Oxaloacetate Decarboxylase from Cod. A Shorter Preparation and Crystallization*

G. W. Kosicki†

ABSTRACT: Crystalline oxaloacetate decarboxylase can be prepared from codfish muscle (*Gadus marrhua*) by the following steps: extraction of the frozen muscle, adjusting the pH to 5.1, heating to 45°, fractionation of the enzyme with $(NH_4)_2SO_4$, chromatography over a $Ca_3(PO_4)_2$-cellulose column, concentration, and crystallization; yield ~80–85 mg from 500 g of frozen muscle. Variations of this method are also presented.

An oxaloacetate decarboxylase from codfish muscle was purified by Schmitt *et al.* (1966). They reported on the stoichiometry of the reaction as

$$\text{oxaloacetate} + H^+ \xrightarrow[\text{pH } 6.6]{Mn^{2+} + \text{oxaloacetate decarboxylase}} \text{pyruvate} + CO_2$$

They showed that their enzyme preparation was not identical with malic enzyme, pyruvate carboxylase, or phosphoenol carboxykinase. It is a metal-activated, nucleotide- and avidin-independent decarboxylase.

This paper describes a short purification of the oxaloacetate decarboxylase to the same final specific activity as that obtained by Schmitt *et al.* (1966). The enzyme can be purified to its final specific activity on the second day with a yield of 47% of the enzyme in the initial extract; preparations from 500 g of frozen codfish muscle yield as much as 85 mg of purified enzyme. This material is then concentrated and set aside to crystallize. Although the crystallization takes days or even weeks, the crystalline enzyme has no higher activity than the purified protein from which crystals may be obtained.

Experimental Section

Materials. The following materials were commercial preparations: oxaloacetic acid (Calbiochem), dithioerythritol (Cyclo-Chemical), and $(NH_4)_2SO_4$ (Mallinckrodt). Other reagents were commercial preparations of analytical grade.

Assay of Oxaloacetate Decarboxylase. The assay depends upon following the decrease in absorption of the enol form of the equilibrated tautomeric mixture of the oxaloacetate–Mn^{2+} complex at 260 mμ. The order of addition and mixing is critical in this assay because of the limiting rate of tautomerization of oxaloacetate at neutral pH (the solid is in the enol form). The substrate is first dissolved in water (1 mg/0.5 ml) and equilibrated for 15 min at room temperature, and then 0.5 ml of 0.1 M potassium phosphate buffer (pH 6.60) is added. The oxaloacetate solution is ready for use after standing at room temperature for 10 min; 0.1 ml of the equilibrated oxaloacetate solution, 0.1 ml of 10^{-2} M $MnSO_4$ (or 10^{-3} M if EDTA is absent), and water to make the final volume 1.0 ml are mixed; then 0.3 ml of 1.0×10^{-1} M potassium phosphate buffer (pH 6.60) is added and mixed, and finally an aliquot (0.05–0.10 ml) of enzyme solution is added and the solution is mixed again. Under these conditions the rate of decrease in absorbance is linear for at least 5 min, provided that the amount of enzyme added gives an absorbance change of less than 0.02/min at 30°, using a cuvet with 1.0-cm light path. The molar absorbancy index (log ε 1.01 × 10^3, pH 6.6) was used to convert the decrease of absorbance into micromoles (Schmitt *et al.*, 1966). The expanded absorbance scale (0.00–0.10 ODU) of a Cary 15 spectrophotometer was used in following the decrease of absorbance with time.

The concentration of enzyme given in milligrams per milliliter is based on the 280 to 260 mμ absorbance ratio (Warburg and Christian, 1942) and the units of enzyme activity are given in micromoles of oxaloacetate decarboxylated per minute at 30°.

Calcium Phosphate–Cellulose. The calcium phosphate–cellulose was prepared according to the method of Massey (1960) with some modifications. A mixture of 453 g of sucrose and 75 g of CaO (reagent grade, with no further treatment) in 2.0 l. of distilled water was stirred overnight. The suspension was filtered to give a clear yellowish solution and chilled to 5°. To the chilled and stirred solution, 60 ml of 85% *o*-phosphoric acid was added dropwise to a pH of 8.5. The white gel was centrifuged and resuspended in 2.0 l. of water (repeated three times) until the pH dropped to 7.2. The weight of the calcium phosphate gel was measured on an aliquot of the sample that was dried at 100° and then the concentration was adjusted to 30 mg/ml. Calcium phosphate gel suspension (1 l.) was added slowly to a stirred suspension of 300 g of fibrous

* From the James Bryant Conant Laboratory of the Department of Chemistry of Harvard University, Cambridge, Massachusetts 02138. *Received July 25, 1968.* This work was supported by Grant GM-04712-12 from the National Institute of General Medical Sciences of the National Institutes of Health.

† On sabbatical leave from the University of Windsor, Department of Chemistry, Windsor, Ontario, Canada, and supported by a special fellowship (1-F3-AM-9760-01) from the National Institute of Arthritis and Metabolic Diseases.

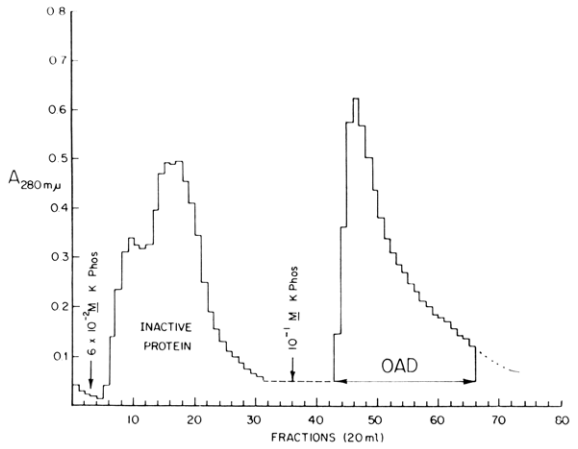

FIGURE 1: Adsorption chromatography on calcium phosphate–cellulose. Fractions 44–72 were combined (560 ml) as shown in Table I.

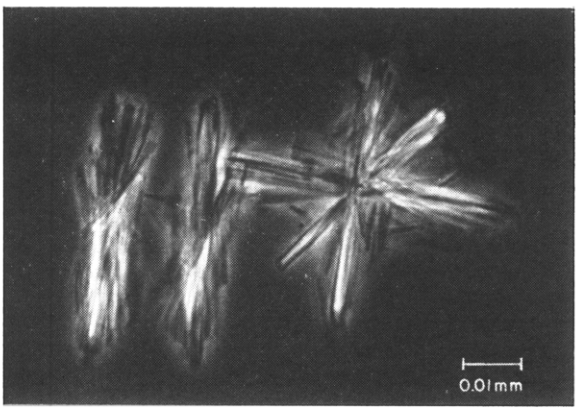

FIGURE 2: Photomicrograph of crystals of oxaloacetate decarboxylase from codfish.

cellulose powder (Whatman No. CF 11) in 2.0 l. of water. The cellulose was previously sized to improve flow rates. The calcium phosphate–cellulose can be stored at 3°. Before use the slurry was degassed under partial vacuum.

Extraction. Freshly frozen codfish (*Gadus morrhua*) muscle[1] (500 g) broken into pieces was homogenized in a Waring Blendor with 1.5–2.0 l. of a 1% NaCl solution containing 10^{-5} M EDTA (disodium salt)–10^{-5} M dithioerythritol–10^{-5} M potassium phosphate buffer (pH 6.8). The homogenization was usually carried out in four batches to ensure thorough extraction. A second extraction is not needed. During the 2-min blending the temperature rose to about 20°. The white suspension was centrifuged for 10 min in a large-capacity centrifuge to pack the sediment.

Acid and Heat Fractionation. To the turbid white supernatant fluid 1.7 N acetic acid was added slowly with stirring until the pH dropped to 5.1, and a white precipitate formed. The suspension (in a 3- or 4-l. Pyrex beaker) was placed in a water bath at 65–70° until the stirred suspension reached a temperature of 45°. The suspension was then cooled in ice below 30° and centrifuged (10 min at 5°) in a large-capacity centrifuge to pack the sediment. Heating can be extended to 55° with some further purification but usually with some loss in total quantity of enzyme.

$(NH_4)_2SO_4$ Fractionation. To the stirred clear supernatant fluid, solid EDTA (disodium salt) was added to a final concentration of 0.01 M and then solid $(NH_4)_2SO_4$ was added slowly to 60% saturation (390.0 g/l.). The suspension was allowed to stir for 1 hr at room temperature, and then centrifuged for 20–30 min (3000*g*, 5°) to pack the precipitate. The precipitate was dissolved in a minimal amount of cold potassium phosphate buffer (0.01 M, pH 6.8) containing 10^{-5} M dithioerythritol and 10^{-5} M EDTA (disodium salt). (The dithioerythritol and EDTA were added to all buffers unless otherwise stated.) Increasing the $(NH_4)_2SO_4$ concentration to 70% saturation or packing the extract in ice did not increase the yield of enzyme. The dissolved $(NH_4)_2SO_4$ fraction (35–50 ml) was dialyzed overnight (4°) against 20-fold excess of 10^{-2} M potassium phosphate buffer (pH 6.8).

Adsorption Chromatography. The dialyzed enzyme was cleared by centrifugation and further purified by chromatography using a column set up with an automatic fraction collector in a cold room at 4°. A column (4.0-cm diameter) was filled with 400 ml of degassed calcium phosphate–cellulose suspension (see Materials) and allowed to settle. The settled cellulose was covered with a filter paper disk and then flushed with 1 l. of 10^{-2} M potassium phosphate buffer (pH 6.8). The protein was applied to the column and then subjected to a stepwise elution. The column was first washed with 1×10^{-2} M potassium phosphate buffer (pH 6.8) (about 100 ml) to ensure that the enzyme had adsorbed to the gel, and then washed with 6×10^{-2} M potassium phosphate buffer (pH 6.8) until no more material absorbing at 280 mμ appeared (see Figure 1). The enzyme was then eluted with 10^{-1} M potassium phosphate buffer (pH 6.8) in a well-defined peak of final purity (see Figure 1). The slope of the curve was characteristic of elution patterns from an adsorbent-type column. The flow can be quite fast (20-ml fractions, 4 ml/min). The eluate (about 500 ml) was concentrated by a second calcium phosphate–cellulose adsorption. The sample was diluted to lower the concentration of potassium phosphate to 0.05 M (pH 6.8) and then quickly (1 l. in 2.5 hr) put on to a smaller column (2.8-cm diameter, 50 ml of calcium phosphate–cellulose). The adsorbed enzyme was then eluted with 15% saturated $(NH_4)_2SO_4$ (83.7 g/l.) containing 10^{-2} M potassium phosphate buffer (pH 6.8). Fractions (3 ml) were collected (1 ml/min) and those containing the 280-mμ-absorbing material were combined. Other methods of concentration were also used: reverse dialysis with dry Sephadex G-25 applied to the enzyme solution in a dialysis bag, or $(NH_4)_2SO_4$ fractionation (0–70%), although recovery is poor by the latter method because of low protein concentration.

Crystallization. To the concentrated eluate containing

[1] No difference in behavior of the enzyme was noticed among the half-dozen codfish used. Only one species of Atlantic cod (*G. morrhua*) is known in the waters of Cape Cod, Mass., where the fish were caught.

TABLE I: Shorter Purification of Oxaloacetate Decarboxylase.

Step	Vol (ml)	Total Protein (mg)	Total Units	Sp Act.[a]
Extraction, 500 g of muscle	1300	4.43×10^3	309 (228–309)	0.07 (0.04–0.07)
Acid, heat	1200	2.1×10^3	252 (228–290)	0.12 (0.10–0.12)
$(NH_4)_2SO_4$, 0–60% saturated and dialysis	43	391	237 (163–237)	0.60 (0.6–0.8)
Calcium phosphate–cellulose column	560	88.5	150 (110–150)	1.70 (1.7–1.8)
Calcium phosphate–cellulose concentration	49	86.5	147 (100–147)	1.72 (1.7–1.8)

[a] Micromoles of oxaloacetate decarboxylated per minute at 30°.

TABLE II: Purification of Oxaloacetate Decarboxylase with Additional Chromatography.

Step	Vol (ml)	Total Protein (mg)	Total Units	Sp Act.[a]
Extraction, 1000 g of muscle	2750	1800	241	0.013
Acid, heat	2500	1300	235	0.18
$(NH_4)_2SO_4$, 0–70% saturated	120	900	220	0.24
Sephadex G-50, 4.7 × 45 cm; eluent: 10^{-3} M sodium acetate (pH 5.5)	295	1000	205	0.20
Sephadex CM-25, 7 × 35 cm; wash: 10^{-3} M sodium acetate (pH 5.5); eluent: 10^{-1} M potassium phosphate (pH 6.8) (diluted to to 0.5 M potassium phosphate)	348	149	131	0.88
Calcium phosphate–cellulose column, 2.6 × 30 cm; wash: 0.06 M potassium phosphate (pH 6.8); eluent: 0.09 M potassium phosphate (pH 6.8) (diluted to 0.05 M potassium phosphate)	375	24.4	43	1.75
Calcium phosphate–cellulose concentration; eluent: 15% saturated $(NH_4)_2SO_4$	19.7	18.4	33.4	1.82

[a] Micromoles of oxaloacetate decarboxylated per minute at 30°.

15% saturated $(NH_4)_2SO_4$ (packed in ice and stirred), solid $(NH_4)_2SO_4$ was added slowly to 50% saturation (219 g/l.). About 25 ml of saturated $(NH_4)_2SO_4$ solution was added slowly to each 100 ml of eluate, so as to bring the $(NH_4)_2SO_4$ concentration to 60%. The solution was set aside at 3° to crystallize. Additions of EDTA (disodium salt, 10^{-3} M) and $MnSO_3$ (10^{-4} M) to the solution during crystallization had no obvious effect. The crystals (Figure 2) prepared by the purification procedure with additional chromatographic steps (Table II) appeared identical with those prepared by the shorter method (Table I).

Results

Typical results obtained with the shorter purification of the enzyme are summarized in Table I; the details of the steps have been presented in the Experimental Section. The numbers within the brackets represent a range of values for several preparations.

Several variations of the shorter method of purification were made. The use of Sephadex G-50 and Sephadex CM-25 column chromatography prior to the calcium phosphate–cellulose column did not increase the final specific activity (see Table II). The calcium phosphate–cellulose can also be used in bulk adsorption to purify the enzyme; the gel is then filtered on a Büchner funnel. This variation was carried out at room temperature on the diluted enzyme (below 0.05 M in potassium phosphate) and thus avoided ammonium sulfate fractionation (see Table III). The chromatography and concentraion steps were carried out as in Table I and described in the Experimental Section.

The schlieren patterns during the ultracentrifugation

TABLE III: A Batch Procedure Using Calcium Phosphate Cellulose in Place of $(NH_4)_2SO_4$ Fractionation.

Steps	Vol (ml)	Total Protein (mg)	Total Units	Sp Act.[a]
Extraction				
750 g of muscle, 2.0 l., 0.5% NaCl	1,750	12,000	380	0.03
Acid, heat				
Dilution to 0.05 M	1,550	6,000	372	0.06
Calcium phosphate–cellulose				
1.0-l. filtrate, three eluents 0.15 M potassium phosphate (pH 6.8) (dilution to 0.05 M potassium phosphate)	8,000 1,500	5,000 585	0 150	0.26
Calcium phosphate–cellulose column;				
Wash 5×10^{-2} M potassium phosphate (pH 6.8)				
Eluent 7×10^{-2} M potassium phosphate (pH 6.8) (dilution to 0.05 M potassium phosphate)	1,185	89	118	1.33
Calcium phosphate–cellulose				
Concentration				
Eluent 15% saturated $(NH_4)_2SO_4$	60	57	108	1.82

[a] Micromoles of oxaloacetate decarboxylated per minute at 30°.

(Spinco Model E) of a sample at 10, 7.5, and 5.0 mg per ml over 1.5 hr at 170,000*g* (20°) indicated a homogeneous preparation.

The instability of the enzyme causes some difficulties at pH values above 8 and below 4.5, where the protein is quickly inactivated (25°). At pH 7.4 (0.01 M potassium phosphate buffer and 0.38 mg of oxaloacetate decarboxylase/ml) the enzyme loses one-third of its activity in 1 hr (25°). The enzyme is not inactivated at pH 5.1 during the purification. The chromatography was carried out at pH 6.8 which is the lower limit of calcium phosphate gel stability. In 0.1 M potassium phosphate buffer at pH 6.8, the dilute enzyme has been found to be stable overnight at 4°.

The enzyme was found to be inactivated extensively on chromatography (room temperature) on carboxymethylcellulose, carboxymethyl-Sephadex G-50, DEAE-cellulose, and DEAE-Sephadex A-25 even in presence of EDTA (disodium salt) and dithioerythritol.

The concentrations of salt also affect the stability of the enzyme. Dialysis against 10^{-3} M potassium phosphate buffer (pH 6.8) at 4° for 12 hr inactivated the enzyme, but it remained active when dialyzed at 4° against 10^{-2} M potassium phosphate buffer (pH 6.8) or when passed through Sephadex G-25 in this buffer. Under the best conditions the enzyme has been stored for a few weeks as $(NH_4)_2SO_4$ suspensions (70% saturated) or in 10^{-1} M potassium phosphate buffer (pH 6.8) at −15° in the presence of 10^{-5} M EDTA (disodium salt) and 10^{-5} M dithioerythritol. Even the crystalline suspension of enzyme was found to partially lose its activity, with a drop in specific activity from 1.7 to 1.0, when stored at 3° for 1 week in 60% saturated $(NH_4)_2SO_4$, 10^{-2} M potassium phosphate buffer (pH 6.8), 10^{-5} M dithioerythritol, and 10^{-5} M EDTA (disodium salt).

The activity of the enzyme has been found to be restored, however, when a solution of the purified enzyme (inactivated by storage) is incubated for 15 min at room temperature in 0.1 M solution of the disodium salt of EDTA, and the enzyme is then precipitated by the addition of $(NH_4)_2SO_4$ to 70% saturation.

Acknowledgments

I wish to thank Professor F. H. Westheimer for suggesting this problem and for his continuing interest in it. I gratefully acknowledge the skillful technical assistance of Mr. Jerome V. Connors in preparing the enzyme and Mr. Malcom Smart for ultracentrifuge measurements. I would also like to thank Mr. Burton Tinker of the U. S. Bureau of Fisheries in Gloucester, Mass., for a generous supply of freshly frozen cod, from which the enzyme was extracted.

References

Massey, V. (1960), *Biochim. Biophys. Acta 37*, 310.

Schmitt, A., Bottke, I., and Siebert, G. (1966), *Z. Physiol. Chem. 347*, 18.

Warburg, O., and Christian, W. (1942), *Biochem. Z. 310*, 384.