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ISOLATION AND CHARACTERIZATION OF CATECHOL OXIDASE FROM *SOLANUM MELONGENA*

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Key Word Index—*Solanum melongena*; Solanaceae, eggplant, catechol oxidase; brinjal; enzyme; purification and properties.

Abstract—Catechol oxidase was distributed in soluble and particulate fractions of *Solanum melongena*. The purified preparation appears to be homogeneous by polyacrylamide gel electrophoresis. The enzyme shows two pH maxima—with catechol, 6.5 and 7.5; and with dopa, 6.5 and 7.9. The latent form of the enzyme does not occur in *S. melongena*. The preparation resembles the enzyme from other sources in substrate specificity towards various mono- and diphenols, having a higher affinity for catechol than dopa; this tendency increases on purification. The cresolase activity decreases with purification and a lag period with *p*-cresol is observed. The oxidation of mono- and diphenols is inhibited by ascorbic acid, sulphydryl compounds and chelating agents.

INTRODUCTION

Catechol oxidase, CO (*o*-diphenol: O_2 oxidoreductase, EC 1.14.18.1) catalyses the aerobic oxidation of various mono- and diphenols to produce pigments. CO has been purified and characterized from a number of sources [1–5]. In most of these CO exists in particulate forms [6, 7], although the extent of binding differs. The latent form of CO has also been reported [8–10]. In the present study the CO from *Solanum melongena* was purified to apparent homogeneity and compared with the CO from other sources.

RESULTS AND DISCUSSION

Considerable CO activity was found in the soluble fraction obtained after centrifugation at 20 000 g for 30 min at 0° and the activity in crude extracts increased when treated with 2% (v/v) Triton X-100 (Table 1). After adding Triton X-100, the extract was subjected to $(NH_4)_2SO_4$ fractionation followed by chromatography on Sephadex G-200 column. The enzyme was eluted just after the void volume of the column as a single peak. On disc electrophoresis with 7.5% polyacrylamide gel at pH 9 a single protein band was observed. On incubation with dopa in 50 mM phosphate buffer, pH 6.5, the CO could be detected only in the protein corresponding to the stained band. Poux [11] has reported the particulate nature of CO in grapes and observed that the greater part of the activity precipitates on centrifugation at 15 000 g for 6 min. It is apparent that the enzyme is located in plastids [4]. In spinach beet, the enzyme is closely associated with the chloroplast lamellae [12].

pH-Activity curve

The activity of CO towards catechol and dopa was investigated as a function of pH over the range 3–9 (Fig. 1). With catechol, the CO shows two pH maxima at 6.5 and 7.5; however, when the substrate was dopa, the pH maxima were 6.5 and 7.9. It seems that CO of *S. melongena* exists in two catalytically active forms. Two pH maxima have also been reported for CO of Red Delicious apple peel [3], grapes [4], potato [13] and for partially purified phenolase from potato pulp tissue [14]. For most of the studies of this CO pH 6.5 was chosen.

UV spectrum

CO in 0.1 M phosphate buffer, pH 6.5, exhibited a typical protein spectrum with an additional shoulder at *ca* 285 nm. The spectrum was similar to that reported for mushroom CO [1, 15]. However, in the present preparation the shoulder was observed at 285 nm instead of 290 nm reported for the mushroom enzyme. There was no significant absorption above 300 nm.

Stability in the presence of detergents

Storage of crude extracts in the absence and presence of different detergents at 0° for 35 days shows a fast decrease in CO activity for 5 days, except with SDS with which comparatively less inactivation was found (Fig. 2). The gradual decrease in activity continues up to 35 days when the experiment was terminated. The stored extracts darkened with time and there was a precipitate after 1 week but no sign of microbial growth or decomposition [8]. Although SDS protected the CO, it could not be used for the extraction because it has been reported to bind with the proteins [16]. The initial fast inactivation of CO on storage might be due to the irreversible binding of the oxidized products to the enzyme protein [17]. The

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Table 1. Purification of catechol oxidase from *Solanum melongena*

Step	Total protein (mg)	Total activity (μkat)	Specific activity (μkat/mg)	Purification (fold)	Recovery (%)
Particulate fraction	1250	208	0.166	1.0	100
Triton X-100 extract	1310	308	0.235	1.4	148
Ammonium sulphate fraction	140	139	0.99	6.0	67
Elution on Sephadex G-200	9.6	54	5.6	33.9	26

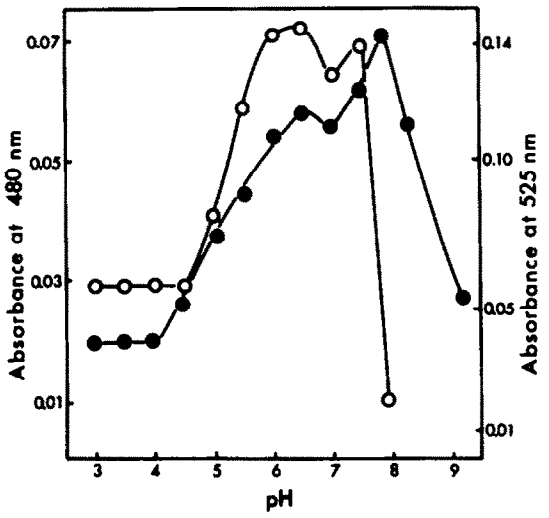

Fig. 1. Effect of pH on the catalytic activity of catechol oxidase. Enzyme preparation was 40% ammonium sulphate fraction dissolved in 0.1 M phosphate buffer, pH 6.5 and dialysed against the same buffer. ○, Activity with catechol (10 mM) as substrate; ●, dopa (8 mM) as substrate.

oxidized products are themselves formed by the oxidation of endogenous substrates as evident by the darkening of the crude extract with time. At a later stage, the activity becomes stable, probably due to exhaustion of the endogenous substrates. No increase in the initial activity of CO on storage indicates that the latent form of the enzyme does not occur in *S. melongena*. Contrary to our observations, the latent form of the enzyme has been reported in beet leaf chloroplasts [12], bananas [18], broad-bean leaves [9, 10], and also in certain insects [19]. Keilin and Mann [20] reported an increase in CO activity in mushroom enzyme preparations and suggested that the increase might be due to liberation of an active enzyme from an inactive complex. SDS activated the latent enzyme in broad beans [21] and cultivated mushrooms [8].

Substrate studies

The relative activity of the CO towards different mono- and diphenols using both crude and purified preparation was studied (Table 2). For diphenols, the activity was greatest with catechol followed by dopa and chlorogenic acid. Little activity was found with pyrogallol and hydroquinone. With monophenols, the CO was active only towards *p*-cresol, whereas with *m*-cresol, tyrosine and tyramine little activity was observed both in crude and purified preparations. The CO from *S. melongena* resembles the enzyme from other plant sources in the substrate specificity towards various mono- and diphenols [4, 20, 22, 23], in contrast to mammalian tyrosinase where the substrate specificity is highly restricted [24]. The enzyme hydroxylates only *p*-cresol and *o*-diphenols to corresponding quinones while *p*-diphenols such as hydroquinone are not oxidized. The cresolase activity of the enzyme decreases with the purification of enzyme. This is supported by our earlier observation [25] that the cresolase site of the enzyme is more labile than that for catecholase, and so becomes inactivated with purification of the enzyme. The enzyme shows a lag

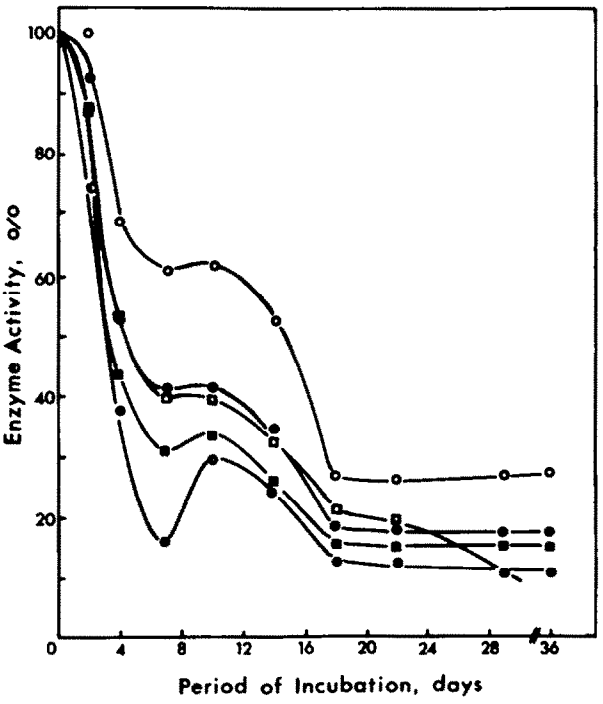

Fig. 2. The stability profile of catechol oxidase in the presence and absence of detergents. Homogenates were prepared in 0.05 M phosphate buffer, pH 6.5 containing 2% (v/v) of Triton X-100, Tween-20, Tween-80, and 0.2% (w/v) SDS. The resultant slurry was filtrated through cheesecloth and centrifuged at 4000 rpm for 25 min at 0° and stored at this temperature for 35 days when the experiment was terminated. The enzyme activity was measured using dopa as described in the text. □, Control; ○, SDS; ■, Tween-20; ●, Tween-80; ◐, Triton X-100.

Table 2. Substrate specificity of catechol oxidase

Substrates	Relative activity (%)* Crude extract	Purified enzyme
Diphenols		
Catechol (10 mM)	100	100
Dopa (8 mM)	58.8	46
Chlorogenic acid (2.5 mM)	19.5	—
Pyrogallol (10 mM)	10.5	4.6
Hydroquinone (10 mM)	1.6	2.7
Monophenols		
p-Cresol (10 mM)	100	100
m-Cresol (10 mM)	0.5	0
Tyrosine (2 mM)	0	0
Tyramine (2 mM)	0	0

* 100% corresponds to sp. act. of 1.2 mmol/min/mg protein.

The concentration of substrates are shown in parentheses. Per cent activity is expressed for di- and monophenols with respect to catechol and *p*-cresol respectively. The enzyme activity was measured in 0.1 M phosphate buffer, pH 6.5 at 37° as described in the text.

period when the substrate is *p*-cresol and the activity increases as the product is accumulated. This is probably due to the fact that the cresolase activity is catalysed by its own products [26].

Inhibitor studies

The effect of various inhibitors on crude and purified enzyme preparation was determined (Table 3). Thiol compounds like cysteine, 2-mercaptoethanol, 2,3-dimercaptopropanol and reduced glutathione inhibit the CO almost completely. EDTA shows no inhibition in a crude CO preparation. The inhibition by sodium azide, thiourea and ascorbic acid is greater on purified enzyme than on crude preparations. The inhibition caused by ascorbic acid might be due to the reduction of dopaquinone to dopa. The quinones are known to react with the sulphydryl compounds; however, Lerner *et al.* [27] observed that the thiol compounds exert most of their inhibitory action by combining with the copper required for enzymatic activity, which could be reversed by the addition of an excess of cupric ions. Seiji *et al.* [28] have shown that glutathione on combination with dopaquinone, an intermediate of tyrosinase–tyrosine reaction, forms new compounds and thereby inhibits the formation of dopachrome and melanin.

Table 3. The effect of various inhibitors at 2×10^{-3} M on catechol oxidase activity

Inhibitors	Inhibition (%)* Crude extract	Purified enzyme
Control	0	0
Ascorbic acid	62.3	78.1
Cysteine	98.2	95.2
Glutathione reduced	99.2	96.3
2-Mercaptoethanol	99.2	93.1
2,3-Dimercaptopropanol	94.6	93.1
Thiourea	29.9	36.4
Sodium azide	12.8	23.6
EDTA	0	3.2

* The enzyme was preincubated with inhibitor at 37° for 30 min.

The enzyme activity was measured in 0.1 M phosphate buffer, pH 6.5 with 8 mM dopa solution (see Experimental).

Michaelis–Menten constants

The K_m was determined from double-reciprocal plots of initial velocity versus substrate concentration [29]. The K_m (Table 4) indicates that the enzyme has higher affinity for catechol than for dopa. This tendency increases on further purification of enzyme. The K_m for catechol is in agreement with the value obtained for 4-methyl-catechol with grape enzyme [4]. It is slightly higher when compared with the mushroom enzyme [30]. Similarly the K_m value obtained for dopa is higher than that reported for the other enzyme preparations [31], indicating that the catechol oxidase from *S. melongena* has a comparatively lesser affinity for the substrate employed.

Table 4. Michaelis constants of catechol oxidase

Substrates	K_m (M) Crude extract	Purified enzyme
Catechol	1.08×10^{-3}	7.04×10^{-4}
Dopa	6.66×10^{-3}	6.25×10^{-3}

Enzyme activity was measured in 0.1 M phosphate buffer, pH 6.5 using 10 mM catechol and 8 mM dopa solutions as described in the text.

EXPERIMENTAL

Material. Fresh purple long brinjals were obtained locally.

Enzyme assays. CO activity with dopa as substrate was assayed by measuring the formation of the coloured product 2-carboxy-2-3-dihydroindole quinone-5,6 (dopachrome). The molar extinction coefficient of dopachrome is 3.6×10^3 [32]. The reaction mixture consisted of 2 ml of 8 mM DL-dopa, 1.8 ml of 0.1 M Pi buffer, pH 6.5 and 0.2 ml of CO soln. After incubation at 37° for 5 min, the formation of dopachrome at 480 nm was measured. The enzymatic activity with L-tyrosine and tyramine (2 mM) as substrate was measured by the same procedure. The CO activity with catechol as substrate was assayed by measuring the formation of quinone which reacts with proline to produce a red compound having an A_{max} at 525 nm [33]. The reaction mixture consisted of 1 ml of 10 mM catechol, 1 ml of 20 mM proline, 1.9 ml of Na–Pi buffer, pH 6.5 and 0.1 ml of CO soln. After incubation at 37° for 5 min, the A was measured at 525 nm. A similar protocol was adopted for activity measurements with 10 mM solns each of hydroquinone, pyrogallol, *m*-cresol and *p*-cresol. The assay of CO activity with chlorogenic acid was essentially as described in ref. [34]. The reaction mixture consisted of 0.1 ml of 2.5 mM chlorogenic acid, 3.88 ml of 0.1 M Na–Pi buffer, pH 6.5 and 0.02 ml of enzyme soln. Decrease in A at 326 nm was observed.

Effect of inhibitor. The CO soln was preincubated with inhibitor at 37° for 30 min and the enzymatic activity was measured at pH 6.5 using dopa soln according to the method described above.

Protein was measured by the Folin–Ciocalteu method as described in ref. [35] with BSA as standard.

Polyacrylamide disc electrophoresis of the CO was performed using Tris buffer of pH 9 according to ref. [36]. Large pore sample gel was omitted. The siliconized gel tubes (0.5 × 9.0 cm) filled with *ca* 2.2 ml of the small pore soln were used. The protein sample (*ca* 100 μg) in 40% sucrose was directly applied on to the top of the gel tubes. A current of 5 mA per tube was maintained for 2 hr. The gel was removed and stained in 1% amido-schwarz 10B in 7% HOAc. The background stain was removed by successive treatment with 7% HOAc.

Purification of CO. The peeled fresh purple long brinjals were used for the extraction of CO. The homogenate (1:3) was prepared in 50 mM Pi buffer, pH 7.3 containing 50 mM ascorbic acid. After squeezing through cheesecloth, the soln was centrifuged at 4500 g for 30 min at 0°. For the solubilization of the particulate enzyme, 2% (v/v) Triton X-100 was added in the buffer soln used for the extraction of enzyme. After centrifugation, the residue was discarded and the extract was brought to 40% $(NH_4)_2SO_4$ satn. After the complete pptn, the resultant suspension was centrifuged at 4000 g for 30 min at 0°. The residue, dissolved in 0.1 M Pi buffer, pH 6.5 was applied on to the Sephadex G-200 column and eluted with 0.05 M Pi buffer, pH 6.5 at a flow rate of 0.25 ml/min. Fractions (2 ml) were collected and CO activity and protein concn were estimated. The purification procedure is summarized in Table 1.

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REFERENCES

1. Kertesz, D. and Zito, R. (1965) *Biochim. Biophys. Acta* **96,** 447.
2. Harel, E., Mayer, A. M. and Shain, Y. (1965) *Phytochemistry* **4,** 783.
3. Stelzic, D. A., Akhtar, S. and Ribeiro, S. (1972) *Phytochemistry* **11,** 535.
4. Harel, E. and Mayer, A. M. (1971) *Phytochemistry* **10,** 17.
5. Ruis, H. (1972) *Phytochemistry* **11,** 53.
6. Ivanov, T. (1967) *Ann. Technol. Agric.* **16,** 35.
7. Mayer, A. M. (1966) *Phytochemistry* **5,** 1297.
8. Yamaguchi, M., Hwang, P. M. and Campbell, J. D. (1970) *Can. J. Biochem.* **48,** 198.
9. Bailey, J. L. (1961) *Biochem. J.* **79,** 514.
10. Kenten, R. H. (1957) *Biochem. J.* **67,** 300.
11. Poux, C. (1966) *Ann. Technol. Agric.* **15,** 149.
12. Mayer, A. M. and Friend, J. (1960) *Nature* **185,** 464.
13. Patil, S. S. and Zucker, M. (1965) *J. Biol. Chem.* **240,** 3938.
14. Alberghina, F. A. M. (1964) *Phytochemistry* **3,** 65.
15. Nakamura, T., Sho, S. and Ogura, Y. (1966) *J. Biochem.* **59,** 481.
16. Tung, J. S. and Knight, C. A. (1972) *Analyt. Biochem.* **48,** 153.
17. Wood, B. J. and Ingraham, L. L. (1965) *Nature* **205,** 291.
18. Palmer, J. K. (1963) *Plant. Physiol.* **38,** 508.
19. Ashida, M. and Ohnishi, E. (1967) *Arch. Biochem. Biophys.* **122,** 411.
20. Keilin, D. and Mann, T. (1938) *Proc. R. Soc. London Ser. B* **125,** 187.
21. Kenten, R. H. (1958) *Biochem. J.* **68,** 244.
22. Harel, E., Mayer, A. M. and Shain, Y. (1964) *Physiol. Plant.* **17,** 921.
23. Sharma, R. C. Ali, R. and Yamamoto, O. (1979) *J. Radiat. Res.* **20,** 186.
24. Brown, F. C., Word, D. N. and Griffin, A. C. (1959) in *Pigment Cell Biology* (Gordon, M., ed.) pp. 525–535. Academic Press, New York.
25. Sharma, R. C., and Ali, R. (1976) *Radiat. Res.* **66,** 33.
26. Malnstrom, Bo G. and Ryden, L. (1968) in *Biological Oxidations* (Singer, T. P., ed.) pp. 415–438. Interscience, New York.
27. Lerner, A. B., Fitzpatrick, T. B., Calkins, E. and Summerson, W. H: (1950) *J. Biol. Chem.* **187,** 793.
28. Seiji, M., Yoshida, T., Itakura, H. and Irimajiri, T. (1969) *J. Invest. Dermatol.* **52,** 280.
29. Lineweaver, H. and Burk, D. (1934) *J. Am. Chem. Soc.* **56,** 658.
30. Keyes, M. H. and Somersky, F. E. (1972) *Arch. Biochem. Biophys.* **148,** 256.
31. Pomerantz, S. H. (1963) *J. Biol. Chem.* **238,** 2351.
32. Horowitz, N. H. and Shen, S. C. (1952) *J. Biol. Chem.* **197,** 513.
33. Jolley, R. L., Jr. and Mason, H. S. (1965) *J. Biol. Chem.* **240,** 1489.
34. Sisler, E. C. and Evans, H. J. (1958) *Biochim. Biophys. Acta* **28,** 638.
35. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.
36. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121,** 404.