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CATECHOL OXIDASE: ENZYMIC LIBERATION FROM SUGAR BEET CHLOROPLASTS

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Abstract—Catechol oxidase present in isolated sugar beet chloroplasts is activated by means of proteolytic enzymes. The most effective treatment is a combination of trypsin and carboxypeptidase a. This gives a fourfold activation and releases the catechol oxidase from its bound form. The molecular weight of the enzyme liberated in this way is of the order of 10,000. Possible location of the enzyme on membranes is discussed.

INTRODUCTION

ISOLATED chloroplasts of sugar beets contain a catechol oxidase (E.C. 1.10.3.1.).[1] In order to study this enzyme it is essential to liberate the enzyme from the chloroplasts. Although this enzyme can be activated by a variety of detergents, previous attempts to solubilize it have been unsuccessful.[2]

In contrast, the enzyme present in tobacco leaves[3] and tea-shoot tips[4] can be solubilized and extracted fairly readily. Latency of catechol oxidase in bean leaves has been reported by Kenten[5,6] and liberation of the latent enzyme has been ascribed to changes in tertiary protein structure.[7,8] The phenomenon of latency of catechol oxidase in certain animal tissues and their release by enzyme action has been reported.[9,10] Mayer[2] suggested that the catechol oxidase present in sugar beet chloroplasts is bound tightly to a membrane structure and activation involves release from or breakage of this structure. Gressel and Avron[11] were able to demonstrate the destruction of the photoreductive and photophosphorylative activity of isolated Swiss chard chloroplasts by sonication, pancreatic lipase and phospholipase A (cf. Ref. 12). Since this is probably due to damage of certain structures within the chloroplasts, it was decided to attempt to liberate the catechol oxidase by similar means.

RESULTS AND DISCUSSION

Sonication of chloroplast suspension for periods from 30 sec to 4 min failed to activate or solubilize the enzyme. Four ml of suspensions were used for these experiments and the

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sonicator employed at maximum output. Treatment of chloroplast suspensions with between 50 and 500 μg lecithinase D for 1 hr in the presence of 0·1 M $CaCl_2$ and 0·05 ml ether/ml at pH 5·6 in acetate buffer failed to activate or solubilize the enzyme. No activation was obtained when chloroplast suspensions were treated with wheat germ lipase at pH 7·4 for up to 1 hr at concentrations between 250 μg and 4 mg/ml of chloroplast suspension. Similarly, treatment with emulsin, 800 μg/ml at pH 5·6, lipoxidase, 100 μg/ml at pH 6·5, or β-galactosidase, 500 μg/ml at pH 7·4 for 1 hr, failed to induce solubilization or activation of the catechol oxidase present in the chloroplasts. Various combinations of these treatments were also ineffective. None of the treatments had any effect on enzyme activity.

Thus enzymes, which attack the lipids of chloroplast membranes, are unable to either activate or liberate the catechol oxidase present in sugar beet chloroplasts. However when the chloroplasts were treated with pancreatin, both activation and solubilization was achieved (Table 1). Numerous repetititions of this experiment gave similar results. Activation was 2–4-fold depending on the amount of pancreatin used and the time of treatment. About half of the total activity after activation was released into 100.000 *g* supernatant. On fractionation

TABLE 1. EFFECT OF PANCRLATIN ON ACTIVATION AND SOLUBILIZATION OF CATECHOL OXIDASE ACTIVITY OF ISOLATED CHLOROPLASTS

Treatment	Activity/ml original chloroplast suspension	Activity of supernatant after 100,000 *g*/ml of original chloroplast suspension
Control	64	3·0
Pancreatin treated 48 mg/ml chloroplast	248	152
Pancreatin treated 24 mg/ml chloroplast	206	130

Preparation contained 0·43 mg chlorophyll/ml. Chloroplasts were diluted with 3 ml of buffer 0·1 M phosphate pH 6·3. Incubation 1 hr at room temperature. Substrate 4-methylcatechol 5·0 mM.

of the soluble enzyme with ammonium sulphate, about two-thirds of the activity precipitated between 0 and 40 per cent saturation and one-third between 40 and 75 per cent saturation.

Electrophoresis on starch gel of the two ammonium sulphate fractions, at pH 7·2, using tris-buffer, 0·04 M, 7·5 V/cm, resolved each fraction into two bands. One band showing high catechol oxidase activity moved very slightly to the anode and a second band, showing low activity, moved far out to the cathode. Clearly ammonium sulphate fractionation was not effective in separating these two activities.

Separation of the enzyme, solubilized by pancreatin treatment, by thin-layer gel filtration on Sephadex G 200, superfine, at pH 6·4 in 0·04 M phosphate buffer indicated that the enzyme so prepared had a molecular weight of between 30,000 and 60,000. As markers for estimating molecular weight mushroom polyphenol oxidase (Worthington), cytochrome *c* (Sigma, type II) and purified apple chloroplast catechol oxidase,[13] were used. In addition, in other experiments in this laboratory, estimates of molecular weight obtained from Sephadex gel filtration, were compared with values obtained from Sephadex filtration in columns, using as additional markers crystalline Bovine serum albumin (Sigma) and crystalline soybean trypsin inhibitor. These results confirmed the estimates of molecular weight obtained by thin-layer

[13] E. HAREL, A. M. MAYER and Y. SHAIN, *Phytochem.* 4, 783 (1965).

gel filtration. The catechol oxidase was detected by a reaction with substrate used for electrophoresis[13] and cytochrome *c* was detected by its characteristic colour on the plates.

The fractionations obtained with ammonium sulphate were not clean and the Sephadex separations were not sufficiently clear. The enzyme was dark brown in colour and contaminated with products of secondary reactions due to the pancreatic digestion of chloroplast proteins. This brown coloration could only be partially removed by dialysis, during or immediately after treatment.

Since pancreatin contains a mixture of various enzymes, it was decided to clarify which was the active part of the pancreatin. For this reason the effect of trypsin, chymotrypsin, lipase and carboxypeptidase on the catechol oxidase was studied. Incubation of the chloroplast for 1 hr with trypsin resulted in liberation of the enzyme into the supernatant and appreciable activation of between one and a half- to three-fold depending on conditions. Addition of lipase to the reaction mixture did not modify the effect of trypsin alone. Treatment of chloroplasts with α-chymotrypsin alone caused some solubilization but did not

TABLE 2. EFFECT OF TRYPSIN AND CARBOXYPEPTIDASE a ON ACTIVATION AND SOLUBILIZATION OF CATECHOL OXIDASE ACTIVITY OF ISOLATED CHLOROPLASTS

Treatment	Activity/ml original chloroplast suspension	Activity of supernatant after 100,000 *g*/ml of original chloroplast suspension
Trypsin 1 mg/ml chloroplasts	240	172
Carboxypeptidase a 7·0 mg/ml chloroplasts	76	20
Trypsin + carboxypeptidase	280	200
Trypsin followed by carboxypeptidase	316	216
Control	96	—

Chloroplast preparation contained 0·67 mg chlorophyll/ml and 2·2 mg/ml protein. 1 ml chloroplasts was diluted with 3 ml buffer, pH 6·3, 0·1 M phosphate, 1·0 M NaCl. Period of incubation 1 hr at 37°. In case of trypsin followed by chymotrypsin incubation was 1 hr followed by a second hour. Substrate 4-methylcatechol 5·0 mM.

activate the enzyme. In combination with trypsin, results were obtained essentially similar to those obtained by trypsin alone. When chloroplasts were treated with carboxypeptidase a, the catechol oxidase activity of the chloroplasts was reduced, but appreciable solubilization was obtained. Treatment with trypsin and carboxypeptidase simultaneously, or successively, resulted in increased activation and better solubilization (Table 2). Some activity invariably remained in the precipitates and could not be solubilized.

Thus a combination of trypsin and carboxypeptidase gave essentially the same degree of activation and solubilization of the catechol oxidase in the chloroplasts as incubation with pancreatin. The soluble enzyme prepared by treatment either with trypsin, carboxypeptidase or combinations of them precipitated from the solution on saturation with ammonium sulphate, between 33 and 66 per cent. The liberated enzyme moved as a single band towards the anode upon electrophoresis at pH 7·2.

The solubilized enzymes obtained by treatment with trypsin, carboxypeptidase or carboxypeptidase and trypsin were fractionated with ammonium sulphate and the fraction obtained in each case between 33 to 66 per cent saturation examined using Sephadex G 200

superfine gel filtration. Conditions were the same as previously described and the same markers were used. The fraction obtained by ammonium sulphate precipitation after treatment with either trypsin or carboxypeptidase showed the presence of three active zones, one with a molecular weight of around 12–16,000, a minor one of molecular weight above 200,000 and one moving only very slightly, indicating low molecular weight peptides on the thin-layer plates. The ammonium sulphate fraction obtained from the supernatant after treatment with both trypsin and carboxypeptidase gave the same zones on the Sephadex as those obtained by either alone, but in addition an active zone appeared with molecular weight of between 5–10,000.

From this work a number of conclusions can be drawn. In order to effectively liberate and activate catechol oxidase from chloroplasts the enzyme must be cleaved at various points. The linkages cleaved are, in the first instance, those involving peptide bonds of non-aromatic amino acids. The enzyme can be degraded appreciably and still retain considerable activity. Molecular weights of about 10,000 are much smaller than those reported till now for the single polypeptide chains constituting part of the catechol oxidase tetramer.[14,15] The results are fully consistent with the previous suggestion[2] that the enzyme is linked to some membrane structure in the chloroplast. It can be removed from this structure by cleavage with proteolytic enzymes. These presumably leave part of the peptide chain attached to the membrane, but remove the bulk of the enzyme, which is then randomly broken down. Carboxypeptidase a can further degrade the enzyme. The active centre of the enzyme seems to be fairly resistant to attack by trypsin. Possibly attack by carboxypeptidase on the enzyme, while still attached to the membrane, impairs its activity, but some solubilization can be achieved. The implication of this form of enzyme release to the normal function of catechol oxidase in intact plant tissues remains to be elucidated.

EXPERIMENTAL

Chloroplasts were prepared as previously described[16] in the presence of 0·01 M sodium ascorbate. Catechol oxidase activity was determined using an oxygen polarographic electrode.[17] Results are expressed as relative units of activity/ml of original chloroplast suspension/minute.

The following enzyme preparations were used for treatment of the chloroplasts: Trypsin (Sigma bovine pancreas Type II 3333 BAEE units/mg); Pancreatin (Sigma porcine grade VI having trypsin activity of 116 BAEE units/mg); Wheat germ lipase (Sigma type II); Lecithinase D (Sigma type II); Lipoxidase (Sigma); β-Galactosidase (Sigma grade III); Emulsin (M.B. Co.); Chymotrypsin (Sigma bovine pancreas 3000 ATEE units/mg); Carboxypeptidase a (Sigma 2 × crystallized, 35 units/mg).

For treatment, the chloroplasts were incubated with enzyme preparation, after dilution with a suitable buffer (see Text and Tables 1 and 2). No catechol oxidase activity was found in any of the enzymes used. As a control chloroplasts were incubated without the addition of enzymes.

If the enzyme was still present in the supernatant after centrifugation at 100,000 *g* for 1 hr, it was regarded as solubilized. An additional criterion for solubility was the ability to

[14] M. FLING, N. H. HOROVITZ and S. F. HEINEMANN, *J. Biol. Chem.* **238**, 2045 (1963).
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[17] E. HAREL, A. M. MAYER and Y. SHAIN, *Physiol. Plantarum* **17**, 921 (1964).

precipitate the enzyme from solution with ammonium sulphate and redissolve the precipitate in 0·1 M phosphate buffer, pH 6·3.

Sonication of the chloroplast was carried out using a Branson sonic ultra-sonicator Model S 110, 20 kc/s.

Thin-layer gel filtration on Sephadex G 200 superfine was carried out according to Andrews.[18] The Sephadex gel was imbibed in 0·04 M phosphate buffer, pH 6·4, for 3 days. It was spread as a 0·5 mm layer in the plates and phosphate buffer, pH 6·4, run through the layer for 18–20 hr to equilibriate the system. This was done in a moist chamber at 20° inclination. The samples were then applied and the plates developed, with the same buffer for 4–6 hr using a flow rate of 2–2·5 cm/hr. The presence of the enzyme on the plates was detected by spraying the plates with substrate, as described by Harel *et al.*[13]

Electrophoresis on starch gel was carried out as described by Harel *et al.*[13]

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