Purification of laccase

Despite a relatively extensive literature, recently reviewed by BONNER[1], our knowledge of the chemical nature and mode of action of the copper-containing oxidases, tyrosinase and laccase, is rather fragmentary. One of the main causes of this has been the lack of well-defined preparations of these enzymes (*cf.* BONNER[1]). The earlier finding by one of us[2] that the mycelia of *Polyporus versicolor* can be induced to form large amounts of laccase as an exoenzyme offered a new possibility for the purification of this enzyme. As will be shown in the present communication, laccase constitutes the major protein component in the medium and, following ammonium sulfate precipitation, a further purification can easily be achieved by zone electrophoresis in cellulose columns[3,4] or by chromatography on a cellulose anion exchanger[5]. The enzyme so obtained appears homogeneous, as judged by electrophoresis, ultracentrifugation, end-group determinations, and copper analyses.

On the basis of results published earlier[2,6,7], the following procedure was adopted for the production of crude enzyme solutions. A strain of *Polyporus versicolor* Fries (No. 11a) was cultured on the following basal medium: glucose, 20 g; L-asparagine, 2.5 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2$, 0.01 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $MnSO_4 \cdot 4H_2O$, 0.001 g; $ZnSO_4 \cdot 7H_2O$, 0.001 g; $CuSO_4 \cdot 5H_2O$, 0.001 g; DL-phenylalanine, 0.15 g; thiamin·HCl, 50 μg; distilled water, 1000 ml. (pH about 5 after sterilization.)

1-l Erlenmeyer flasks, each containing 500 ml of this medium, were sterilized in the autoclave at 120° for 20 min. They were inoculated with a mycelial suspension obtained by shaking mycelia of *P. versicolor* with glass beads in a glass-stoppered bottle. The size of the inoculum corresponds to about 20 mg dry weight per flask.

The cultures were incubated on a rotary shaker (110 rev/min, 5 cm radius) at 24°. 4 days after inoculation an "inducer" was added, normally 2.5-xylidine (Eastman Kodak No. 758) at a concentration of $2 \cdot 10^{-4}$ *M*. This substance induces a strong formation of laccase, which is almost quantitatively secreted from the mycelium into the medium. Without this addition, the formation of laccase is negligible.

The oxidase activity of the medium was followed daily by colorimetric tests (catechol). When the maximum had been reached, usually after 2 or 3 days, the mycelia were filtered off, the filtrates being combined and cooled. The enzyme was precipitated by saturating the solution with solid ammonium sulfate. The precipitate, which rose to the surface as a compact layer, was easily separated from the solution. Excess solution was removed by centrifugation (1500 × *g*). The precipitate was finally dialyzed in the cold (4°) against several changes of dilute acetate buffer, pH 5 (0.01 *M*), during 2–3 days. After dialysis, the solution often showed a marked green color. The dialyzed solution was frozen, lyophilized, and the dry powder obtained (Fraction A) was used for further purification of the enzyme.

Fraction A was studied in paper electrophoresis at a number of different pH values (5.0, 6.8, and 8.2). Acetate, phosphate, and phosphate–borate buffers, all having $\mu = 0.05$, were used. In all three buffer systems, only two protein components were obtained. The activity could be localized in the paper by spraying with a catechol solution, and it was found that both protein spots possess oxidase activity. The two components were separated in cellulose column electrophoresis in an apparatus of the type described by PORATH[8]. A typical pattern for Fraction A is shown in Fig. 1; the experimental details are given in the caption. The samples in tubes Nos. 18–24 (Fig. 1) were

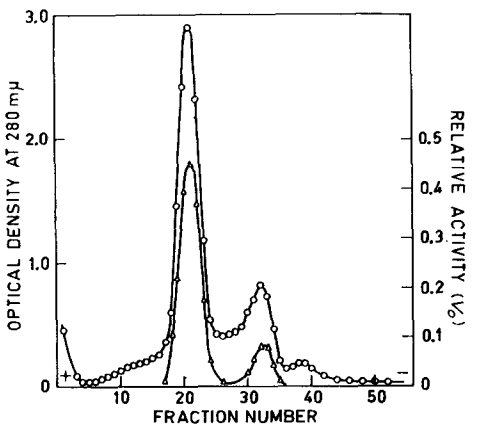

Fig. 1. Cellulose column electrophoresis of laccase (Fraction A). Column dimensions: 1.8 cm diameter, 50 cm length; buffer: phosphate, pH 6.8, $\mu = 0.05$; sample: 2 ml of solution having an optical density at 280 mμ of 95 (about 60 mg/ml); current: 15 mA; time: 33 h; fraction volume: approx. 3 ml. ○ = protein concentration, measured as optical density at 280 mμ; △ = enzyme activity. The activity was measured by adding 10 μl of sample to 3 ml of substrate in a 1-cm cuvette in a Beckman DU spectrophotometer and following the extinction change at 415 mμ as a function of time at 20°; substrate solution: 0.1 *M* catechol in phosphate buffer, pH 5.8, $\mu = 0.05$. The (+) and (—) at the bottom of the diagram indicate the position of the anode and cathode, respectively.

combined, dialyzed against water, and lyophilized (Fraction B). In general, only this main fraction has been studied further. The smaller component has a considerably lower specific activity, but it is impossible to say, as yet, if it constitutes a different enzyme or a form of microheterogeneity, often observed with enzymes[9,10].

Fraction A can also be purified by chromatography on the chloride form of DEAE cellulose[5]. A column having 1.5 cm dia. and 18 cm length was used. It was equilibrated with a 0.05 *M* tris-(hydroxymethyl)aminomethane(Tris)–HCl buffer, pH 7.3, and 60 mg of Fraction A, dissolved in 1 ml of the same buffer, were applied to the column. Stepwise elution was performed with the following concentrations of Tris: 0.05, 0.1, 0.2 and 0.3 *M*. The main component emerged at 0.2 *M* Tris, while the smaller component was not adsorbed even at the lowest ionic strength. The samples of zone emerging at 0.2 *M* Tris were combined, dialyzed against water, and lyophilized (Fraction C). In paper electrophoresis, fraction C showed the same mobility as fraction A.

Fraction B (or C) appears to represent pure laccase as evidenced by several criteria. The specific activity across the zone in Fig. 1 is constant. One component only is obtained in the ultracentrifuge ($S_{20} = 6.0$ s for a 0.8% solution). The protein yields only one N-terminal amino acid (glycine), as determined both with the phenylisothiocyanate and with the fluorodinitrobenzene method[11]. Fraction A contains more than 16% carbohydrate (determined by the orcinol method[12] with lactose as standard), while the carbohydrate content of Fraction B is less than 1%. The highest specific activity, expressed as Q_{O_2} with catechol as substrate[1], is 36000 μl/mg/h, which is 1.8 times higher than that of laccase prepared by the method of KEILIN AND MANN[13]. The copper content of Fraction B is 0.40% (analysis by the carbamate method[14]; the protein concentration was determined by micro Kjeldahl analysis, assuming a N content of 16%), *i.e.* 1.7 times that found in the preparation of KEILIN AND MANN[13]. The molecular weight has not yet been determined but the copper content, coupled with the sedimentation constant, makes it likely that the molecule contains 4 atoms of copper. Electron-spin resonance measurements have demonstrated the presence of Cu(II), but the spectrum shows two resonance lines, which may indicate that the copper atoms are not all bound in the same way. This question is being studied further. In addition, attempts are being made to isolate copper-containing fragments after partial hydrolysis with proteolytic enzymes.

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