PHENOL OXIDASE OF THE COTTON PLANT

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Phenol oxidases form a class of enzymes that is widely distributed in plants. The phenol oxidases of tea [1-3], the potato [4, 5], the apple [6, 7], tobacco [8], fungi [9], etc., have been studied. The enzyme has been isolated from various plant organs, as a result of which the number of known phenol oxidases has increased. They differ in molecular weight, amino-acid composition, copper content, and enzymatic properties.

A phenol oxidase is also present in the leaves of a perennial cotton plant of the species Gossypium hirsutum [10]. We have investigated young leaves of an annual cotton plant of this species (variety 108-F). The phenol oxidase from the leaves of the cotton plant was studied in planned fashion according to the periods of vegetation of the plant. The enzyme was not found in the seeds and in the seed leaves: It is formed and accumulates in the young leaves and, as we have found, possesses its maximum specific activity in the period of vigorous growth of the cotton plant (between pinching-out and July) [11]. Consequently, for a far-reaching investigation of the plant we took the leaves gathered in the pinching-out period and stored them in a freezing chamber at −20° C. The phenol oxidase was extracted from the leaves with citrate−phosphate buffer, pH 7.8-8.0, in the presence of various reducing agents of phenols [12, 13]. The best results were obtained on extraction with a 1% solution of ascorbic acid. Then the solution was dialyzed against 0.001 M phosphate buffer, pH 7.8-8.0 and was chromatographed twice on a column of DEAE-cellulose.

After lyophilization, the preparation was light yellow. On rechromatography on DEAE-cellulose it gave one symmetrical peak coinciding with the activity peak. On ultracentrifugation no peak of a sedimenting substance was found by the rate of sedimentation method, while by the method of unestablished equilibrium its molecular weight was found to be about 11,700.

Disk electrophoresis in 15% gel at pH 8.9 of the enzyme obtained showed two protein bands, the less mobile band being considerably stronger. Both bands were more active when the gel was incubated in a solution of 3,4-dihydroxyphenylalnine (DHPA). The preparation of phenol oxidase investigated was incubated in a 2% solution of sodium dodecyl sulfate (SDS) at 37° C for 2 h, and was then subjected to disk electrophoresis in 15% gel. This gave one broad band migrating with the Bromophenol Blue label. The electrophoresis of this solution in a 30% gel gave one narrow protein band migrating approximately one third of the whole length of the gel. These results indicated the existence of a quaternary structure of the phenol oxidase.

To confirm the results obtained, a solution of phenol oxidase after incubation in 2% SDSNa was cooled in a refrigerator for the partial elimination of the SDS. The precipitate of SDS that deposited was separated off by centrifuging, and the supernatant liquid was studied electrophoretically in 15% gel at pH 8.9. Two protein bands were marked: one migrating with the dye and the other in the upper part of the gel.

The solution used for the experiments after the partial elimination of the SDSNa was centrifuged on an ultracentrifuge (Fig. 1).

The molecular weight of the substance shown in Fig. 1b was 5300 which again confirms the existence in the enzyme of two subunits with identical or very close molecular weights.

The amount of copper in the phenol oxidase was determined spectrophotometrically [14]. Analysis was performed twice with different amounts of protein. The copper content was approximately 0.9%. Calculated from the copper content, the minimum molecular weight is about 7000, i.e. there is one copper ion to one subunit of the enzyme.

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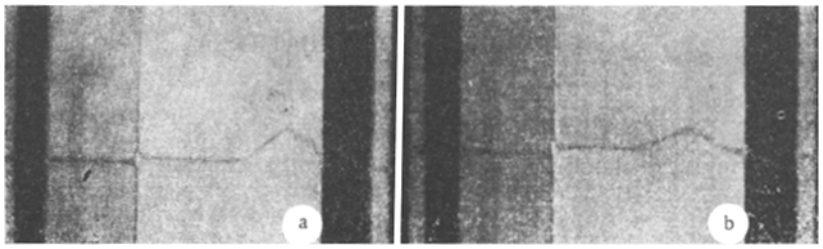

Fig. 1. Ultracentrifugation of the phenol oxidase of the cotton plant after incubation in 2% SDS and partial elimination of the SDS: a) beginning of centrifugation; b) end of centrifugation.

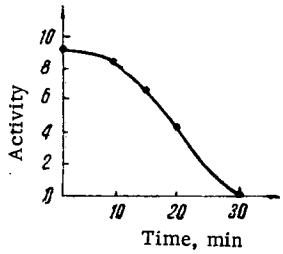

Fig. 2

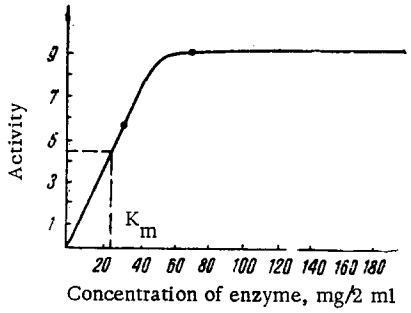

Fig. 3

Fig. 2. Stability of the phenol oxidase at 70° C.

Fig. 3. Dependence of the activity of the phenol oxidase on the concentration of catechol.

The results of an investigation of the dependence of the activity of phenol oxidase on the pH showed that the enzyme has a pH optimum at 7.6-7.8. In this range there are two temperature optima (at 30 and at 60° C); the activity at 60° C is 2.5 times greater than at 30° C. The increased activity of phenol oxidase at 60° C enabled us to investigate its stability at 70° C (Fig. 2). It can be seen from Fig. 2 that the enzyme is inactivated with time, and on incubation for 30 min it loses its activity completely.

In a study of the dependence of the activity on the concentration of phenol oxidase we found that with an increase in the concentration of protein from 0.02 to 0.2 mg/ml the relationship concerned is linear.

The value of K_m, approximately $2.3 \cdot 10^{-4}$ M, was determined from a graph (Fig. 3). The substrate specificity was investigated with very different phenols; however, the phenol oxidase exhibited activity only with respect to catechol and DHPA, its activity with respect to DHPA being twice that for catechol. Consequently, this phenol oxidase belongs to the orthodiphenol oxidases (EC 1.10.3.1).

EXPERIMENTAL METHOD

Determination of Activity. A. According to Drawert [15]. To 2 ml of a 10% solution of catechol were added 2 ml of a 2% solution of proline and 1 ml of enzyme solution. After 20 min, the reaction was stopped by the addition of 0.5 ml of a 5% solution of TCA. The intensity of color was determined on photoelectric colorimeter (violet filter). A similar mixture was used as the standard solution, but 1 ml of 0.1 M phosphate buffer, pH 7.8, was used instead of 1 ml of the enzyme solution. As the unit of activity we took a change in absorption by 0.001 in 1 min. All the solutions were prepared in 0.1 M phosphate buffer, pH 7.8.

B. Polarographic method. The activity of the enzyme was determined in a polarographic cell of the design due to Shol'ts and Ostrovskii [16]. A Czech polarograph, type LP-7, was used. The solution consisted of a mixture of 1 ml of a 0.05 M solution of the substrate, 0.5 ml of enzyme solution, and 1.5 ml of 0.1 M phosphate buffer, pH 7.8.

Isolation ond Purification. The lyophilized leaves (23 g) were extracted with 1150 ml of 0.01 M citrate–phosphate buffer, pH 7.8-8.0, containing 1% of ascorbic acid and 50 g of Kapron [polycaproamide] powder. The mixture was filtered through a layer of absorbent cotton and then through a layer of filter

paper. The green-pink extract was dialyzed against 0.001 M phosphate buffer, pH 7.8-8.0, until the dialysis solution no longer absorbed at 260 nm. The dialyzate was deposited on a column (4 × 40 cm) of DEAE-cellulose equilibrated with the same buffer. The rate of flow of the solution in the deposition of the protein on the column was 60 ml/h. The protein was sorbed as a narrow band. It was eluted in a potassium chloride gradient. The rate of elution was 50 ml/h. The fraction with the highest specific phenol oxidase activity was redialyzed against 0.001 M phosphate buffer, pH 7.8-8.0, and was deposited on a column (1 × 27 cm) of DEAE-cellulose equilibrated with the same buffer. Elution was performed in a potassium chloride gradient. The rate of elution was 12 ml/h. The concentration of protein was determined by the biuret method and from the absorption at 280 nm.

Electrophoresis. Disk electrophoresis was performed in 15% and 30% polyacrylamide gels at pH 8.9. The substance was revealed with a 0.25% solution of Coomassie Blue. The substrate activity was determined by incubating the gel in 0.001 M DHPA solution at room temperature for 2 h. Before development, the gel was fixed in 80% ethanol cooled to +4° C for 20 min.

Determination of Copper. The protein was dissolved in distilled water which had been redistilled in a glass apparatus with a quartz condenser and was dialyzed against the same water for two days. Then the protein solution obtained was lyophilized. After this, 1.5 mg of the protein was dissolved in a quartz test tube in a mixture of 1 ml of concentrated cp H_2SO_4 and one drop of concentrated cp HNO_3. The solution was placed in an oven at 120° C for 3 h. After digestion, the total volume of the solution was brought to 10 ml with distilled water, and 5 ml of this solution was diluted to 30 ml, mixed with 5 ml of 40% citric acid, and then made alkaline with concentrated ammonia solution. To this mixture was added 10 ml of a 0.1% solution of sodium diethyldithiocarbamate which had been twice recrystallized from ethanol. The mixture was shaken well and was extracted with CCl_4 (cp 4 × 2.5 ml). After the extract had been dried over anhydrous sodium sulfate, the absorption at 440 nm was measured. The amount of Cu^{2+} in the solution was determined from a standard curve obtained under the same conditions as the sample using the same solutions.

For a check we determined the amount of copper in the remaining 5 ml, as well. Then a second experiment was performed with a 2-mg sample. The mean copper content in 1 mg of protein was derived from the results obtained. It was approximately 9.1 μg, which corresponds to about 0.9% of copper in the protein.

Determination of K_m and Study of the Stability of the Enzyme at 70° C. To determine K_m we studied the dependence of the activity of the phenol oxidase on various concentrations of catechol by Drawert's method. Then we found the concentration of catechol at which the rate of the reaction was half the maximum. The stability of the phenol oxidase at 70° C was studied by incubating the enzyme for a predetermined time in the water bath and, after bringing the temperature to that of the room in an ice bath, determining the activity (Drawert's method).

Ultracentrifugation was performed by the method of an established equilibrium at 22,000 and 8000 rpm on an MOM 3170 ultracentrifuge.

SUMMARY

An investigation of the phenol oxidase present in the leaves of the cotton plant according to its vegetation periods has been performed. From the leaves at the period of maximum phenol oxidase activity so found an enzyme with a molecular weight of 11,700 has been extracted, the homogeneity of which was shown by the rechromatography of DEAE-cellulose, ultracentrifugation, and disk electrophoresis.

2. It has been established that the enzyme has a quaternary structure.

3. The properties of the phenol oxidase have been studied. It belongs to the class of orthodiphenol oxidases (EC 1.10.3.1). It contains approximately 0.9% of copper.

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