FINAL PURIFICATION OF A LATENT PHENOLASE WITH MONO- AND DIPHENOLOXIDASE ACTIVITY FROM TENEBRIO MOLITOR

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Although several purified preparations of an active tyrosinase from different sources have been described, only one paper deals with a nearly homogeneous preparation of the latent preenzyme. Karlson, Mergenhagen and Sekeris (1964) obtained a preparation the main component of which had a sedimentationcoefficieht $s_{20,w} = 15,4$ S.

Recently, Kertesz and Zito (1965) reported a purification of an active mushroom polyphenoloxidase with a molecular weight of $\pm$ 125 000. This enzyme contained 0.20 per cent of copper, exclusively or prevalently in the cuprous form.

In this paper we describe a procedure for the final purification of the protyrosinase from the mealworm, and some of its chemical and physical properties.

Methods.

Partially purified latent phenolase was prepared from living last instar mealworms using the procedure of Aerts and Vercauteren (1964). Conditions of activation and determination of activity have been reported previously (Heyneman and Vercauteren, 1964).

Protein was estimated with the biuret method of Robinson, et al. (1940) or in more purified fractions with a micromethod described by Mattenheimer (1960).

Total copper was determined with bathocuproinesulfonate (Fluka AG) after destruction and evaporation until dryness with perchloric acid in specially designed micro-Kjeldahl flasks of 1 ml. Further conditions for optimal results were based upon the method of B. Zak (1958). In this way down to 0.1 µg of copper could be detected. All determinations were done in duplicate and a standard curve was determined simultaneously under identical conditions. Cuprous copper was estimated by a modification of the method described by Poillon and Dawson (1963). Under our experimental conditions best results were obtained with the following final concentrations of the reagents : bathocuproinesulfonate, $4,5 \cdot 10^{-4}$ M; EDTA and p-chloromercuribenzoate, $1 \cdot 10^{-4}$ M.

Column electrophoresis on Sephadex G-25 was done with the LKB apparatus type 3340. Micro agar gel electrophoresis according to Wieme (1959) was carried out in 0.05 M phosphate buffer (pH 7.5) containing 0.05 M ammoniumsulfate, required to preserve the enzyme activity (Aerts and Vercauteren, 1964). The latent enzyme was activated by incubating the gel in the same buffer containing 30 per cent methanol and 1 µmole oleate/ml. Methanol was necessary for dissolving the activator as otherwise the micelles of oleate appeared to be unable to enter the agar gel. Under these conditions even a superactivation was obtained (Heyneman and Vercauteren, 1964).

Sedimentation ultracentrifugation was performed in the Spinco Model E ultracentrifuge equipped with schlieren phase plate optics.

Experimental Results and Discussion.

Purification procedure: Experiments of final purification started with a DEAE-preparation as developed in our laboratory by Aerts and Vercauteren (1964). We obtained an average specific activity of 70 µmoles O_2/min/mg N. Five ml of this partially purified latent enzyme, coming off the DEAE-column and containing 11.3 mg protein/ml in 0.066 M phosphate buffer (pH 7.5) and 0.066 M $(NH_4)_2SO_4$, were introduced at the anodic upper side of the electrophoresis column. Several experiments were performed in a number of buffer solutions at different pH values and concentrations, and with widely different currents. Conditions for optimal separation were: 0.015 M phosphate buffer (pH 7.5) containing 0.015 M ammoniumsulfate, 280 V, 42 mA, 48 h. The active fractions (7 to 11) were combined and brought up to saturation with ammoniumsulfate. The precipitate was redissolved in a minimal volume of 0.066 M phosphate buffer (pH 7.5) and 0.066 M ammoniumsulfate, the basic medium used in all further steps of operation. After removal of the excess sulfate on a Sephadex G-25 column, further fractionation was obtained by centrifugation in the Spinco ultracentrifuge on a sucrose density gradient using the swinging bucket rotor, SW 39 or SW 25 (for larger amounts). With the SW 39 rotor the gradient was made up by layering 1 ml aliquots of sucrose solutions (in the mentioned medium) of the following percentages: 10, 15, 20 and 25. Tubes were topped with 0.4 ml of the enzyme solution. After 7 h running at 120 000 g the bottom was punctured and the fluid was collected in several fractions of 10 drops each. Fig. 2 represents the distribution of protein and the enzymic activity measured after removal of sucrose by exhaustive dialysis against the usual buffer. Renewed sucrose gradient centrifugation showed only one protein peak con-

taining the entire enzyme activity. All steps of purification were performed at 4°.

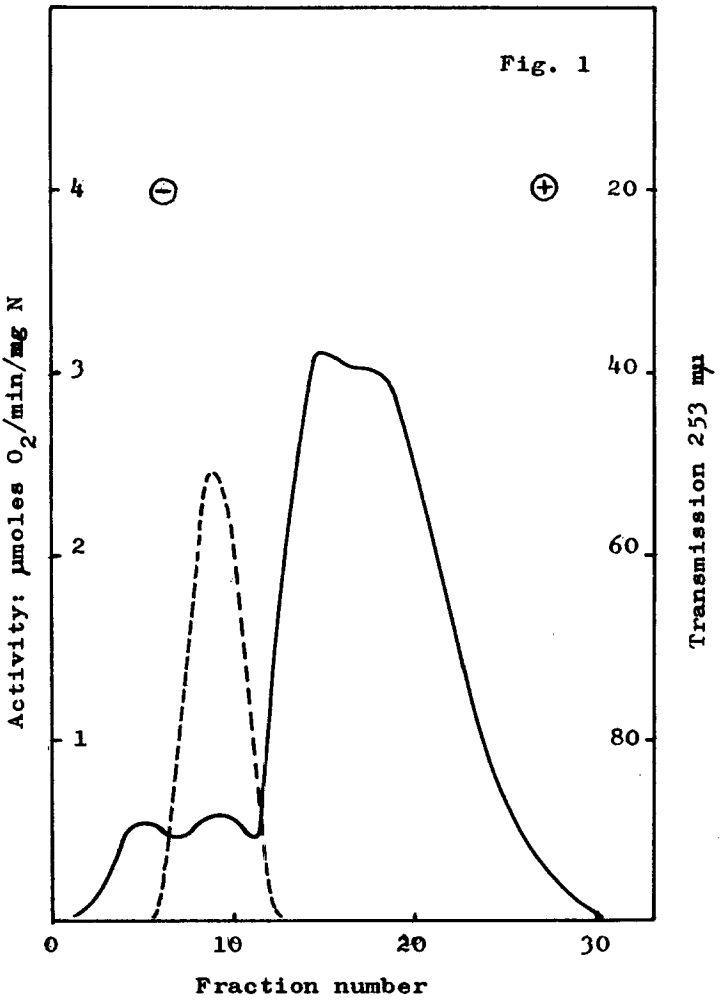


Fig.1. Column electrophoresis of partially purified protyrosinase after DEAE-chromatography. Initial concentration: 11,3 mg of protein per ml; 0.015 M phosphate buffer (pH 7.5) containing 0.015 M ammoniumsulfate; 280 volts; 42 ma. The elution curve after 48 h of running shows the % transmission at 254 mµ as recorded by the LKB Uvicord (solid line), and the enzymic activity (dashed line).

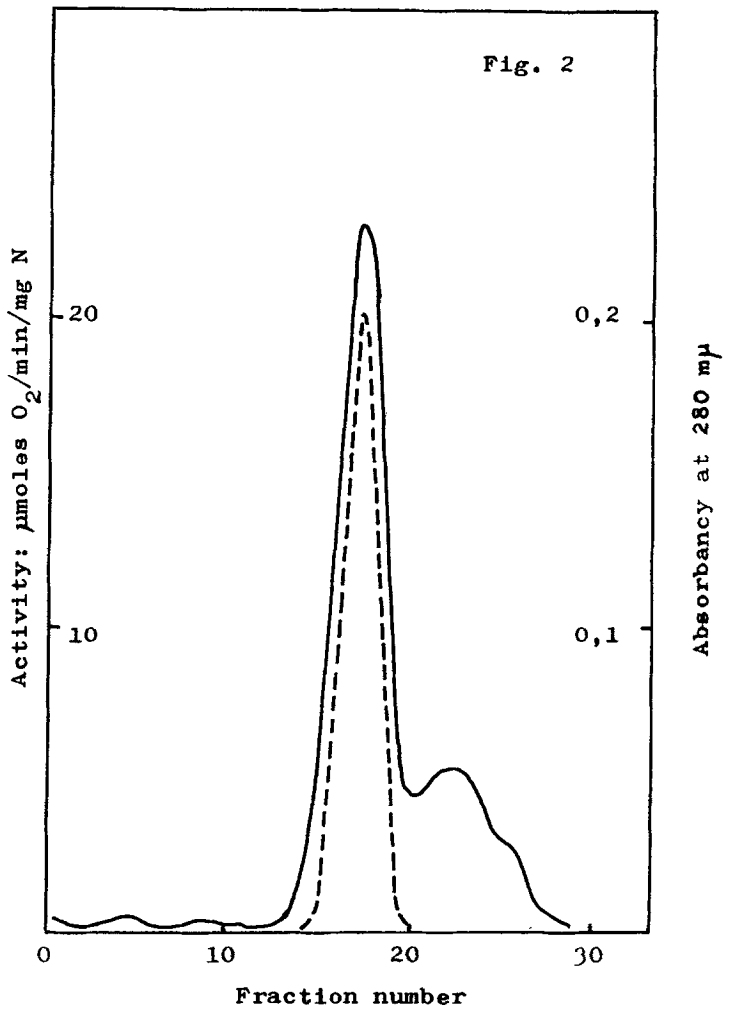


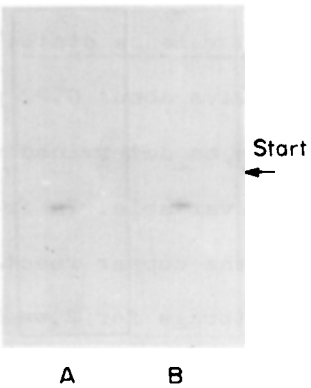
Fig.2. Sucrose density gradient centrifugation of highly purified protyrosinase after concentration with ammoniumsulfate and gelfiltration on Sephadex G-25. Sucrose gradient, 10 to 25 %; rotor speed, 39 000 rpm; time, 7 h; solid line, absorbancy at 280 mµ measured in the microcells of the Beckman DU spectrophotometer; dashed line, enzymic activity.

The specific activities (µmole O_2/min/mg N) measured at subsequent steps of the purification process are expressed in the following table:

Crude extract	7
DEAE-preparation	65
Electrophoresis preparation	546
Gradient preparation	710

The ratio cresolase/catecholase activity, determined at optimal conditions of activation and assay (pH 6.5), was 1/2.

Homogeneity tests: Fig.3 shows the electropherogram of the purified preenzyme. Only one protein band was observed which corresponded exactly to the darkbrown band resulting from the enzymic oxidation of dihydroxyphenylalanine.



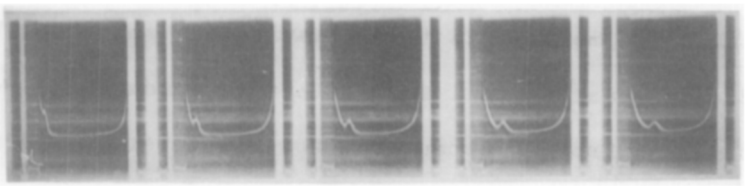


Fig.4

Fig.3. Electropherogram of latent phenolase after concentration in a special concentration cell (Colover, 1961). Conditions: 70 volts, 60 ma, 20 min. A: protein fraction coloured with amidoblack. B: enzymic activity with DOPA.

Fig.4. Sedimentation pattern of latent phenolase. Concentration 1.6 mg/ml. Solution in 0.066 M phosphate buffer (pH 7.5) and 0.066 M $(NH_4)_2SO_4$. Rotor speed 56 000 rev./min. The interval between the photographs is 8 min.

Another proof for the homogeneity of our preparation was furnished by analytical ultracentrifugation experiments. The protein fraction moved as a single component (Fig.4). Several determinations of $s_{20,w}$, extrapolated to zero concentration, gave a value of 7.3, which is in general agreement with the findings on mushroom tyrosinase by Bouchilloux et al. (1963) and Kertesz and Zito (1965), but differs from the value of 15.4 S obtained by Karlson et al. (1964) for the preenzyme of Calliphora larvae. It is noted, however, that their sedimentation pattern shows a minor component having $\sim$ 7 S, which might be the true preenzyme.

Copper content and valence state: The total copper content of our preparation was always about 0.22 per cent. The valence state of copper in the enzyme, as determined with bathocuproinesulfonate, was found to be highly variable. In fresh preparations, between 88 and 72 per cent of the copper reacted as if it were in the cuprous state. After storage for 2 weeks at 4°, a fraction of the metal, 53 per cent of the total was found to be Cu^{+}. However, the specific activity decreased with only 9 per cent.

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