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CHLOROGENIC ACID OXIDASE FROM POTATO TUBER SLICES: PARTIAL PURIFICATION AND PROPERTIES

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Abstract—An *o*-diphenol:O_2 oxidoreductase has been purified about 40-fold from potato tuber slices. The enzyme differs from previously described polyphenoloxidases purified from potato for the fact that chlorogenic acid and 4-methylcatechol are much better substrates than DOPA and catechol. The name of chlorogenic acid oxidase is proposed for this enzyme. The K_m values for chlorogenic acid and for O_2 are, respectively, $1{\cdot}7.10^{-4}$ M and $1{\cdot}5.10^{-4}$ moles/l. The action of inhibitors and of the pH on the activity of the enzyme have been studied.

ALTHOUGH polyphenoloxidases (*o*-diphenol:O_2 oxidoreductase) are known to occur in a variety of plant tissues, their metabolic function is not clear.[1] A number of attempts have been made to implicate these enzymes in respiratory metabolism, by postulating a physiologically significant transfer of electrons from reduced coenzymes to quinones.[2] As early as 1937, it was possible to reconstruct *in vitro* a system transferring electrons from $NADPH_2$ to oxygen, through catechol and its oxidase,[3] but no clear-cut evidence exists that this system functions in the intact cell.

Recent work shows the presence of both NADP-chlorogenic acid oxidoreductase and chlorogenic acid oxidase activities in the potato tuber tissue.[4] Since chlorogenic acid is one of the *o*-diphenols most widespread in plant tissue[5] and the major phenolic component of potato tuber,[6] it was thought that a characterization of the chlorogenic acid oxidase activity of potato tuber could be of interest in regard of the above mentioned problem.

This paper is a report of the purification and partial characterization of the enzyme from potato tuber slices.

RESULTS

Enzyme Purification

Potato tuber slices, incubated on wet filter paper for 48 hr, were used for the extraction and purification of the enzyme, because these slices have a chlorogenic acid oxidase activity several times greater than that of the fresh slices.[4] All the operations of the purification were carried out in the cold. A known weight of potato slices (generally about 300 g) was ground in a pre-chilled mortar with quartz sand in 5.10^{-2} M phosphate buffer (2 ml/g) containing 5.10^{-5} M versene and 10^{-2} M ascorbate, pH 6·7. The homogenate was filtered through cheese-cloth and centrifuged at 10,000 *g* for 30 min. The clear supernatant was collected and centrifuged at 60,000 *g* for 90 min. In the pellet ("microsomal" fraction) there was about

[1] W. D. BONNER, *Ann. Rev. Plant Physiol.* **8**, 427 (1957).
[2] W. WOSILAIT and A. NASON, *J. Biol. Chem.* **206**, 255 (1954).
[3] F. KUBOWITZ, *Biochem. Z.* **293**, 308 (1937).
[4] E. MARRÈ, F. ALBERGHINA and O. SERVETTAZ, *G. Bot. It.* **69V**, 250 (1962).
[5] T. A. GEISMAN, *Encyclopaedia Plant Physiol.* Vol. 12/2, p. 543, Springer-Verlag, Berlin (1960).
[6] K. R. HANSON and M. ZUCKER, *J. Biol. Chem.* **238**, 1105 (1963).

25 per cent of the whole chlorogenic acid oxidase activity and about 40 per cent of the whole NADP-chlorogenic acid oxidoreductase activity. In attempts to solubilize the particle-bound enzyme the best results were obtained by treatment with 1% digitonin, which solubilized about a third of the microsomal oxidase activity, while nearly all the NADP-chlorogenic acid oxidoreductase was left bound to the particles; but the digitonin solubilized enzyme is not stable, so, for the further purification steps, the 60,000 *g* supernatant (fraction 1) was employed. It was brought to 35 per cent saturation with solid ammonium sulphate, recrystallized from a 10^{-3} M versene solution. The precipitate was discarded by centrifugation

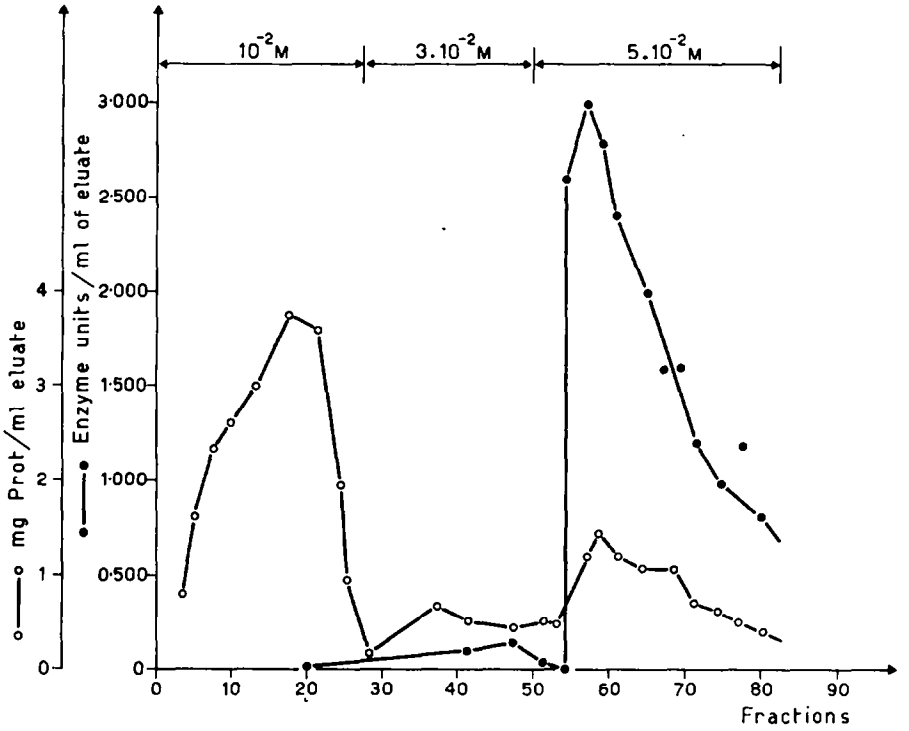

FIG. 1. DEAE-CELLULOSE CHROMATOGRAPHY OF DIALYSED AMMONIUM SULPHATE FRACTION.

Step-wise elution was performed with 10^{-2} M, 3.10^{-2} M, 5.10^{-2} M phosphate buffer (pH 7) as indicated. The assay system contained, in a final volume of 3 ml, chlorogenic acid 5.10^{-5} M in 0·05 M phosphate buffer (pH 6).

and solid ammonium sulphate added to the supernatant until 55 per cent saturation was reached. After 20 min with stirring, the precipitate present was separated by centrifugation and dissolved in about 20 ml of 10^{-4} M phosphate buffer, versene 10^{-5} M, pH 7 (fraction 2). This fraction was dialysed for 36 hr against several changes of 10^{-4} M phosphate buffer containing 10^{-5} M versene and 10^{-5} M ascorbate, pH 7.

After dialysis, the precipitate formed was removed by centrifugation. The supernatant (fraction 3) was applied to a DEAE-cellulose column equilibrated with 10^{-3} M phosphate buffer, pH 7. After washing with 10^{-3} M phosphate buffer, a step-wise gradient elution with increasing concentrations of buffer (pH 7) was performed. The bulk of proteins was eluted by 10^{-2} M buffer, while the activity was eluted by 5.10^{-2} M buffer (Fig. 1). The more active

fractions of the DEAE-cellulose eluate were pooled and used for the characterization of the enzyme. Occasionally, they were fractionated on a second DEAE-cellulose column; a further 2-fold purification was achieved with low recovery. The results of a typical purification are shown in Table 1.

TABLE 1. PURIFICATION OF CHLOROGENIC ACID OXIDASE FROM POTATO TUBER SLICES

Fract. No.	Purification step	Volume, ml	Activity, units/ml	Total, units	Protein, mg/ml	Specific activity, units/mg	Purification
1	Supernatant after 60,000 *g* for 90 min	610	137	84000	3·5	39	1
2	35–55% $(NH_4)_2SO_4$ pellet	23	2610	60000	41·3	63	1·6
3	Fraction 2, dialysed and centrifuged	27	1550	42000	15·0	102	2·6
4	I DEAE cellulose eluate (pooled fractions)	36	234	8400	0·22	1060	27
5	II DEAE cellulose eluate (pooled fractions)	12	105	1260	0·06	1830	47

Substrate Specificity

A variety of compounds were tested as substrates for the purified enzyme preparation. Table 2 gives a survey of the activities obtained with different *o*-diphenols at varying concentration. Chlorogenic acid and 4-methylcatechol appear the most effective, while catechol and DOPA, generally considered the best substrates for polyphenoloxidase, are less active. Ascorbate is not oxidized by the preparation. As chlorogenic acid is a normal constituent of potato tuber,[6] whilst methyl catechol is not, it appears convenient to call the enzyme chlorogenic acid oxidase.

TABLE 2. ACTIVITY OF CHLOROGENIC ACID OXIDASE WITH VARIOUS SUBSTRATES

Substrate	Concentration, M.10^5	Activity, μl. O_2/mg prot. for 10′
Chlorogenic acid	40	120
	20	92
	10	70
	2	20
4-Methylcatechol	20	80
Catechol	400	22
	40	20
DOPA	10	11
	2	7
Caffeic acid	2	5·6
	1	5·3
Protocatechuic acid	20	0
Hydroquinone	120	50*
Ascorbic acid	5,000	0

* After a 1-hr lag period.

In the crude extract of potato tuber, the ratio of catechol oxidase activity/chlorogenic acid oxidase activity is close to 1, while in the final preparation it is very markedly shifted in favour of chlorogenic acid (Table 3). Furthermore the purified enzyme preparation appears inhibited by high concentration of catechol, while the crude homogenate does not. These observations could be explained on the basis of two hypotheses: (1) more than one polyphenoloxidase is present in the crude homogenate; (2) a single polyphenoloxidase is present, but in the crude homogenate conditions prevail which prevent its inhibition by catechol.

TABLE 3. OXIDASE ACTIVITIES AT DIFFERENT STAGES OF PURIFICATION

Fraction	Substrate			
	Catechol 4.10^{-4} M	Chlorogenic acid 4.10^{-4} M	4-Methylcatechol 4.10^{-4} M	DOPA $1{\cdot}67.10^{-4}$ M
	(μl. O_2/min/mg prot.)			
Supernatant of 10,000 *g*	1·78	2·25	2·50	0·57
0·35–0·65 Ammonium sulphate fraction dialysed	0·60	1·41	1·41	0·10
DEAE cellulose eluate	2·65	10	9·2	2·0

Effects of pH and Inhibitors on Enzyme Activity

As shown in Fig. 2, the chlorogenic acid oxidase activity of the enzyme preparation has a maximum at pH 4·3, and a narrow plateau between pH 5 and 6. The oxidase activities towards catechol and 4-methylcatechol show wide plateaux: the former from pH 5 to pH 8, the latter from pH 4·5 to pH 6 (Figs. 3 and 4).

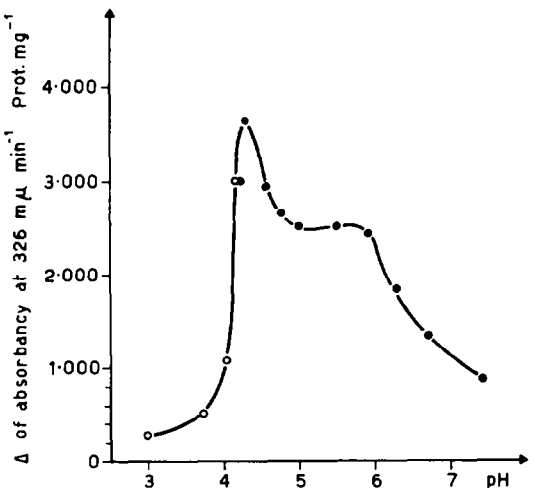

FIG. 2. EFFECT OF pH ON ACTIVITY OF PURIFIED CHLOROGENIC ACID OXIDASE ACTIVITY.
The assay system contained, in a final volume of 3 ml, chlorogenic acid 10^{-4} M in 0·05 M phosphate buffer (●) or in 0·05 M citrate buffer (○).

The effect of a number of inhibitors on the enzyme is shown in Tables 4 and 5. Diethyldithiocarbamate (DIECA) at 10^{-5} M and KCN 5.10^{-5} M gave 50 per cent inhibition of the enzyme activity. Thiourea and *o*-phenanthroline were less inhibitory.

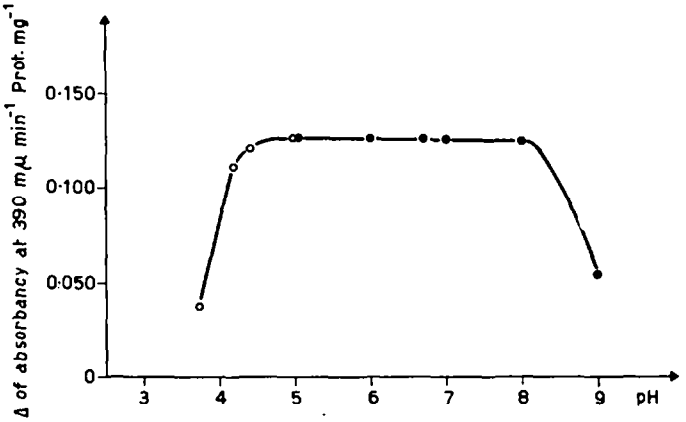

FIG. 3. EFFECT OF pH ON ACTIVITY OF CATECHOL OXIDASE ACTIVITY OF PURIFIED PREPARATION.
The assay system contained, in a final volume of 3 ml, catechol 4.10^{-4} M in 0·05 M phosphate buffer (●), or in 0·05 M citrate buffer (○).

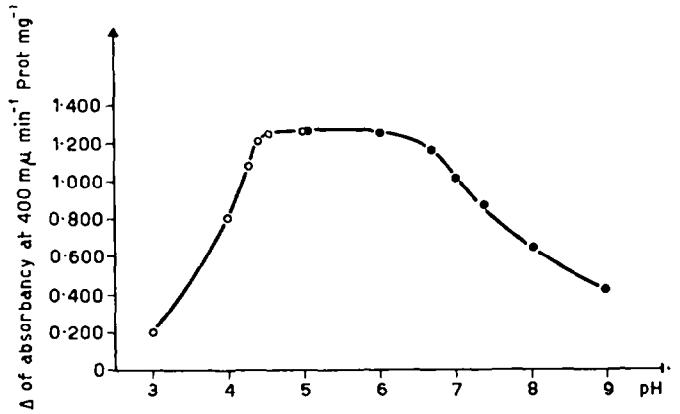

FIG. 4. EFFECT OF pH ON ACTIVITY OF METHYLCATECHOL OXIDASE ACTIVITY OF PURIFIED PREPARATION.
The assay system contained, in a final volume of 3 ml, methylcatechol 2.10^{-4} M in 0·05 M phosphate buffer (●), or in 0·05 M citrate buffer (○).

Effect of Substrate Concentration on Enzyme Activity

Data were obtained as oxygen uptake in the manometric assay at pH 6·3, with air as gas phase. Figure 5 is a Lineweaver–Burk plot of the enzyme activity on chlorogenic acid, catechol, 4-methyl-catechol. The Michaelis constants (K_m) found are: $1{\cdot}7.10^{-4}$ M for chlorogenic acid; $2{\cdot}5.10^{-4}$ M for 4-methylcatechol and $1{\cdot}4.10^{-3}$ M for catechol. The rates of oxygen uptake in the determination of K_m for catechol had to be extrapolated at zero time because of the above-mentioned inhibition of oxidase activity by catechol. Chlorogenic acid oxidase affinity for oxygen is low, K_m value being $1{\cdot}5.10^{-4}$ M (Fig. 6).

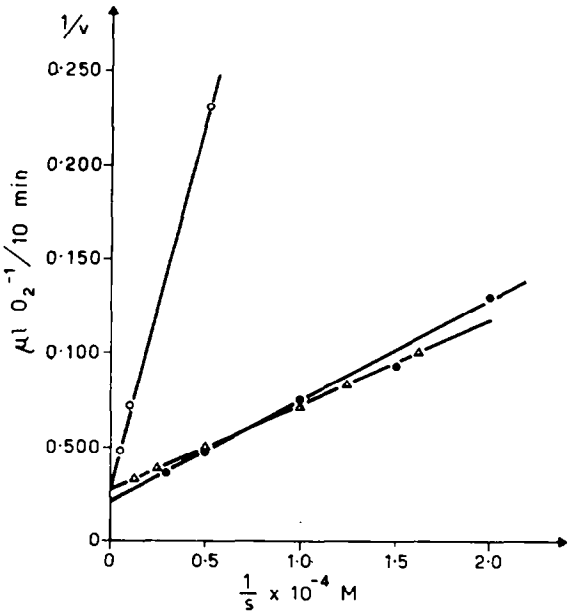

FIG. 5. LINEWEAVER–BURK PLOT OF OXIDASE ACTIVITY ON CHLOROGENIC ACID (Δ) 4-METHYLCATECHOL (●) AND CATECHOL (○) AS FUNCTION OF *o*-DIPHENOLS CONCENTRATION.

Data obtained with air in gas phase.

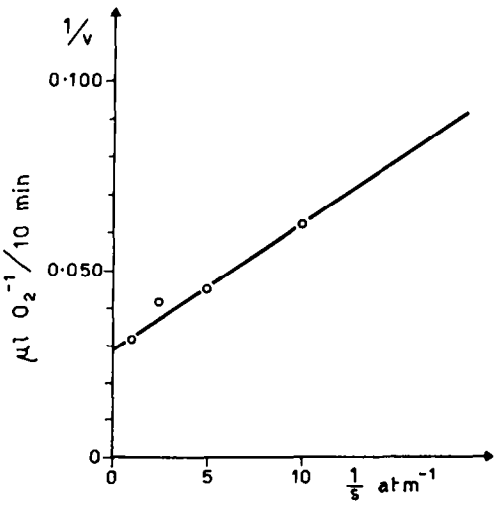

FIG. 6. LINEWEAVER–BURK PLOT OF CHLOROGENIC ACID OXIDASE ACTIVITY AS FUNCTION OF OXYGEN PRESSURE.

The assay system contained chlorogenic acid 3.10^{-4} M in 0·05 M phosphate buffer, pH 6·3

TABLE 4. THE EFFECT OF SOME INHIBITORS ON CHLOROGENIC ACID OXIDASE

Inhibitor	Concentration, M.10^5	Activity, Δ OD at 326 mμ/min/mg protein
None	—	2·100
KCN	100	0·000
	30	0·170
	10	0·660
	10*	0·330
	5	1·170
DIECA	100	0·000
	20	0·000
	10	0·250
	2	0·500
	1	1·500

Chlorogenic acid, at final concentration of 10^{-4} M, was added, 2 min after the inhibitor, to the enzyme preparation (0·12 mg of protein for cuvette), in 5.10^{-2} M phosphate buffer, 10^{-5} versene pH 6, in a final volume of 3 ml.

* Enzyme pre-incubated with KCN for 30 min before the determination of activity.

TABLE 5. THE EFFECT OF SOME INHIBITORS ON CHLOROGENIC ACID OXIDASE

Inhibitor	Concentration, M.10^5	Activity, μl. O_2/mg protein/10 min
None	—	57
DIECA	5	16
	2·5	26
	1	29
o-Phenanthroline	300	51
	50	63
Thiourea	100	54

Chlorogenic acid was added from the side arm in final concentration of 10^{-3} M, 30 min after the inhibitor, to the enzyme preparation (0·6 mg of protein for each vessel).

DISCUSSION

The enzymatic preparation here described appears to correspond closely to the "polyphenoloxidase" obtained by Sisler and Evans from tobacco roots,[7] as far as the K_m value for chlorogenic acid and the ratio of the activities towards chlorogenic acid and catechol are concerned. On the other hand, it differs from the polyphenoloxidase from potato tuber described by Neumann *et al.*,[8] who found a lower K_m for chlorogenic acid and a much higher activity towards catechol. This difference is possibly due to the fact that Neumann *et al.*

[7] E. C. SISLER and H. J. EVANS, *Plant Physiol.* **33**, 255 (1958).
[8] J. NEUMANN, G. LEGRAND, G. LEHONGRE and J. LAVOLLAY, *Compt. rend.* **251**, 3091 (1960).

did not purify their enzyme beyond the ammonium sulphate fractionation step, while in our procedure a further 20-fold increase of specific activity was obtained in the succeeding steps.

The characteristics of this partially purified enzyme activity appear to justify the name *chlorogenic acid oxidase*; as both its affinity and its maximal activity are higher with respect to this substrate than with respect to catechol, DOPA and the other polyphenols investigated.

Chlorogenic acid appears to be a normal metabolite present in the potato tuber according to results by Geisman[5], Hanson and Zucker[6] and Zucker and Levy[9] which have been recently confirmed and extended in this laboratory. Moreover, potato tuber homogenates show a considerable NADP-chlorogenic acid oxidoreductase activity.[4] Therefore we suggest that in potato tuber an electron transfer chain including chlorogenic acid and chlorogenic acid oxidase could be involved in the reoxidation of $NADPH_2$ formed at the level of the pentose phosphate pathway. It is interesting to observe in this connection that a system, with the same low affinity for oxygen as the chlorogenic acid oxidase described here, has been reported as being involved in the respiration of the potato tuber by Burton and Mapson.[10]

EXPERIMENTAL

Chemicals

Chlorogenic acid and caffeic acid were purchased from California Corporation for Biochemical Research; 4-methylcatechol, protocatechuic acid and dihydroxyphenylalanine (DOPA) were supplied by the British Drug House; DEAE-cellulose by Whatman. All other products used were obtained from Merck.

Enzyme Assay

Oxidase activity was determined either manometrically or spectrophotometrically.

The manometric assay was carried out in the Warburg apparatus in air at 25°C. In a typical experiment 1 ml 0·1 M potassium phosphate buffer pH 6·3, 0·1 ml 10^{-3} M versene, 0·5 ml 0·1 M ascorbate, 1·4 ml enzyme (about 0·18 mg protein), were used with 1 ml substrate, in suitable concentration, in the side-arm. Manometric readings were made at 5-min intervals for 30 min; during this period oxygen uptake was found to proceed at a constant rate.

Spectrophotometric assay for the chlorogenic acid oxidase activity was essentially that of Sisler and Evans.[11] The reaction mixture contained 0·05 M phosphate buffer, pH 6; 5.10^{-5} M chlorogenic acid in a final volume of 3 ml. The amount of enzyme added to start the reaction was such as to give a decrease of absorbancy at 326 mμ of about 0·100 in 1 min. The absorbancy was recorded at 15-sec intervals for 2 min. One unit of enzyme activity was defined as the amount of enzyme which catalyses the oxidation of 1 mμmole of chlorogenic acid in 1 min in the spectrophotometric assay; specific activity is expressed as units/mg of protein.

Catechol oxidase activity was determined by measuring the increase of absorbancy at 390 mμ during the oxidation of catechol. The reaction mixture contained catechol 4.10^{-4} M, 0·05 M phosphate buffer in a final volume of 3 ml.

4-Methylcatechol oxidase activity was determined by measuring the increase of absorbancy at 400 mμ during the oxidation of the substrate. The reaction mixture contained 4-methylcatechol 2.10^{-4} M, 0·05 M phosphate buffer in a final volume of 3 ml.

Protein was determined by a spectrophotometric assay.[12]

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[11] E. C. SISLER and H. J. EVANS, *Biochim. Biophys. Acta* **28**, 638 (1958).
[12] H. M. KALCKAR, *J. Biol. Chem.* **167**, 461 (1947).