, pH 5.8, or distilled water, at a protein concentration of 5 mg per ml or higher, the crystallization usually began within a few minutes, and was virtually complete within a few hours. The crystals appeared as long, thin, white needles (Fig. 3B). The needle-shaped crystals could be formed from Fractions VII and VIII, and from Fraction VI if the latter solution was dialyzed against the standard buffer. After the needle-shaped crystals had been formed, they were removed from the supernatant by centrifugation and suspended in the standard buffer. The activity remaining in the supernatant after formation of Fraction VIII from a solution with an initial protein concentration of 10 mg per ml was estimated to be 12% of the initial activity.

The needle-shaped crystalline material was slightly soluble in H₂O and buffers in the pH range of 5.0 to 11.0. The following compounds had no effect on the solubility of the crystals: sodium lauryl sulfate, sodium deoxycholate, octyltrimethyl ammonium bromide, dimethyl sulfoxide, dithiothreitol, EDTA, NaCl, sodium pyruvate, decarboxylase, and 1 M phosphate, pH 6 to 8.

Since the crystals were not put into soluble form and assayed successfully, the specific activity could not be determined. A washed crystal suspension (even stored in the dry state at room temperature for several months) showed "high" specific activity. When the crystals were brought into solution by decreasing or increasing pH, low specific activity was determined. The stability properties and homogeneity of the enzyme (see below) indicate that the enzyme has been inactivated by this treatment, rather than that the crystals are artifacts and not the enzyme protein.

The observation that the needle-shaped crystals were formed from Fraction VI if the solution had previously been dialyzed against the standard buffer led to the speculation that (NH₄)₂SO₄ prevented the formation of the insoluble crystals and favored the formation of the soluble plate-shaped crystals. When the plate-shaped crystals were dissolved in the standard buffer containing 0.2 M (NH₄)₂SO₄ at a protein concentration of 5 mg per ml or higher, the needle-shaped crystals were not immediately formed. After a week, microcrystals of the insoluble material could be observed.

Test for Homogeneity—Further recrystallization of the plate-shaped crystals did not increase the specific activity. The crystalline pH 6 acetolactate-forming enzyme moves as a single component in electrophoresis experiments carried out on cellulose acetate strips (4.8 × 19.7 cm) at pH 7.0 and 150 volts (Fig. 4). The protein band on the strip, visualized with an aqueous trichloracetate solution of Ponceau S dye, coincides with the band of enzyme activity. The protein moves as one peak in the analytical ultracentrifuge. No impurities could be detected by these methods.

Stability—Preliminary experiments with crude and partially purified enzyme showed that cocarboxylase, MgCl₂, and sodium pyruvate had a marked stabilizing effect on the enzyme. This is shown in Fig. 5 with an enzyme preparation purified ten times. Similar results were observed when the enzyme was heated to 60° for 3 min in the absence of cocarboxylase, MgCl₂, and sodium pyruvate, or when only one of them was present. In the presence of all three compounds, approximately 50% of the activity was intact after heating.

Partially purified enzyme has also been stored in the standard buffer containing 50% glycerol at 0° for 18 months without significant change in enzyme activity. Suspensions of the plate-shaped crystals have been stored successfully for several months before recrystallization.

DISCUSSION

The procedure described above is a simple and reproducible method for isolating highly purified pH 6 acetolactate-forming enzyme with good yield. The preparation was free from acetolactate decarboxylase activity. Hydroxylapatite allows good resolution of the protein components which on DEAE-Sephadex elute together.

Comparing the activity of the recrystallized enzyme with the activity of the crude extract, the pH 6 acetolactate-forming enzyme would account for about 0.6 to 0.9% of the total protein in the bacterial cell.

The peculiar behavior of the enzyme in yielding an insoluble crystalline form seems without parallel in the literature. Attempts to get it into solution without decrease in specific activity have been unsuccessful. At the present time, the following criteria indicate that the needle-shaped crystals are a crystalline form of the pH 6 acetolactate-forming enzyme. (a) The insoluble crystals have been formed from plates recrystallized three times. (b) No impurities have been detected by electrophoresis and ultracentrifugation of the plate-shaped crystals. (c) A washed suspension of the insoluble crystals showed "high" specific activity.

Further studies on the physical properties of the enzyme are in progress.

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1 Unpublished results.
2 Can be replaced by MnCl₂.
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Fredrik C. Störmer


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