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Isolation and Purification of Polyphenol Oxidase from a New Variety of Potato

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ABSTRACT

Polyphenol oxidase (E.C. 1.10.3.1) was extracted from potato (Solanum tuberosum *var. Tebere) tissue and purified using the techniques of ammonium sulphate fractionation, DEAE-cellulose chromatography and filtration through Sephadex G-200. Four isoenzymes with different relative mobilities were detected by polyacrylamide gel electrophoresis. The enzyme had a molecular weight of 112 000 as estimated by gel filtration on Sephadex G-200 and an apparent* Km *of* $8{\cdot}68 \times 10^{-5}$M *for chlorogenic acid as substrate, and pH optimum of 6·6. The heat inactivation plot at 65 °C at different pH values was biphasic. It was also found that the Arrhenius plot for chlorogenic acid oxidation was biphasic showing a discontinuity at 8 °C with an increased slope as temperatures were reduced to 1 °C. Cinnamic and ferulic acids were competitive inhibitors with respect to chlorogenic acid with* Ki *values of* 2×10^{-5} *and* 5×10^{-6}M, *respectively.*

INTRODUCTION

Polyphenol oxidase (PPO) (*o*-diphenol:O_2 oxidoreductase, E.C.1.10.3.1) is currently the most studied enzyme concerned with the enzymatic browning in fruits and vegetables. One reason for this attention by biochemists and food technologists is that browning is commercially undesirable because it impairs the sensory properties and, hence, the

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marketability of a product (Vámos-Vigyázó, 1981; Walter & Purcell, 1980).

The enzyme belongs to the group of oxidoreductases and catalyses the hydroxylation of monophenols and the oxidation of *o*-dihydroxyphenols to *o*-quinones. Polyphenol oxidase is a copper-containing enzyme present in most tissues in substantial amounts, and is generally of broad substrate specificity, being inhibited by a considerable number of substances. Many workers have shown that plants contain multiple forms of polyphenol oxidase separable by electrophoresis and column chromatography. Rivas & Whitaker (1973), using DEAE-cellulose and hydroxyapatite chromatography purified two proteins with polyphenol oxidase activity. Kahn (1976) reported the presence of six active enzymes in the 30–90% ammonium sulphate fraction of Fuerte avocado and the catechol oxidase from apple chloroplasts was resolved by DEAE-cellulose into three peaks. Galeazzi *et al.* (1981) isolated four isoenzymes from a dwarf variety of banana with a molecular weight (mol. wt) of 62 000 and a polypeptide mol. wt of 31 000. In contrast with the above observations Roudsari *et al.* (1981) were able to obtain a 15-fold purification with a preparation of polyphenol oxidase from eggplant. The enzyme was eluted in a single active fraction after chromatography on DEAE-cellulose and Sephadex G-100 gel filtration.

This work is part of a study on polyphenol oxidase of a new variety of potato selected by a plant breeding program conducted at the Agronomic Institute of Campinas, Brazil. Our purpose is to correlate PPO activity and phenolic level with browning rate. We now report on the isolation and purification of the enzyme.

MATERIALS AND METHODS

Extraction and purification of PPO

The 'Tebere' potato variety was obtained at harvest from the Agronomic Institute of Campinas, Brazil. After washing and grading the tubers were cured at 10 °C. The tubers were peeled and cut into small pieces. One hundred gram samples were homogenised in 0·2 M phosphate buffer, pH 7·5, containing 1 mM L-cysteine and Amberlite IRA 938 (2 g/10 g tissue), in an Ultraturrax homogeniser for 1 min. The homogenate was filtered through four layers of cheesecloth and centrifuged at 25 000 *g* for 40 min.

The supernatant was collected and used as a crude enzyme extract. Solid $(NH_4)_2SO_4$ sufficient to give 20% saturation was added to the extract with continuous stirring. After stirring for 60 min the preparation was centrifuged at 25 000 *g* for 40 min and the pellet was discarded. To the clear supernatant $(NH_4)_2SO_4$ was added to give 90% saturation. The precipitate obtained between 20 and 90% was collected by centrifugation, dissolved in a minimal volume of the same buffer and applied to a column of Sephadex G-25 (35 × 2·5 cm) equilibrated with the extraction buffer. The enzyme was eluted with 5 mM phosphate buffer, pH 7·5 and loaded into a DEAE-cellulose column (24 × 1·5 cm) previously equilibrated with the same buffer. The column was washed with 3 bed volumes of the buffer and the enzyme was eluted with a linear KCl gradient (0–0·4 M) in 5 mM phosphate buffer, pH 7·5. The active fractions constituting three peaks were pooled and combined. To the combined fractions, solid ammonium sulphate to give 90% saturation was added and the suspension was kept with continuous stirring for precipitation. The precipitate was collected by centrifugation at 25 000 *g* and dissolved in 3 ml of 5 mM phosphate buffer, pH 7·5. The enzyme fraction was dialysed against 1 mM phosphate buffer, pH 7·5, with three buffer changes. A Sephadex G-200 column (94 × 1·5 cm) was prepared and equilibrated with 5 mM phosphate buffer, pH 7·5. The enzyme fraction after dialysis was loaded onto the column and subsequently chromatographed with the above buffer. Fractions of 3 ml were collected at a flow rate of 12 ml/h and the active fractions pooled. The whole procedure was carried out at 4 °C.

Measurement of PPO activity

The polyphenol oxidase activity was measured with a Varian recorder spectrophotometer. The standard reaction mixture at 30 °C consisted of 0·4 ml of freshly prepared chlorogenic acid (5-caffeoylquinic acid, the IUPAC 1976 nomenclature has been employed for numbering the quinic acid ring), 0·2 ml enzyme sample and 0·5 ml of phosphate buffer, pH 6·6 in a total volume of 3 ml. The unit of enzyme activity was defined as a change of 10^{-3} absorbance units/min at 420 nm.

Protein determination

Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. Absorbance at 280 nm was used to monitor protein in the column's eluates.

Polyacrylamide gel electrophoresis

Electrophoresis was carried out according to the method of Davis (1964) using 7% polyacrylamide gel in Trisglycine buffer, pH 8·6, at 4°C. After the run the proteins in the gel were stained with Coomassie brilliant blue. For the enzyme activity, the gels were incubated in a solution containing 2 mM 4-methyl catechol and 0·1 M phenylenediamine for 1 h.

Molecular weight determination

The molecular weight of the enzyme was determined using Sephadex G-200 in a 90 × 1·5 cm column equilibrated with 5 mM phosphate buffer, pH 6·6, at 4°C. Cytochrome C (mol. wt 12 000), soyabean trypsin inhibitor (mol. wt 21 500), ovalbumin (mol. wt 45 000), alkaline phosphatase (mol. wt 100 000), aldolase (mol. wt 158 000) and urease (mol. wt 480 000) were used as molecular weight standards.

Determination of energy of activation (*Ea*).

The *Ea* for conversion of the substrate to product was calculated from the slope of Arrhenius plot obtained by plotting the logarithm of initial velocity *vs* reciprocal of reaction temperature.

RESULTS AND DISCUSSION

To isolate polyphenol oxidase from potato tissue a combination of a macro-reticular adsorbent, Amberlite IRA 938, and L-cysteine were required to obtain a clear extract preventing the enzymic browning. When ammonium sulphate was added to 90% saturation, 40% of the original activity was lost, and this loss of activity was higher considering that the increase in the specific activity was negligible (Table 1). The passage of the dissolved precipitate through a Sephadex G-25 column separated the enzyme from phenolics and low molecular weight compounds and gave a preparation reliable for further purification. Three fractions (I, II and III) with polyphenol oxidase activity were eluted from DEAE-cellulose (Fig. 1). Although they account for approximately 8, 6 and 7% of the extracted activity, the great increase in the specific activity shows that this step was efficient in removing inert protein. The three active fractions were pooled

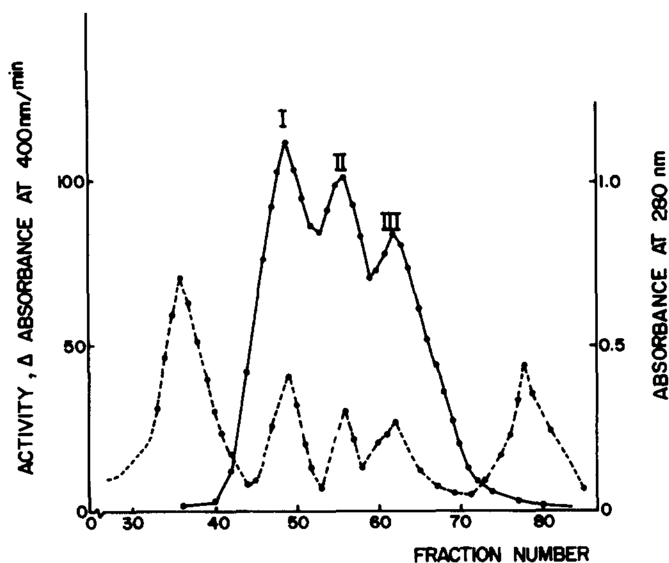

Fig. 1. Chromatography of potato PPO var. Tebere in DEAE-cellulose column. The enzyme was eluted with a linear KCl gradient (0–0·4 M) in 5 mM phosphate buffer pH 7·5. ●– –● absorbance at 280 nm; ●—● PPO activity (units/ml).

TABLE 1
Purification of PPO from Potato Tubers var. Tebere

Purification steps	*Volume (ml)*	*Total activity*	*Protein content (mg)*	*Specific activity (units/mg protein)*	*Purification (fold)*
Crude extract	152·0	140 760	270·3	520·7	1·0
20–90% $(NH_4)_2SO_4$ fractionation	17·0	88 200	160·4	549·4	1·05
Sephadex G-25	23·5	89 300	101·9	876·3	1·6
DEAE-cellulose					
peak I	3·0	11 530	0·57	21·700	41·7
peak II	3·0	8 210	0·91	9·000	17·3
peak III	3·0	6 920	1·01	6·800	13·1
Sephadex G-200	3·0	6 200	0·17	35·300	67·7

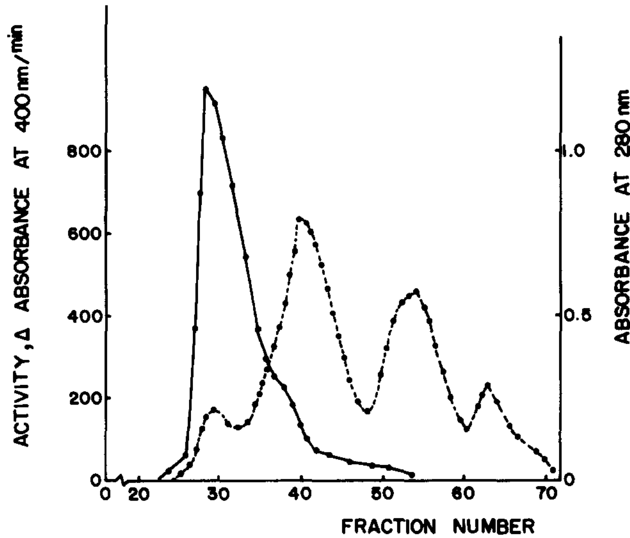

Fig. 2. Sephadex G-200 chromatography of the potato PPO var. Tebere. The enzyme was eluted with 5 mM phosphate buffer, pH 7·5. ●-- ● absorbance at 280 nm, ●—● PPO activity (units/ml).

together as previously described and applied onto a Sephadex G-200 column. The active fractions were eluted in a single peak (Fig. 2), free of peroxidase. The electrophoresis on polyacrylamide gel showed that this fraction contained six protein bands, four of them with polyphenol activity (Fig. 3). These results indicate that the fraction eluted from Sephadex G-200 has a high degree of purity and consists of four isoenzyme forms with different relative mobilities.

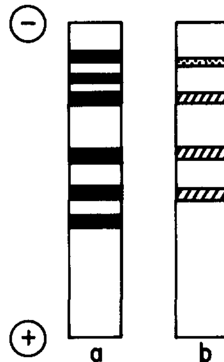

Fig. 3. Polyacrylamide gel electrophoresis of the potato PPO preparation eluted from Sephadex G-200 column. (a) protein bands; (b) PPO activity bands.

Physicochemical properties

The apparent molecular weight of the enzyme was determined on Sephadex G-200 and was estimated as described by Whitaker (1963). The distribution of activity revealed a single peak indicating the presence of only one molecular species, or more than one species with a molecular weight of about 112 000. Roudsari *et al.* (1981), studying the polyphenol oxidase from eggplant, determined a molecular weight of 79 000. More recently Interesse *et al.* (1983) reported, for a highly purified isoenzyme of wheat, a mol. wt of 115 000. The molecular weight reported here is somewhat similar to the predominant forms of catechol oxidase extracted from mushroom (Mayer & Harel, 1979). The polyphenol oxidase showed the optimum pH value at 6·6 with citrate–phosphate buffer (pH 4·0–8·0). Alberghina (1964), in a study on the properties of polyphenol oxidase from potato tuber slices, reported a pH value of 4·3 with chlorogenic acid as substrate. The results of substrate specificity study with purified enzyme samples are shown in Table 2. Readings were taken at wavelengths of maximum absorption and at optimum pH for each of the substrates. Monophenolase activity towards *p*-coumaric acid was detected with the crude extract; however, the enzyme lost its ability to hydroxylate this substrate during the purification. The lability of cresolase activity is a well known phenomenon and it has been suggested that this behaviour results from changes in the structure of protein during the purification (Walker & Purcell, 1980). In contrast, Macrae &

TABLE 2
Substrate Specificity of PPO from Potato Tubers var. Tebere

Substrates	*Specific activity (units/mg protein)*	%
4-methyl cathecol	7 124	100
Chlorogenic	1 034	15
Caffeic acid	1 020	14
Cathecol	917	12
DL Dopa	496	6
p-Coumaric acid [a]	260	

[a] The enzyme activity was determined with crude extract.

Duggleby (1968) point out that the potato tuber variety, Orion, shows no capacity to catalyse the hydroxylation of *p*-coumaric acid. The data presented here indicate that the crude extract from var. Tebere is not devoid of *p*-coumaric hydroxylating activity. Rhodes & Wooltorton (1976) and Rhodes *et al.* (1979) have demonstrated, with extracts from tomato fruit and potato tuber, the enzymic potential for conversion of *p*-coumaric acid to *p*-coumarylquinic acid and Hanson & Zucker (1963) reported the enzymic hydroxylation of *p*-coumarylquinic to chlorogenic acid by a polyphenol oxidase preparation from potato. Steck (1968) presented evidence that caffeic acid, formed by hydroxylation of *p*-coumaric acid, is also a precursor of chlorogenic acid synthesis. These results suggest that the *p*-coumaric hydroxylating activity could have some significance in chlorogenic acid synthesis in some species of higher plants.

The data presented in Table 2 show that the enzyme rapidly oxidised 4-methyl catechol followed by chlorogenic and caffeic acids. These results agree with those obtained by Abukharma & Woolhouse (1966). They reported that 4-methyl catechol, chlorogenic and caffeic acids are the most active substrates for the enzyme from potato tuber variety, King Edward. The effect of substrate concentration on enzyme activity was determined and a Michaelis constant of $8{\cdot}6 \times 10^{-5}$M was found. This value is lower than those obtained by Macrae & Duggleby (1968) and

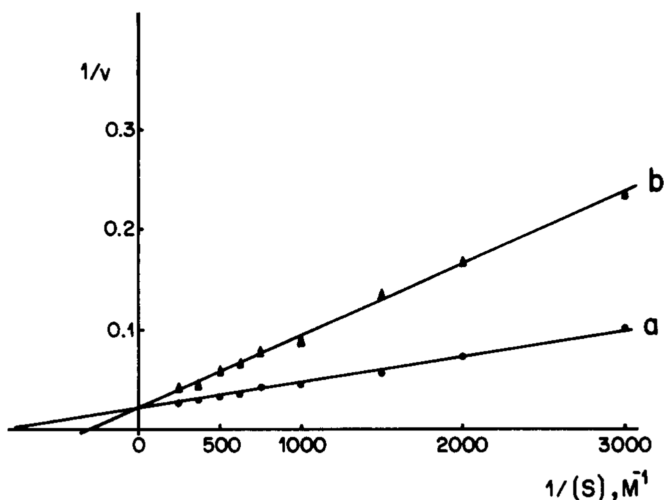

Fig. 4. Lineweaver–Burke plot for potato PPO at different concentrations of cinnamic acid. ●—●, 0·16 mM; ▲—▲, 0·33 mM.

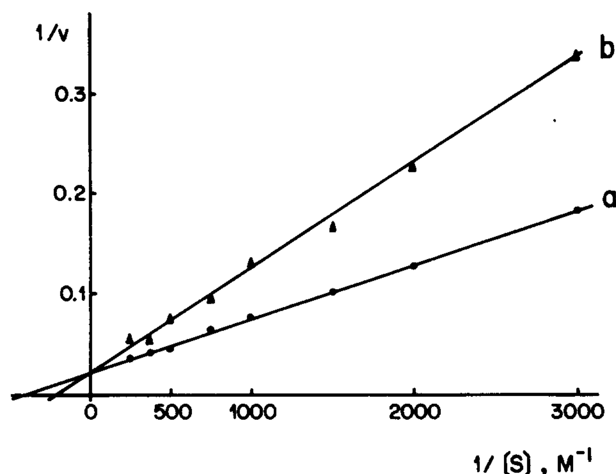

Fig. 5. Lineweaver–Burke plot for potato PPO at different concentrations of ferulic acid ●—●, 0·16 mM; ▲—▲, 0·33 mM.

similar to those found by Abukharma & Woolhouse (1966) for potato polyphenol oxidase from the varieties Orion and King Edward. The inhibition kinetics of polyphenol oxidase by cinnamic and ferulic acids were determined by Lineweaver–Burke plots of $1/v$ *vs* $1/S$ at two levels of inhibitor and confirmed by Dixon plots of $1/v$ *vs* i. Figures 4 & 5 show that cinnamic and ferulic acids inhibited the polyphenol oxidase competitively with *Ki* values of 2×10^{-5} and 5×10^{-6}M, respectively. These findings are in keeping with the observations of Walker & Wilson (1975) who reported that cinnamic and ferulic acids are competitive inhibitors of solubilised enzyme from apple. On the other hand, non-competitive inhibition for ferulic acid and mixed type inhibition for cinnamic acid in the presence of chlorogenic acid as substrate, was found by Macrae & Duggleby (1968). In contrast with the above observations, protocatechuic acid showed different inhibitory behaviour, inhibiting non-competitively the enzyme (*Ki* 8×10^{-4}M), which indicates that the substrate and the inhibitor bind at different sites on the enzyme molecule (Fig. 6). Protocatechuic acid has also been found to be an inhibitor of polyphenol oxidase from *Prunus avium* fruits (Pifferi *et al.*, 1974).

The effect of heating the polyphenol oxidase at 65 °C for various times and different pHs (5·0, 6·0 and 7·0) on the enzyme activity is presented in Fig. 7. The plot of heat inactivation was biphasic and the decrease of PPO activity did not follow first-order kinetics and the enzyme showed a

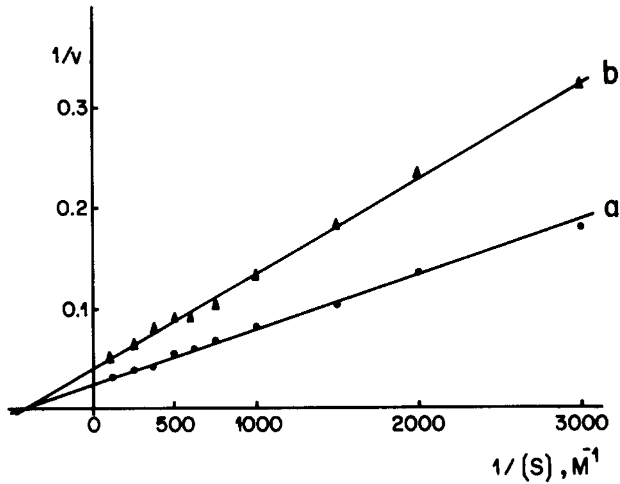

Fig. 6. Lineweaver–Burke plot for potato PPO at different concentrations of protacatechuic acid. ●—●, 0·16 mM, ▲—▲, 0·33 mM.

relatively high stability, with 40 % of the original activity remaining after 10 min heating at pH 7·0. According to Yamamoto *et al* (1962), this kinetic behaviour suggests the presence of a heat-labile and heat-resistant isoenzyme. The results of this study demonstrate that acidification from pH 7·0 to 5·0 decreases the heat resistance of the potato enzyme.

Figure 8 shows the Arrhenius plot of the effect of temperature on the

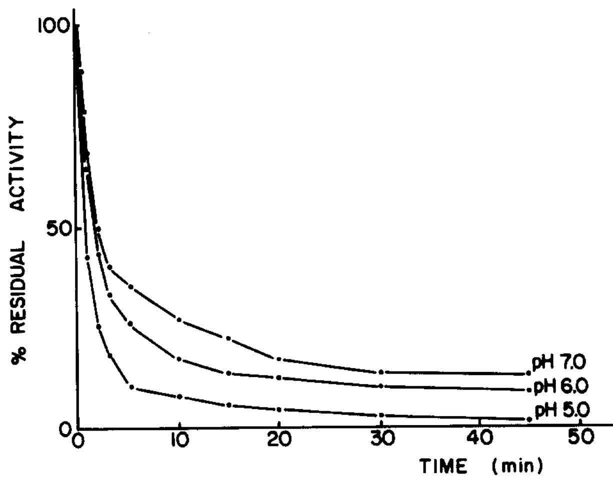

Fig. 7. Effect of pH on heat stability of potato PPO at 65 °C.

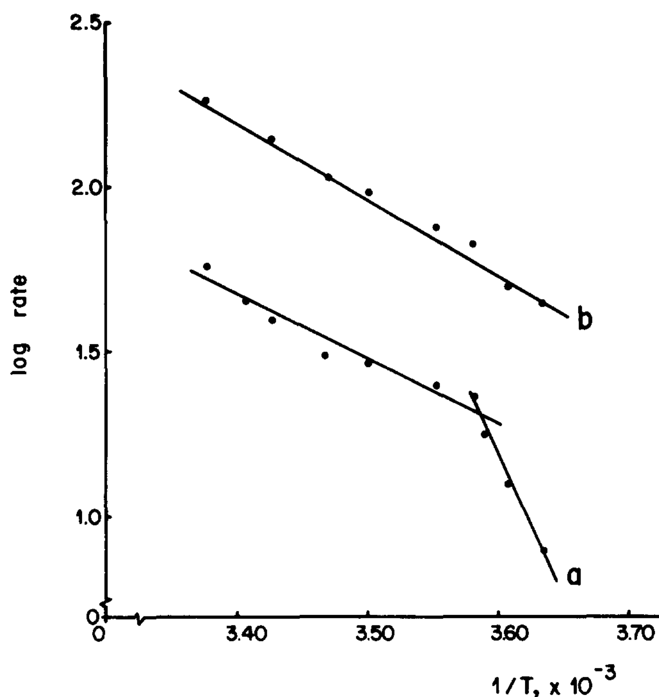

Fig. 8. Arrhenius plot of potato PPO (a) oxidation of chlorogenic acid; (b) oxidation of 4-methyl catechol.

enzyme activity. The most important feature of Fig. 8 is the difference between the shapes of curves A and B. The oxidation of chlorogenic acid by PPO shows a biphasic Arrhenius plot with a marked discontinuity in the plot below 8 °C with an increase in *Ea* (curve A). In contrast the oxidation of 4-methyl catechol shows a linear Arrhenius plot with a constant *Ea* over the entire temperature range studied (curve B). Although biphasic plots have been obtained for other enzymes, it was unexpected to get a difference in the shape of the curves, for a purified enzyme, apparently free of lipids. Since the same enzyme oxidising 4-methyl catechol does not show a discontinuity of Arrhenius plot, it is possible that the change in *Ea* for chlorogenic acid oxidation is a result of a thermally induced conformation change in the enzyme associated with the binding of a specific substrate (Levy *et al.*, 1962). Thus, chlorogenic acid does not seem to be able to stabilise one conformation of active site over the temperature range studied and further work will be needed to explain the interaction of this substrate with the enzyme molecule.

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