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Partial purification and properties of plantain polyphenol oxidase

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Free and bound polyphenol oxidase activities were identified in plantain; the soluble form increases with the maturity of the fruit. No difference was found between the two fractions during the purification process. An eight-fold partially purified preparation was obtained after gel filtration and ion-exchange chromatography. The enzyme has an optimum pH of 6·5. The apparent molecular weights, 30 000 ± 5000 and 70 000 ± 500, estimated by gel filtration are indicative of the presence of multiple forms. The thermal inactivation pattern agrees with the presence of two isoenzymes with different thermal stabilities, the values obtained for E_A being 18 kJ/mol for the heat-resistant form. The greatest activity was found when 4-methyl catechol, catechol, pyrogallol, dopamine and L-dopa were used as substrates. The presence of trace quantities of dopamine in plantain pulp (2 μg/g of fresh matter) was established by high-performance liquid chromatography (HPLC). The plantain-polyphenol oxidase is inhibited by copper complexing agents and by reducing agents. One-hundred per cent inhibition was noted in the presence of sodium diethyl dithiocarbamate, L-cysteine, sodium metabisulphite and ascorbic acid (10^{-3} M). At this concentration only 40% of inhibition was obtained with potassium cyanide. Determination of browning during flour preparation shows that the enzyme may be efficiently inactivated using sodium metabisulphite. This compound also prevents non-enzymatic browning during the slicing and drying steps of preparation of plantain flour.

INTRODUCTION

It is generally recognized that the enzymatic browning of fruits and vegetables is the result of the action of polyphenol oxidases. *o*-Quinones are produced by oxidation of plant phenolics catalysed by this enzyme. The non-enzymatic polymerization of these highly reactive compounds produces brown pigments called melanins.

Several workers have noted the presence of a polyphenol oxidase activity in banana (Palmer, 1963; Galeazzi *et al.*, 1981; Mowlah *et al.*, 1982; Ngalani & Crouzet, 1986). On the other hand only a preliminary report (Nglani & Crouzet, 1986) has been published on soluble plantain polyphenol oxydase. However, the occurrence of enzymatic browning during the preparation of plantain purée has been observed (Ngalani, 1989).

In the present work, the study of plantain polyphenol oxidase—isolation and characterization of the enzyme and its thermal and chemical inhibition—has been performed to discover how to prevent enzymatic browning during processing of plantain pulp.

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MATERIALS AND METHODS

Fruits

Plantains ('French sombre' variety) were kindly supplied by Station de Recherche Agronomique I.R.A., Nyombe (Cameroon). Fruits were gathered at maturity, as defined by the general shape of the bunch and estimated degree of finger filling.

Crude extract preparation

Plantain pulp, obtained from fruits with yellow peel, stage 4–5 (Anon., 1964) was used. All steps of the procedure described below were carried out at 4°C.

Soluble form
Plantain pulp (150 g) was blended for 1 min in a Waring blender in the presence of 166 ml of 0·1 M McIlvaine buffer (pH 6·5). This buffer, prepared from 0·1 M dibasic sodium phosphate adjusted to the correct pH by addition of citric acid, contains 5 g of insoluble polyvinyl pyrrolidone (Polyclar AT). The homogenate was centrifuged at 20 000 *g* for 30 min and the supernatant filtered through a Buchner funnel. The filtrate obtained was the crude soluble extract.

Bound form
The bound form was extracted by adding 1% Triton X 100 in the extraction medium used for the soluble enzyme.

Enzyme assay

The polyphenol oxidase activity was determined by spectrophotometry at 420 nm according to the method of Joslyn and Ponting (1951). Enzymic extract (100 μl) was rapidly added to a solution of 0·1 M McIlvaine buffer (pH 6·5) and 0·125 M catechol. The total volume of the reaction mixture was 2·5 ml; all determinations were carried out at 25°C. One unit of polyphenol oxidase activity (AU) is defined as a change of 10^{-4} absorbance units a second; thus enzymatic activity was expressed as ΔA_{420nm} 10^{-4}/s. For the relative activity study, the increase of absorbance was measured at 420 nm for the following substrates used as 0·01 M: 4-methyl catechol, catechol, pyrogallol, L-dopa, chlorogenic acid, caffeic acid and *p*-cresol. The increase of absorbance was also measured at 420 nm for tyrosine (0·0015 M) and at 460 nm for dopamine (0·01 M).

Enzyme purification

Ultrafiltration
The crude extracts were concentrated twice by ultrafiltration on a PM 10 Diaflo membrane.

Gel filtration chromatography
Concentrated crude extract (50 ml) was applied to a Sephadex G 100 column (1·5 cm × 85 cm) equilibrated with 0·1 M McIlvaine buffer, pH 7·0. Fractions of 9 ml were collected and assayed for polyphenol oxidase activity.

DEAE–cellulose chromatography
Fractions containing polyphenol oxidase activity collected after gel filtration chromatography (about 25 ml) were applied to a DEAE–cellulose DE 32 (Whatman) column (3·2 cm × 20 cm) equilibrated with 0·1 M McIlvaine buffer, pH 7·5. After elimination of non-retained proteins, elution was performed with a linear gradient of NaCl from 0·01 M to 0·5 M in the same buffer.

Protein denaturation

Proteins were quantitatively analysed according to the method of Lowry *et al.* (1951), using bovine serum albumin as a standard, either directly or after precipitation with trichloracetic acid. If $(NH_4)_2SO_4$ was present in the fractions eluted after chromatographic separations, proteins were estimated by absorbance determination at 280 nm.

Gel electrophoresis

Acrylamide gel electrophoresis (acrylamide 7·5%) was carried out according to Davis (1964) with a Tris–glycine buffer, pH 8·3. Enzymatic activity was revealed using catechol (0·125 M) in 0·1 M McIlvaine buffer, pH 6·5, as substrate.

Optimum pH

The polyphenol oxidase activity was measured under standard conditions in 0·1 M McIlvaine buffer with pH varying from 4·0 to 7·0.

Molecular weight determination

The apparent molecular weight was determined by gel chromatography according to the method of Laurent and Killander (1964). A column of Sephadex G 100 (1·5 cm × 85 cm) was equilibrated with 0·1 M McIlvaine buffer, pH 7·0, and calibrated with chymotrypsinogen (25 000), ovalbumin (45 000) and bovine serum albumin (67 000) monomeric and (134 000) dimeric.

Thermal inactivation

Enzymatic solutions (0·5 ml) in 0·1 M McIlvaine buffer, pH 6·5, were incubated for various times from 5 to 60 min at 60, 70, 75 and 80°C. The residual activity was then determined under standard conditions.

Dopamine extraction

The method described by Todoriki *et al.* (1983) for the determination of catecholamines in rat brain was used.

Dopamine analysis

The extract obtained from plantain pulp was derivatized using *o*-phthalaldehyde (OPA) (Todoriki *et al.*, 1983) and analysed by high-performance liquid chromatography (HPLC). An Analprep 93 pump (Touzart et Matignon) fitted with a Rheodyne 7125 injection valve, a Brownlee C_{18} Spheri 5 column, 250 μm × 4·6 mm, thermostated at 50°C, a UV 50 detector (Varian) operated at 290 nm and a Shimadzu CR 1 B integrator, were used. The mobile phase was acetonitrile–0·05M monochloroacetate buffer, pH 2·5 (35:65, v/v) at 1·0 ml/min. Dopamine was identified by comparison of the retention time for the OPA derivative of the compound present in plantain extract with that of the derivative obtained from an authentic sample of dopamine. The OPA derivative of dihydroxybenzylamine was used as an internal standard for quantitative determination.

Enzyme inhibition

The inhibition effect of metallic ion complexing agents (potassium cyanide and sodium diethyldithiocarbamate) or of reducing agents (L-cysteine, sodium metabisulphite and ascorbic acid), at 10^{-5}–10^{-2} M, was studied under the conditions used for enzymatic assay, with catechol (0·125 M) as substrate.

Browning measurement

The method described by Guadagni *et al.* (1949) was used. Plantain pulp (3·5 g) or plantain flour (10 g) obtained after treatment with sodium metabisulphite (0·1 M), drying (80°C for 12 h) and crushing, or obtained after treatment at 80°C for 8 min, drying (80°C for 12 h) and crushing, was mixed with 25 ml of distilled water in a Waring blender. The homogenate was filtered through a Buchner funnel and 1 ml of the filtrate was transferred to the cuvette of a spectrophotometer. The change in per cent transmittance was followed at 475 nm over a 30-min period at 25°C using distilled water as reference.

RESULTS AND DISCUSSION

Evidence for free and bound activities

The use of detergents, and especially of Triton X 100, shows the presence in plantain of a bound form for this enzyme (Table 1), whereas only the free form of plantain polyphenol oxidase was studied in a previous work (Ngalani & Crouzet, 1986). These two forms are present during the maturation of the fruit; the total activity increases from the green stage to the yellow–brown stage of peel coloration. On the other hand, the difference between the free and bound activities decreases between these two stages. As indicated in Table 1, the activity of the two forms is of the same order of magnitude at the yellow–brown stage, whereas the value obtained for the bound form is more than 10 times the value obtained for the free form at the green stage. The increase of the percentage of soluble activity with increased maturity of the fruits is indicative of the fact that soluble plantain polyphenol oxidase originates in the particulate fraction. This result is in good agreement with previously reported data for apple and olive (Harel *et al.*, 1966).

Table 1. Free and bound polyphenol oxidase activity during the maturation of plantain fruit

Maturity stage	Total activity (AU)		Activity (%)	
	Free	Bound	Free	Bound
Green	11·5	174	6	94
Green–yellow	61	270	18	81
Yellow	150	380	28	71
Brown–yellow	360	432	45	54

The bound polyphenol oxidase is extracted using Triton X 100 (1%). AU—Arbitrary unit; change of 10^{-4} absorbance units a second.

Partial purification of plantain polyphenol oxidase

Gel filtration on Sephadex G 100

The crude extract previously concentrated by ultrafiltration using a Diaflo membrane, PM 10, was layered on the surface of a Sephadex G 100 column equilibrated with 0·1 M McIlvaine buffer, pH 7·0. The elution pattern obtained for the soluble form is given in Fig. 1. This pattern is identical to that obtained for the bound form. Two peaks exhibiting the polyphenol oxidase activity E_1 and E_2 were detected as shown in Fig. 1. The multiplicity of plant polyphenol oxidase resulting from aggregation of subunits of the same enzyme has been reported by several workers (Harel & Mayer, 1971).

Ion exchange chromatography on DEAE–cellulose

The fractions E_1 and E_2 containing the activity were pooled and applied onto a DEAE cellulose column previously equilibrated with 10 mM McIlvaine buffer, pH 7·5. The column, washed with 275 ml of this buffer, was then developed with a linear gradient of NaCl from 0·01 to 0·5 M. The elution pattern obtained (Fig. 2) was the same for the two fractions E_1 and E_2 isolated by gel chromatography.

Three major protein fractions, D_1–D_3, with a polyphenol oxidase activity, were detected: however, two of them were eluted before the application of the concentration gradient. The greatest activity was located in the D_3 fraction. As indicated by gel electrophoresis, only this fraction exhibits a band (R_f value 0·17) with polyphenol oxidase activity matching the greater band detected in the E_2 fraction isolated by gel filtration.

No difference between the free and bound forms of plantain polyphenol oxidase could be found during the two steps of the purification process. These results are

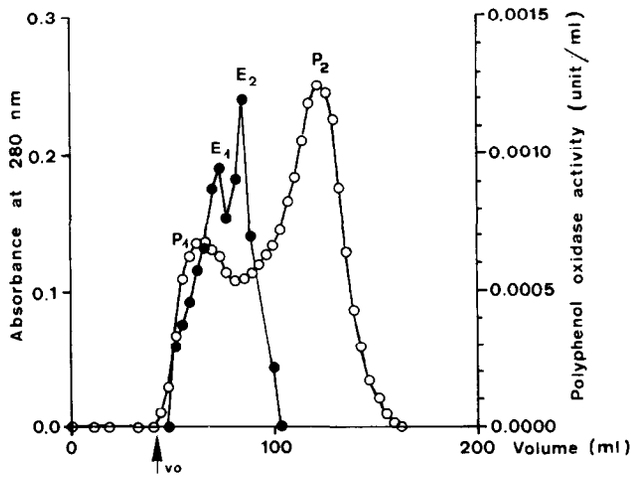

Fig. 1. Elution pattern of enzyme extract on Sephadex G 100 column (1·5 cm × 85 cm). ○ Protein concentration; ●, polyphenol oxidase activity. Elution buffer was 0·1 M McIlvaine buffer, pH 7·0.

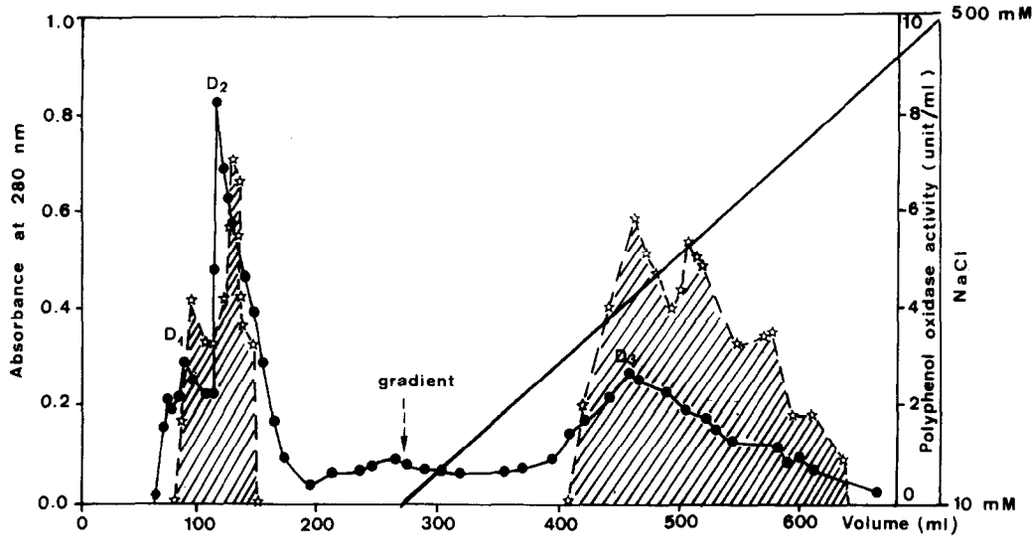

Fig. 2. Elution pattern of active Sephadex G 100 extract on DEAE–cellulose column (3·2 cm × 20 cm). ●, Protein concentration; ☆, polyphenol oxidase activity. Elution buffer was 0·1 M McIlvaine buffer, pH 7·0. D_1, D_2, D_3; fractions with a polyphenol oxidase activity.

in good agreement with those previously reported on the increase of the relative percentage of the free form during maturation of the fruit.

The results of the purification are reported in Table 2; the plantain polyphenol oxidase was partially purified (eight-fold) from the crude extract with a yield of 36%. The purification obtained was considered sufficient for the characterization of the enzyme and the inhibition studies.

Table 2. Purification of plantain polyphenol oxidase

Step	Total proteins (mg)	Total activity (AU)	Specific activity (AU/mg)	Fold purification	Yield (%)
Crude extract	42	50 721	1207	1	100
Sephadex G 100	10·1	44 830	4438	3·6	88
DEAE–cellulose	1·9	18 183	9570	7·9	36

Characterization of plantain polyphenol oxidase

Optimum pH

The optimum pH was 6·5 when catechol was used as substrate. Several studies have shown that optimum pH for polyphenol oxidase is dependent on the nature of the plant tissue studied and the nature of the substrate used. However, the values obtained vary from 4·0 to 7·5 (Palmer, 1963; Kahn, 1977).

Molecular weight

The apparent molecular weights estimated by gel filtration were, respectively, 30 000 ± 5000 for fraction E_1 and 70 000 ± 5000 for fraction E_2. In these conditions, the assumption that plantain polyphenol oxidase is present in multiple forms is strengthened, and this result agrees with previously reported data (Harel *et al.* 1966; Harel & Mayer, 1971).

Thermal stability

The residual per cent activity of the enzyme for various inactivation times and temperatures from 60 to 75°C is shown in Fig. 3. Two phases may be distinguished from the inactivation curves: an initial step with a fast loss of activity, followed by a second step characterized by a slow decrease of the activity as a function of time. This phenomenon has been observed during the thermal inactivation of several fruit polyphenol oxidases (Padron *et al.*, 1975).

These curves can be analysed by assuming the presence of two groups of isoenzymes in plantain polyphenol oxidase, one heat-labile and the other heat-resistant (Ling & Lund, 1978). From the experimental data, only the activation energy for the thermal destruction of heat-resistant (R) isoenzyme, E_{AR} = 18 kJ/mol. could be determined using the Arrhenius equation. This value is in good agreement with those reported for heat-resistant banana enzyme, E_{AR} = 18·4 kJ/mol (Padron *et al.*, 1975).

Relative activity

The relative activities of plantain polyphenol oxidase towards several monophenols and *ortho*-diphenols are given in Table 3. These results, which show that this enzyme had good activity relative to *o*-diphenols,

Table 3. Relative activities of plantain polyphenol oxidase towards some monophenols and *ortho*-diphenols

Substrate	Concentration (M)	Total activity (AU)	Relative activity (%)
4-Methyl catechol	0·01	95	100
Catechol	0·01	32·5	34
Pyrogallol	0·01	25	26
Dopamine	0·01	20·5	21
L-Dopa	0·01	18	19
Chlorogenic acid	0·01	2·5	2·6
Caffeic acid	0·01	1·0	1·05
p-Cresol	0·01	0	—
L-Tyrosine	0·001 5	0	—

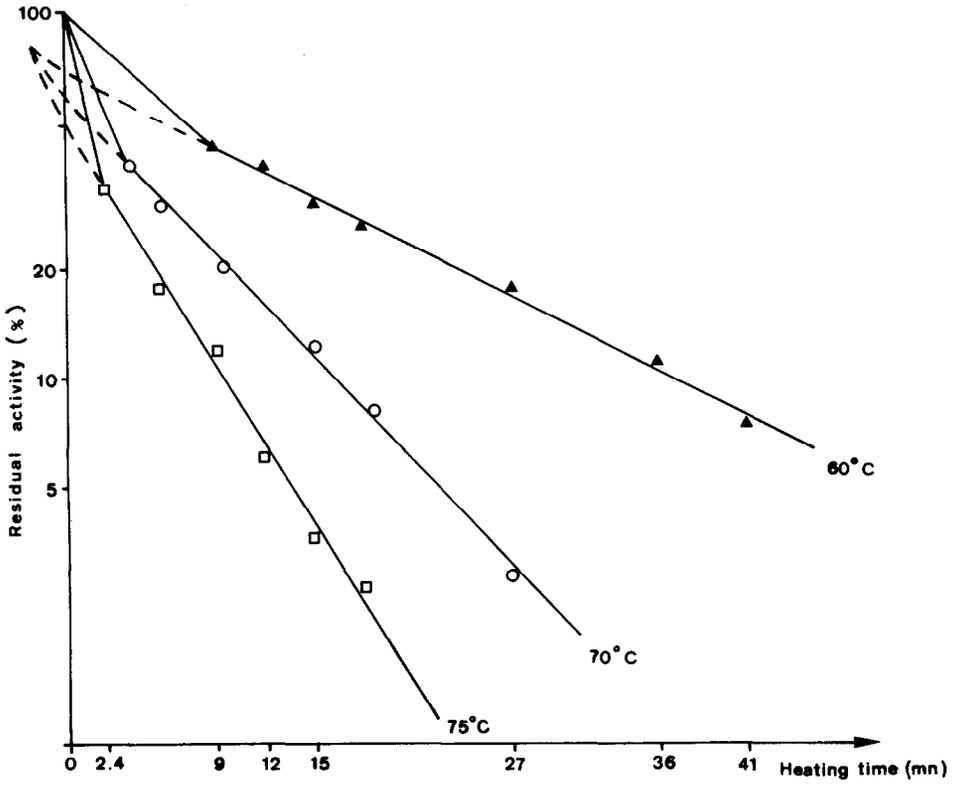

Fig. 3. Thermal inactivation of plantain polyphenol oxidase at 60°C, 70°C, and 75°C.

except chlorogenic and caffeic acids, but not to monophenols such as *p*-cresol and L-tyrosine, are in good agreement with previously reported data for pear or banana (Galeazzi & Sgarbieri, 1981). With regard to *o*-diphenols, the greatest activity was found for 4-methyl catechol. Very low activities were found for chlorogenic and caffeic acids, whereas activities varied from 20 to 35% for the other compounds studied.

The values obtained for dopamine and dopa are of particular interest, in view of the fact that these two compounds have been described as preferential substrates for banana polyphenol oxidase (Palmer, 1963). Dopamine, present at 8 μg/g of fresh matter in banana pulp, is considered as a natural substrate of banana enzyme (Waalkes *et al.*, 1958; Udenfriend *et al.*, 1959; Griffiths, 1961). However, to our knowledge this compound has not been previously reported among amines identified in plantain pulp (Udenfriend *et al.*, 1959). Under these conditions, it was necessary to search for the presence of dopamine in plantain.

Determination of dopamine in plantain

The chromatogram obtained from the extract of plantain pulp prepared as indicated in the experimental section (Todoriki *et al.*, 1983) is given in Fig. 4(a). The presence of dopamine was established by comparison of the retention times of OPA derivatives of the compounds isolated from plantain pulp with an authentic sample of dopamine (Fig. 4(b)).

Quantification using the OPA derivative of dihydrobenzylamine as internal standard shows that dopamine is present in plantain pulp at 2 μg/g of fresh matter. Because of this low content (four times less than the content found for banana) and the low activity of plantain polyphenol oxidase towards dopamine, it may be assumed that this compound is not critical in the enzymatic browning observed during processing of plantain pulp.

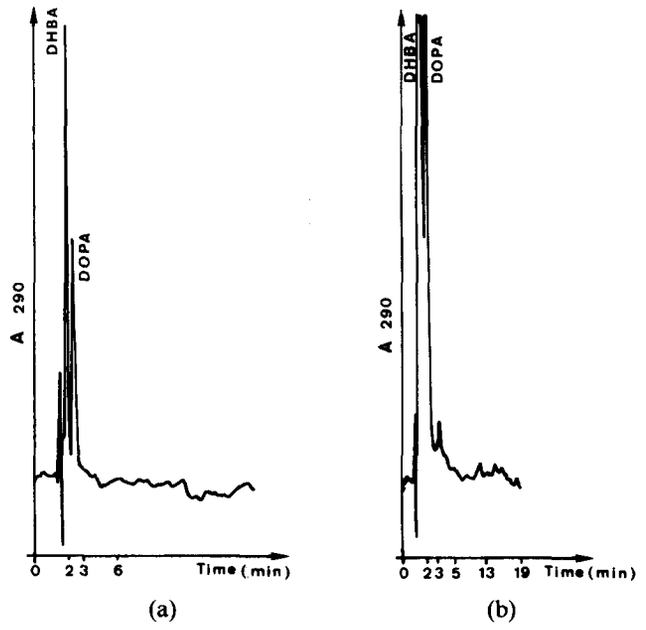

Fig. 4. HPLC chromatograms. (a) *o*-Phthalaldehyde (OPA) derivative of plantain pulp extract with dihydroxybenzaldehyde (internal standard) added; (b) OPA derivatives of dihydroxybenzaldehyde and dopamine. A Brownlee C_{18} Spheri 5 column, 250 mm × 4·6 mm, thermostated at 50°C was used. The mobile phase was acetonitrile–0·05 M monochloroacetate buffer, pH 2·5 (35:65, v/v), at 1·0 ml/min. UV detection at 290 nm.

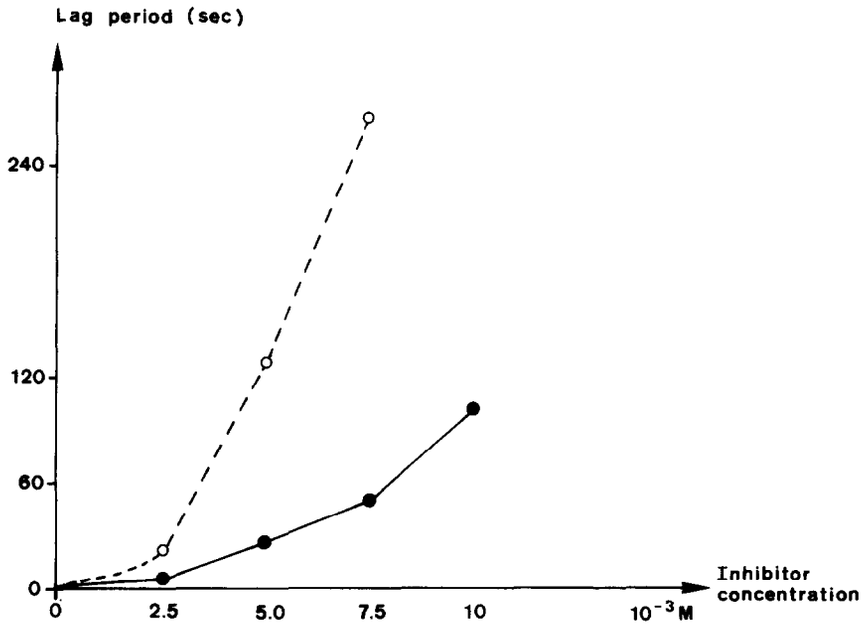

Fig. 5. Effect of inhibitor concentration on the lag period inhibition, for the plantain polyphenol oxidase. ○, Ascorbic acid; ●, sodium metabisulphite.

Action of inhibitors

Two categories of enzyme inhibitors, using catechol as substrate, were studied—metallic ion complexing agents and reducing agents (Table 4). For the first class of inhibitors, the results obtained show that 49% inhibition was obtained for KCN (10^{-2} M), whereas total inhibition was reached for a similar concentration of sodium diethyldithiocarbamate. This result, in agreement with previously reported data (Harel & Mayer, 1971; Roudsari *et al.*, 1980), indicates that plantain polyphenol oxidase could belong to the metalloenzyme class. For reducing agents, total enzyme inhibition was achieved for the various compounds used at 10^{-3} M. At concentrations lower than 10^{-3} M a more or less extensive lag period, followed by an increase of activity, was noted. The extent of the lag period increases with increase of inhibitor concentration (Fig. 5). This phenomenon has been reported previously (Varoquaux *et al.*, 1977).

Table 4. Effect of inhibitors on plantain polyphenol oxidase activity

Inhibitor	Concentration (M)	Total Activity (AU)	% Inhibition
Potassium cyanide	10^{-2}	34	49
	10^{-3}	54	20
	10^{-4}	56	16
	10^{-5}	58	11
Sodium diethyl dithiocarbamate	10^{-2}	0	100
	10^{-3}	0·35	99·5
	10^{-4}	29	57
	10^{-5}	60	10·5
L-Cysteine	10^{-3}	0	100
	10^{-4}	40	40
	10^{-5}	58	13·5
Sodium metabisulphite	10^{-3}	0	100
	10^{-4}	10	84·5
	10^{-5}	35	48
Ascorbic acid	10^{-3}	0	100
	10^{-4}	26	61·5
	10^{-5}	33	55·5

The sodium metabisulphite or thermal treatment effects on plantain pulp or purée browning were determined by measuring the decrease of transmittance at 475 nm (Guadagni *et al.*, 1949). The transmittance determined for the filtrate obtained from pulp treated with sodium metabisulphite (10^{-2} M) was 98%. This value decreases to about 78% after thermal inhibition of the enzyme at 80°C for 8 min and chilling to 4°C. The increase in absorbance occurring during pulp blanching may be attributed to the non-enzymatic browning. In the absence of inactivation treatment, transmittance decreases to 50%. On the other hand, the decrease of transmittance noted for flour obtained from pieces of plantain cut in the presence of sodium metabisulphite and dried at 80°C for 12 h was only 90%. In all cases, no significant change in transmittance was found over an 80-min time period.

It may be assumed that the metabisulphite present prevents non-enzymatic browning during the slicing and drying steps. These results show that the most important problem is enzymatic browning, and that the enzyme may be efficiently inactivated using sodium metabisulphite (Galeazzi & Sgarbieri, 1981). In these conditions, sodium metabisulphite was considered as an acceptable inhibitor, and this compound was used concurrently with thermal inactivation for polyphenol oxidase inhibition in the process of plantain flour preparation (Ngalani, 1989).

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