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Hydrogen donor specificity of mango isoperoxidases

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Purified mango cationic and anionic isoperoxidases catalyse the oxidation of guaiacol, *o*-dianisidine and 2,2'-azino-di-3-ethyl-benzothiazoline sulphonate (ABTS) at different rates. For an anionic isoperoxidase (A1) *o*-dianisidine is the most susceptible substrate, whereas for a cationic isoperoxidase (C1) ABTS is oxidised more rapidly. For indoleacetic acid (IAA), mixtures of anionic isoperoxidases catalysed the oxidation at higher rates than cationic isoperoxidases relative to their peroxidase activity towards *o*-dianisidine. For four purified isoenzymes the greatest rate of oxidation of IAA was found for A1. For dihydroxyfumarate (DHFA), mixtures of cationic isoperoxidases catalysed the oxidation at approximately twice that observed for anionic isoperoxidases per unit of peroxidase activity. Likewise, purified mango cationic isoperoxidases (C1 and C2) oxidised DHFA at a rate three times greater than that for the anionic isoperoxidases of identical peroxidase activity towards *o*-dianisidine. The mango anionic peroxidases have a greater molecular mass than the cationic isoenzymes. Molecular weights determined by gel filtration of 40 000, 44 000, 22 000 and 27 000 were found for mango isoperoxidases A1, A2, C1 and C2.

INTRODUCTION

Peroxidases (E.C. 1.11.7 donor: hydrogen peroxide oxidoreductase) are found as haem enzymes in many fruits and vegetables. They occur in both a soluble and a bound form (Kahn *et al.*, 1981; Wang & Luh, 1983; Da Silva *et al.*, 1990) which is associated with insoluble plant cell wall material and probably certain organelles (Schloss *et al.*, 1987; Ros Barcelo *et al.*, 1989; Abeles & Biles, 1991). In all fruits and vegetables peroxidases seem to occur in a wide range of isoenzyme forms with p*I* values varying from approximately 3.5 to 10.0. Depending on the p*I* value the isoperoxidases have also been described as basic or cationic and anionic. Based on recent research with horseradish peroxidases the main groups of isoperoxidases are acidic, neutral and basic with p*I* values of 4–5, 6–7 and 7–10 respectively (Bartonek-Roxa *et al.*, 1991). Not only have isoperoxidases been shown to differ in their sensitivity to heat (Robinson, 1990), but they also catalyse the oxidation of a number of different hydrogen donors some of which are frequently used as test substrates (Gaspar *et al.*, 1982; Garcia-Florenciano *et al.*, 1991). These have included ascorbic acid, guaiacol, *o*-dianisidine 2,2'-azino-di-3-ethyl-benzothiazoline sulphonate (ABTS), and recently 4-methyl-*a*-naphthol (Ferrer *et al.*, 1990), indole-3-acetic acid (IAA), and dihydroxyfumaric acid (DHFA). For the latter two substrates, oxidation can occur in the absence of hydrogen peroxide via an oxygenase-type reaction where oxygen is used as an electron acceptor. It is generally agreed that both the peroxidatic and oxidatic activity involve the same haem moiety. The oxidation of IAA was first reported by Kenten (1955). The use of labelled oxygen has shown that the products of IAA oxidation are 3-hydroxymethyl-indole and 3-hydroxymethyl-oxindole arising from a skatole-free radical (Munoz *et al.*, 1991) and the incorporation of one atom of oxygen from O_2 and not water (Grambow, 1986). Stonier (1972) and Stonier *et al.* (1979) claimed that IAA catabolism was catalysed by basic isoperoxidases but more recently this has been disputed by Garcia-Florenciano *et al.* (1991). For oxidation of DHFA, one mole of O_2 is taken up per mole of DHFA oxidised and the mechanism of the reaction is believed to involve DHFA free radicals (Halliwell, 1977). The initial amount of hydrogen peroxide required for the formation of compound I is believed to be generated by the non-catalytic autoxidation of DHFA. Recently, a possible general model for the mode of oxidation of DHFA has been proposed by Pedreno *et al.* (1990).

Both the physiological functions and the mechanism of the effect of peroxidase action on food quality are not well documented, due to the complexity of the

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isoenzymes and their differential distribution at the subcellular level. In some plant foods, peroxidase activity is quite high and has been reported in processed products (Buescher & McGuire, 1986; Buescher *et al.*, 1987). During catalysis, peroxidase is first oxidised by hydrogen peroxide to compound I, when the haem is in oxidation state V. A very broad specificity is shown by compound I towards aromatic compounds which are released from the enzyme as oxidised free radicals. This accounts for the wide range of physiological functions and the diverse effects on food quality by peroxidase-catalysed oxidation of phenolics and other compounds susceptible to oxidation and polymerisation by the generated free radicals. It has been proposed for horseradish peroxidase that the aromatic electron donors are oxidised at the haem edge of the enzyme (Ator *et al.*, 1989). The peroxidatic and oxygenase reactions have been reviewed by Grambow (1986) and Robinson (1991) and re-examined by Munoz *et al.* (1991).

Although it is still difficult to purify sufficient amounts of the individual isoperoxidases for compositional analysis directly from plant material, recently the authors have been able to obtain sufficient mango isoperoxidases (Khan & Robinson, 1993*a*) in order to compare their enzymic activity towards test substrates. Their homogeneity has been established by isoelectric focusing. In one case, for the mango A1 isoperoxidase, the absence of other proteins prior to amino acid analysis has been established (Khan & Robinson, 1993*a*). Here, the catalytic activity is reported of a number of purified mango isoperoxidases towards *o*-dianisidine, guaiacol, indole-3-acetic acid and dihydroxyfumaric acid used as test substrates.

MATERIALS AND METHODS

The isoperoxidases were purified chromatographically from fresh ripe mangoes (var. *chaunsa*), assayed and classified electrophoretically as described by Khan and Robinson (1993*a,b*).

Enzyme assays

o-Dianisidine assay
The reaction mixture contained 2.7 ml of 0.03% (w/v) hydrogen peroxide in 10 mM phosphate buffer (pH 6.0) plus 9.2 ml of the peroxidase extract. The enzymic reaction was initiated by the addition of 0.1 ml, 1% (w/v) *o*-dianisidine solution and the initial change in absorbance was recorded at 460 nm at 25°C.

Guaiacol assay
The reaction mixture contained 1.4 ml 0.1% (w/v) hydrogen peroxide in 100 mM sodium acetate buffer (pH 5.6), plus 0.2 ml of the peroxidase extract. The reaction was started by the addition of 1.4 ml 0.5% (v/v) aqueous guaiacol solution and the initial change in absorbance at 420 nm was measured at 30°C.

ABTS assay
The reaction mixture contained 2.2 ml 100 mM sodium phosphate buffer (pH 7.9), 0.7 ml 100 mM aqueous ABTS solution plus 0.1 ml of peroxidase extract. The reaction was initiated by the addition of 0.1 ml of 0.03% (w/v) hydrogen peroxide in distilled water and the initial change in absorbance at 436 nm was measured at 25°C. One unit of peroxidase activity was defined as an increase of 1.0 optical density units at 436 nm/min.

Oxidation of IAA
The oxidation of IAA by mango isoperoxidases was monitored by following the method of Thomas *et al.* (1980). The reaction mixture contained 0.35 ml of 1.2 mM 2,4-dichlorophenol, 2.0 ml of 50 mM sodium phosphate buffer, 0.35 ml of 0.857 mM manganese chloride plus 0.05 ml of enzyme solution. The enzymic reaction at 30°C was started following the addition of 0.35 ml of 4.2 mM IAA. The oxidation product was measured at 247 nm for 10 min.

Oxidation of DHFA
A 0.25 ml sample of 3 mM (w/v) DHFA, 2.65 ml of 10 mM citrate phosphate buffer plus 0.1 ml of enzyme solution were mixed in a cuvette. The oxidation of DHFA was monitored by following the decrease in absorbance at 325 nm for 10 min at 25°C (Halliwell, 1977). The autoxidation of DHFA was also measured at 325 nm and for this purpose the enzyme solution was replaced with citrate phosphate buffer (10 mM, pH 3.2).

Molecular weights

These were determined by gel filtration with Sephadex G-100. The column was calibrated with standard proteins, bovine serum albumin, ovalbumin, chymotrypsinogen, ribonuclease, and the molecular weights of the isoperoxidases calculated from a plot of log molecular weight against elution volume.

RESULTS AND DISCUSSION

Mango isoperoxidases were purified by cell fractionation, gel filtration and ion-exchange chromatography on Q-Sepharose and SP-Tris-acryl as described by Khan and Robinson (1993*a,b*). For peroxidase activity only, each isoperoxidase preparation A1, A2, C1 and C2 were ascertained to be homogeneous by isoelectric focusing, as there was only one stained band for peroxidase activity associated with each preparation (Khan & Robinson, 1993*a*). Although the A1 isoperoxidase preparation was also found to be free of other proteins, as judged by silver staining, the other isoperoxidase preparations, A2 and C2, contained a mixture of other unknown proteins with similar p*I* values. However, the authors consider all the preparations of mango isoperoxidases to be sufficiently pure for the present purpose, for the comparison of their peroxidase and oxygenase activities. The presence of a mixture of other proteins

Table 1. Enzymic activity of mango peroxidases (optical density change per mg of protein)

Substrate	Isoperoxidases[a]			
	A1	A2	C1	C2
o-Dianisidine (OD 460 nm/min/ml)	24·6 (6·15)	3·4 (0·94)	21·6 (0·58)	36·1 (1·93)
Guaiacol (OD 420 nm/min/ml)	6 (1·5)	0·1 (0·03)	0·5 (0·01)	2·7 (0·14)
ABTS (OD 436 nm/min/ml)	4 (1)	3·6 (1)	37·5 (1)	18·7 (1)

[a] The numbers in parentheses represent the relative peroxidatic oxidatic actvity towards the three test substrates.

revealed by silver staining, after isoelectric focusing for the C2 and A2 isoperoxidases, of course indicated that these two isoperoxidase preparations were still unsuitable for amino acid analysis and sequencing, as previously reported (Khan & Robinson, 1993*a*).

As shown in Table 1 the purified cationic and anionic isoperoxidases catalysed the oxidation of the test substrates, guaiacol, *o*-dianisidine and ABTS at different relative rates. The calculated relative peroxidatic activity of the purified isoperoxidases towards the test substrates for a common activity of unity towards ABTS is given in parenthesis. For the anionic isoperoxidase (A1), *o*-dianisidine was the most susceptible substrate, whereas for the cationic isoperoxidase (C1), ABTS was oxidised more rapidly than either *o*-dianisidine or guaiacol. For the isoperoxidases, A2 and C1, the rate of oxidation of guaiacol was relatively slow.

When IAA was used as a test substrate the greatest rate of oxidation per unit of peroxidase activity was found for the isoperoxidase A1 (Table 2). Also, the mixture of the anionic isoperoxidases catalysed the oxidation of IAA approximately five times faster per unit of peroxidase activity, as measured with *o*-dianisidine, than the mixture of cationic isoenzymes. DHFA was oxidised by the mixture of cationic isoperoxidases at approximately twice the rate of that observed for oxidation by the mixture of anionic isoperoxidases per unit of peroxidase activity (Table 2). Likewise, the purified mango cationic isoperoxidases (C1 and C2) also catalysed the oxidisation of DHFA at a rate three to four times greater than that observed for the purified anionic isoperoxidases per unit of peroxidase activity as determined with the *o*-dianisidine assay.

The findings presented in this paper, where the oxygenase activity has been normalised to one unit of peroxidase activity, indicate that the anionic and cationic mango isoenzymes catalyse the oxidation of IAA and DHFA at different rates. IAA was oxidised at the greatest rate by the purified anionic mango isoperoxidase, A1, per unit of peroxidase activity as determined by the *o*-dianisidine assay. The greater capacity of mango isoperoxidase A1 for oxidation of IAA is confirmed if ABTS is used as the reference substrate for peroxidase activity. Although previously it has been suggested, for other plant sources (Grambow, 1986), that mixtures of cationic isoperoxidases have a greater capacity for IAA oxidation than anionic isoperoxidases, the present results for mango isoperoxidases contrast sharply with these earlier claims.

The various mango isoperoxidases described here possess different enzymic properties towards all the synthetic test substrates and the plant hormone and thus the magnitude of the calculated relative activities given in Table 2 depend on the choice of reference substrate. The reason for different capacities to oxidise the test substrates seems almost certainly to be due to variation in the molecular structure of the isoperoxidases and it is postulated that the rates of oxidation might be determined by the rates at which different free radicals can be released from the enzyme–substrate complex. The mechanism of peroxidase activity has been understood generally since the discovery of peroxidase compound I (Chance, 1952), formed by the oxidation of the enzyme with hydrogen peroxide, whereas the peroxidase–oxygenase catalysed reactions have been characterised more recently (Halliwell, 1977; Grambow, 1986). Both the peroxidatic action and peroxidase–oxygenase activity towards a wide range of hydrogen-donor substrates is brought about by abstraction of single electrons by compounds I or II from the substrates to initially form free radicals (Robinson, 1991).

Due to the presence of a number of other proteins in the preparations of isoperoxidases A2 and C2, as shown by isoelectric focusing and also the absence of a

Table 2. Oxidation of indoleacetic acid by mango isoperoxidases

Isoenzyme	Peroxidase activity[a] (1)	Oxidation of indoleacetic acid (OD 247 nm/min/ml)[b] (2)	Oxidase activity per unit of peroxidase $\times 10^{-2}$ (2)/(1)	Mean enzyme activity (OD 325 nm/min/ml)[b] (3)	Oxidase activity per unit of $\times 10^{-3}$ (3)/(1)
Mixture of anionic isoenzymes	7·6	0·433 ± 0·001	5·6	0·008 7 ± 0·003 2	1·12
Mixture of cationic isoenzymes	7·76	0·083 ± 0·004	1·07	0·018 3 ± 0·002 5	2·35
A1	0·3	0·082 ± 0·006	27	0·003 ± 0·001 0	10
A2	0·3	0·28 ± 0·002	9·3	0·002	6
C1	0·3	0·016 ± 0·004	5·3	0·008 7 ± 0·002 5	29
C2	0·3	0·015 ± 0·003	5·0	0·007 7 ± 0·002 9	25

[a] Assayed with *o*-dianisidine.
[b] Mean of three determinations.

confirmatory protein-staining band for isoperoxidase C2, the authors have not attempted to carry out amino acid analysis for the isoperoxidase preparations other than for the mango isoperoxidase A1 previously reported (Khan & Robinson, 1993*a*). However, the molecular weights have been determined by gel filtration using peroxidase activity to measure the elution volumes for the isoenzymes. Values for the molecular weights of 40 000, 44 000, 22 000 and 27 000 were calculated for the mango isoperoxidases A1, A2, C1 and C2 respectively eluted from Sephadex G-100. Surprisingly, the molecular weights of the mango cationic peroxidases are considerably lower than those previously reported for plant peroxidases and also, in this case, for the anionic mango isoperoxidases. Without further information for the amino acid composition and carbohydrate content of the mango isoperoxidases, particularly for the smaller cationic isoenzymes, it is not yet possible to comment in detail on the significance of this finding. However, as the cationic isoperoxidases are still active enzymically, their smaller mass might be due to fewer, or indeed the absence, of *N*-asparaginyl-linked oligosaccharide chains, while the protein moiety may be similar to that present in the larger anionic isoperoxidases. Hu and van Huystee (1989) have described a non-glycosylated peanut peroxidase obtained from cell cultures with a molecular weight of approximately 31 000 representing 78% of that of the glycosylated form. Variability has also been reported recently for the number and composition of glycan chains present in cell cultured peanut peroxidases (van Huystee *et al.*, 1992). Alternatively the mango cationic isoperoxidases could be structurally composed of quite different proteins.

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