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Browning in processed yams: peroxidase or polyphenol oxidase?

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Abstract: Polyphenol oxidase and peroxidase were purified from white yam (*Dioscorea rotundata*) using DEAE-cellulose ionexchange chromatography. Thermoinactivation curves for polyphenol oxidase showed monophasic kinetics, while those for peroxidase were biphasic. Urea partially stabilised peroxidase against irreversible thermoinactivation, but did not do so in the case of polyphenol oxidase. Only peroxidase was capable of regenerating activity after thermoinactivation. The results showed that thermoinactivation of peroxidase was mainly due to conformational changes, while that of polyphenol oxidase was probably due to covalent damage. Peroxidase reactivation might play an important role in the browning of processed yam.

Keywords: *Dioscorea rotundata*; white yam; polyphenol oxidase; peroxidase; thermoinactivation; reactivation

INTRODUCTION

Despite the importance of yam as a major food crop in West Africa, its postharvest biochemistry has been poorly studied.[1] As a consequence, postharvest losses due to sprouting, physical damage, pests and pathogens have remained relatively high.[2]

One relatively successful procedure for improving the postharvest storage of white yam (*Dioscorea rotundata*) tubers involves processing into flour. This consists of (1) peeling off the outer covering, (2) cubing, (3) boiling in distilled water for 10–15 min, (4) drying and (5) milling into flour. Unfortunately, consumer acceptability and the shelf-life of the flour are adversely affected by the inability of the flour to retain the typical white colour of yam which is highly desired by consumers.[3,4]

Colour changes in freshly damaged plant materials have been attributed to the activity of polyphenol oxidase (PPO; *o*-diphenol:O_2 oxidoreductase, EC 1.10.3.2), which catalyses the oxidation of polyphenols to *o*-quinones.[5,6] Peroxidase (Px; donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7) catalyses the oxidation of a number of aromatic compounds and has been associated with darkening in fresh and processed vegetables and fruits.[7] Both PPO and Px are known to be highly heat-resistant but each displays a different capability for reactivation after thermoinactivation.[5,8–11]

In *D rotundata*, which contains PPO,[3,4,12] correlating PPO activity with tissue browning was shown to be contraindicative.[13] The purpose of the present study was to relate the thermoinactivation processes of PPO and Px to the discolouration of processed yam.

MATERIALS AND METHODS

Materials

Freshly harvested and uninfected yams were purchased from the local market.

Methods

Enzyme purification

PPO was extracted and purified in 0.025 M sodium phosphate buffer (pH 7.5) according to the procedure of Chilaka *et al.* [6] In the case of Px, yams were peeled, cubed and homogenised in ice-cold 0.025 M Tris-chloride buffer (pH 8.0). After centrifugation at 20 000 × *g* for 15 min at 4 °C, the supernatant was applied directly to a DEAE-cellulose column (9.5 × 5.0 cm id) pre-equilibrated with buffer. Gradient elution was employed using 0.0–0.5 M NaCl; 5 ml fractions were collected. Active fractions were pooled, desalted on a Sephadex G-25 column (25.0 cm × 2.0 cm id) and stored at −16 °C.

Enzyme assay

Assay of polyphenol oxidase activity was performed as already described.[6] One unit of polyphenol oxidase activity is defined as a change in absorbance of 0.001 min^{-1} at 400 nm using 3.3 mM catechol solution prepared in 0.1 M sodium phosphate buffer (pH 7.0). Assay of peroxidase activity was also performed as previously described.[14] The assay mixture contained 0.1 ml of 5% *o*-dianisidine, 0.1 ml of 0.1 M hydrogen peroxide, 2.7 ml of 0.05 M sodium acetate buffer (pH 5.4) and 0.1 ml of suitably diluted enzyme. One unit of peroxidase activity is defined as a change in absorbance of 1.0 min^{-1} at 460 nm ($\Delta OD_{460}\,min^{-1}$) at 25 °C

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due to oxidation of *o*-dianisidine in the presence of H_2O_2.

Thermoinactivation procedure

Polyphenol oxidase was incubated at 30, 40, 50, 60, 70 and 80 °C in 0.1 M sodium phosphate buffer (pH 7.0) in a temperature-controlled water bath. To minimise the lag phase, 4 ml of buffer was preheated to the required temperature and 1 ml of enzyme was added and rapidly mixed. At suitable intervals, samples (usually 0.1 ml) were withdrawn for activity determinations. At 60 °C the enzyme was incubated in the presence and absence of 8 M urea. This procedure was repeated for peroxidase, but incubation was in 0.05 M sodium acetate buffer (pH 5.40).

RESULTS AND DISCUSSION

Table 1 shows that yam PPO was purified about 35-fold over the crude enzyme extract with about 36% recovery of initial PPO activity. In the case of peroxidase, purification was about 23-fold with about 22% activity recovery. Ion exchange revealed no isoenzymic forms of either PPO or Px. Three isoenzymic forms of PPO were previously found in Amasya apple[16] but only one in yam.[3,17] Peroxidases from many sources are known to exist as isoenzymes.[18,19]

Table 2 presents a summary of the properties of yam PPO and peroxidase. The properties of yam PPO are similar to those already described.[3,17] The properties of yam peroxidase are similar to those of peroxidases from *Ficus gabarata* and mung bean.[18,21] It is important to note that thiourea was a good inhibitor of both PPO and peroxidase, with low K_i values of 0.15 and 0.41 mM respectively. However, while inhibition with PPO was uncompetitive, thiourea inhibited peroxidase competitively. In the case of peroxidase the H_2O_2 and thiourea may be binding to the same site on the enzyme and thus competitive inhibition occurs.

Fig 1 shows the time curves for the thermoinactivation of PPO and peroxidase. The results indicate that the inactivation of polyphenol oxidase was monophasic while that of peroxidase was biphasic, consisting of a fast initial phase and a slower second phase. Also, there was no measurable PPO activity after 20 min at 70 °C, while measurable peroxidase activity was still obtained after 60 min at 80 °C, indicating that the peroxidase was more stable than the PPO. Monophasic inactivation kinetics refers to a single-step irreversible process involving two enzyme forms of the type[22]

native enzyme → denatured enzyme

Biphasic curves suggest a multi-step process involving stable intermediate(s). In this regard, two-step-in-series models have been proposed.[22–24] In each case the native enzyme, in a first step, undergoes a reversible or irreversible transition, yielding an equilibrium distribution or population of at least two intermediate forms of the enzyme with entirely different stabilities and kinetic behaviours. This is then followed by a reversible or irreversible second step yielding the denatured state. For yam peroxidase it is possible that the biphasic inactivation curves might represent a multi-step process involving stable intermediate(s) of different stabilities. However, there are suggestions that biphasic inactivation kinetics can be attributed to the presence of peroxidase isoenzymes with different heat sensitivities.[10,14] The purification of yam peroxidase by DEAE anion exchange chromatography did not reveal the presence of other anionic isoenzymes, thus probably excludings the presence of other isoenzymes as a reason for the biphasic inactivation of yam peroxidase. Perhaps biphasic inactivation kinetics is an inherent property of the enzyme molecule and may reflect an adaptive and protective mechanism towards heat inactivation. The initial fast phase may represent the inactivation of the native enzyme and the slower second step that of the more stable intermediate(s).

Yam PPO can be completely inactivated after 30 min of incubation at 70 °C and yam peroxidase after 2 h of incubation at 80 °C. Fig 2 shows that yam peroxidase was reactivated 36 h after thermoinactivation. However, PPO showed no reactivation. This suggests that while in PPO there was only irreversible inactivation, both irreversible and reversible inactivation occurred in peroxidase. Regeneration of activity after thermoinactivation is common among plant peroxidases and has been associated with deterioration

Purification step	Total protein[a] (mg)	Total activity[b] (kilounits)	Yield (%)	Specific activity (kilounits mg^{-1} protein)	Purification factor
PPO					
Crude enzyme	900	14000	100	15.56	1
DEAE-cellulose	9.2	5000	35.71	54.35	34.9
Px					
Crude enzyme	950	4600	100	4.93	1
DEAE-cellulose	8.0	1000	21.74	125	23.35

Table 1. Purification of polyphenol oxidase (PPO) and peroxidase (Px) from yam tubers

[a] Protein estimation was by the method of Lowry *et al.*[15]

[b] One unit of PPO activity is defined as a change in absorbance of 0.001 min^{-1} at 400 nm. One unit of Px activity is defined as a change in absorbance of 1.0 min^{-1} at 460 nm.

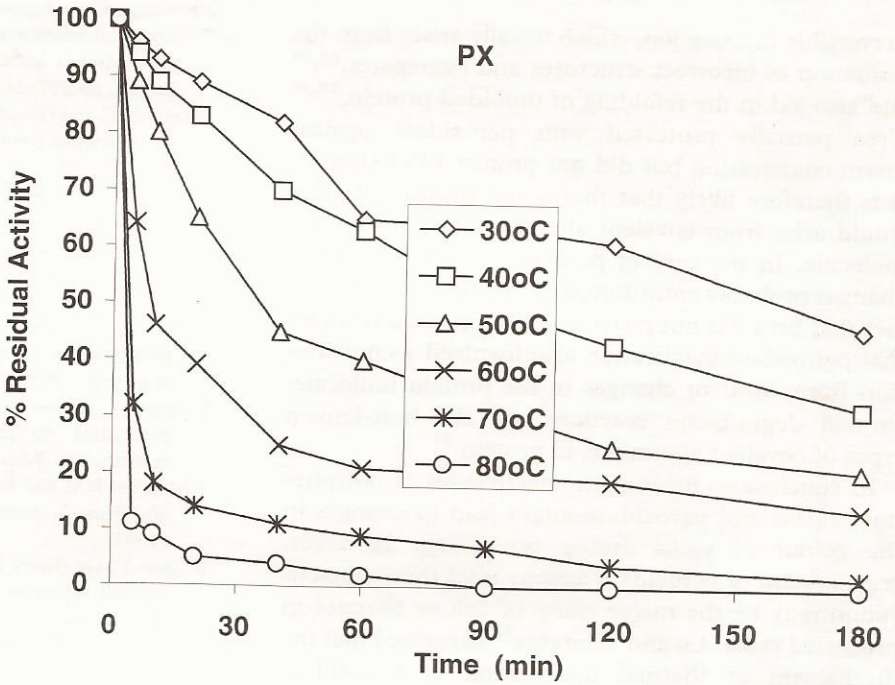
Property[a]	*Polyphenol oxidase*	*Peroxidase*
K_m	10.25 mM	1.03 mM
V_{max}	180 kilounits mg^{-1} protein	21.50 units mg^{-1} protein
Inhibition by thiourea	Uncompetitive	Competitive
K_i(thiourea)	0.15 mM	0.41 mM
M_r	100k	41k
Optimum pH	7.0	6.5
Optimum temperature	40 °C	45 °C

[a] K_m, V_{max}, mode of inhibition and K_i were determined from Lineweaver–Burk plots of initial velocity data and their appropriate replots. Substrates used were catechol for PPO and H_2O_2 for Px. M_r was determined by gel filtration of purified enzyme using a Sephadex G-100 column (67 cm × 2.5 cm id) equilibrated with appropriate extraction buffer.[20]

Table 2. Properties of Polyphenol oxidase and Peroxidase from yam tubers

of food quality during storage.[8–10,25,26] Reports of PPO reactivation are rare.[11]

Irreversible thermoinactivation can be due to either conformational or covalent changes.[27] Reversibly inactivating agents such as urea and guanidine hydrochloride protect enzymes against conformational



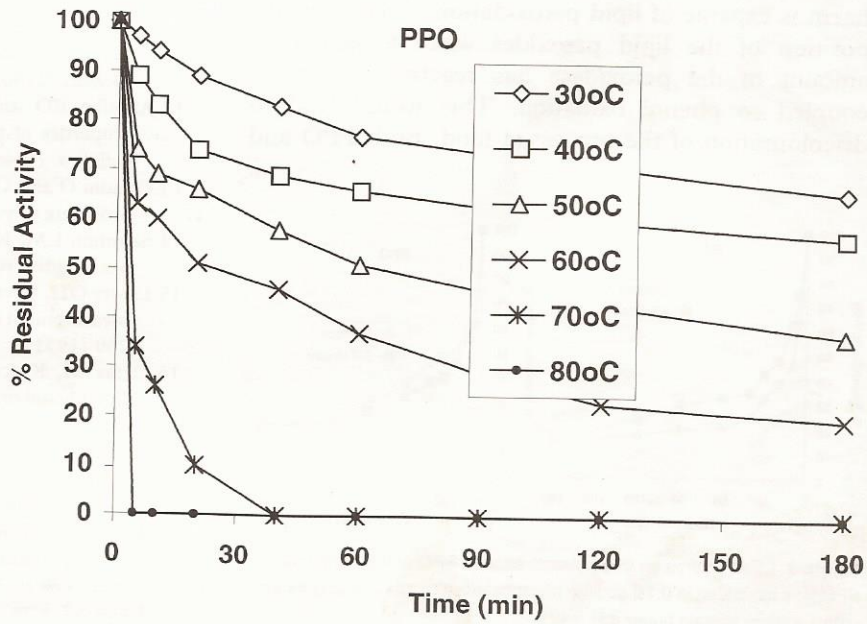


Figure 1. Thermoinactivation of yam PPO and Px. PPO was inactivated in 0.1 M sodium phosphate buffer (pH 7.0) and Px in 0.05 M sodium acetate buffer (pH 5.40).

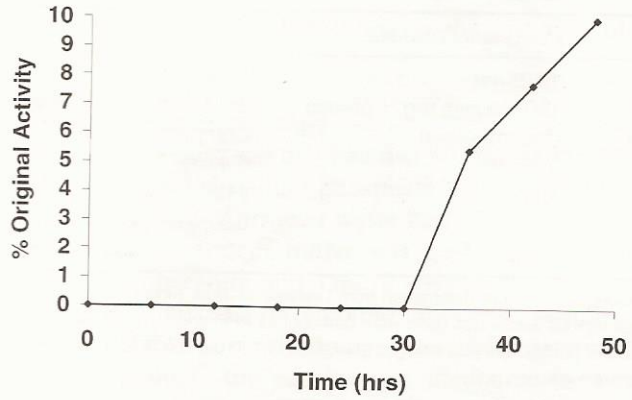


Figure 2. Regeneration of peroxidase activity. Inactivation was by incubation at 80°C for 2h. The enzyme was then cooled directly to 25°C and its activity monitored for 48h.

irreversible inactivation, which usually arises from the formation of incorrect structures and aggregates,[24,28] and also aid in the refolding of unfolded protein.[29,30] Urea partially protected yam peroxidase against thermoinactivation but did not protect PPO (Fig 3). It is therefore likely that thermoinactivation of PPO would arise from covalent alterations in the enzyme molecule. In the case of peroxidase, conformational changes probably contributed to the inactivation. The fact that urea did not provide 100% protection shows that peroxidase inactivation also involved a contribution from covalent changes in the protein molecule. Protein degradation reactions are the best-known types of covalent alterations in protein.[31]

In conclusion, incomplete inactivation of polyphenol oxidase and peroxidase might lead to changes in the colour of yams during processing. However, regeneration of peroxidase activity after thermoinactivation may be the major cause of colour changes in processed yams. Lu and Whitaker[32] suggested that the mechanism of thermal inactivation of peroxidase involves the release of the haem moiety. The exposed haem is capable of lipid peroxidation.[25] The decomposition of the lipid peroxides when a significant amount of the peroxidase has reactivated can be coupled to phenol oxidation. This would lead to discolouration of the processed food. Both PPO and peroxidase are inhibited by thiourea. Thiourea has been used to keep fruits and vegetables from darkening.[25] It is suggested that the use of thiourea could reduce colour changes arising from enzyme-catalysed browning during and after yam processing. Luckily, the low K_i of the enzyme for thiourea means that low concentrations could be used during processing. This ensures that organoleptic consumer idiosyncrasies, arising from high concentrations of chemical preservatives and which may adversely affect acceptability of the processed yam, are not elicited.

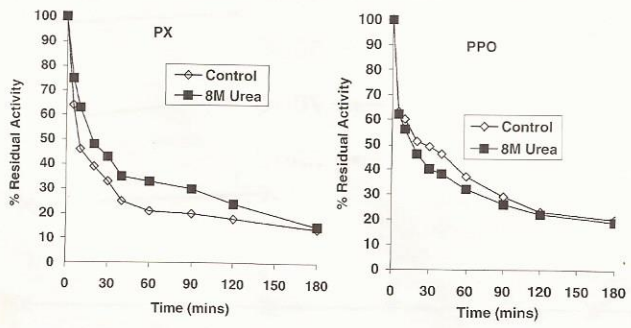


Figure 3. Effect of urea on thermoinactivation of PPO and Px at 60°C. PPO was incubated in 0.1 M sodium phosphate buffer (pH 7.0) and Px in 0.05 M sodium acetate buffer (pH 5.40).

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