CRYSTALLIZATION AND SOME PROPERTIES OF METAPYROCATECHASE*

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Studies on the properties and mechanism of action of oxygenases have been greatly hampered because many oxygenases are too unstable to be extensively purified or prepared in crystalline form (Hayaishi, 1962). Metapyrocatechase which catalyzes the oxidation of catechol to α-hydroxymuconic semialdehyde was first discovered by Dagley and Stopher in an extract of a gram-negative organism (Dagley and Stopher, 1959). Some of the properties of a partially purified metapyrocatechase preparation have been reported (Kojima et al., 1961). The enzyme is extremely sensitive to oxygen and is readily inactivated in the presence of air. The activity of the inactivated enzyme is temporarily restored by the addition of certain reducing agents such as sodium borohydride (Taniuchi et al., 1962). The present paper describes the purification and crystallization of metapyrocatechase from cells of Pseudomonas arvilla and in addition, some of its properties.

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The bacteria were grown on a medium containing 0.25 % $(NH_4)_2HPO_4$, 0.15 % KH_2PO_4, 0.5 % NaCl, 0.02 % $MgSO_4 \cdot 7H_2O$, 0.05 % yeast extract and 0.3 % sodium benzoate for 20 hours at 30° with vigorous aeration. Forty grams of wet cells were suspended in 400 ml of 0.05 M phosphate buffer, pH 7.5, and disrupted by sonic oscillation at 10 kc for 10 minutes. The supernatant fluid (crude extract) was separated from the residue by centrifugation at 15,000 x g for 10 minutes. Although the crude enzyme preparation thus obtained was about 30 times more active than that reported by Kojima et al. (Kojima et al., 1961), its properties were quite similar to those previously reported, especially with respect to its sensitivity to oxygen. However, at a low concentration, an organic solvent such as acetone or ethanol was found to protect the enzyme almost completely from inactivation by air, even at room temperature (30°C). Therefore, both chromatography and crystallization procedures were carried out in the presence of 10 % acetone.

To the crude extract, 200-300 µg of deoxyribonuclease were added and the mixture was incubated for 15 minutes at room temperature. One volume of cold acetone was then added and the resulting precipitate was removed by centrifugation at 10,000 x g for 10 minutes. The same volume of acetone was added to the supernatant to give a final concentration of 66 % acetone. The resulting precipitate was collected by centrifugation and suspended in about 20 ml of 0.05 M phosphate buffer, pH 7.5, containing 10 % acetone (hereafter referred to as acetone-buffer). The insoluble material was removed by centrifugation and the clear supernatant solution was dialyzed against acetone-buffer overnight in a cold room. The dialyzed enzyme was applied to a DEAE-cellulose column (2 x 15 cm), which had previously been equilibrated with acetone-buffer. After washing the cellulose column with acetone-buffer, the enzyme was

eluted with a linear gradient established between acetone-buffer (500 ml) and acetone-buffer containing 5 % ammonium sulfate (500 ml). The enzyme was eluted at a concentration of between 1.5 and 2.5 % ammonium sulfate.

To the combined active fractions (320 ml), 2 volumes of cold acetone were added to precipitate the enzyme. The precipitate was collected by centrifugation at 10,000 x g for 10 minutes, and dissolved in a small amount of acetone-buffer (about 5 ml). Finely powdered ammonium sulfate was then gradually added to the solution until it became slightly turbid. White, needle-like crystals appeared after several hours at 0°. Recrystallization was carried out by repeating the crystallization process described above. The yield and specific activity obtained at each step of the procedure are shown in Table I.

The three times recrystallized enzyme preparation was completely homogeneous upon ultracentrifugation. The sedimentation constant ($S_{20,w}$) and diffusion constant ($D_{20,w}$) of the enzyme were 5.54×10^{-13} (cm/sec) and 3.92×10^{-7} (cm^2/sec), respectively. The molecular weight was calculated to be approximately 140,000 assuming a partial specific volume of 0.75.

Preliminary results obtained from colorimetric determinations with o-phenanthroline suggest that the enzyme contains one atom of iron per molecule of enzyme protein. However, the mode of binding of the iron is uncertain. Quantitative studies on the content of iron and other metals are now in progress. The absorption spectrum of the three times crystallized enzyme showed no significant peaks in the visible range and no evidence for the presence of heme was found.

Although the crystallization procedure represents only an overall purification of about 30 fold, the specific activity (116) and the molecular activity (16,000) are several times higher than

TABLE I

Purification of Metapyrocatechase

Enzyme fraction	Volume (ml)	Units*	Specific** activity	Recovery (%)
Crude extract	530	26,700	4.0	100
Acetone fraction	20	21,400	20.9	81.0
DEAE cellulose chromatography	320	13,700	105.0	51.7
First crystallization	5	6,000	110.0	22.4
Second crystallization	3	5,300	114.0	19.8
Third crystallization	3	4,000	116.0	15.0
Supernatant from third crystallization	3	500	102.0	2.0

* The assay was performed spectrophotometrically by measuring the increase in optical density at 375 mμ (Kojima et al., 1961). One unit of enzyme is defined as that amount which oxidizes 1 μmole of catechol per minute at 20°.

** Specific activity is defined as the number of enzyme units per mg of protein. Protein was determined by the method of Lowry et al. (Lowry et al., 1951).

those (20.0 and 1,600, respectively) previously reported for the most purified preparation of pyrocatechase which was homogeneous upon ultracentrifugation and electrophoresis (Hayaishi et al., 1954). The details of the properties of the crystalline metapyrocatechase will be reported elsewhere.

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