Food Chemistry **46** (1993) 61–64

Purification of an anionic peroxidase isoenzyme from mango (*Mangifera indica* L. var. *chaunsa*)

A. A. Khan & D. S. Robinson*

Procter Department of Food Science, University of Leeds, Leeds LS2 9JT, UK

(Received 18 March 1991; revised version received and accepted 24 May 1991)

A mango anionic peroxidase isoenzyme (A1) has been purified to homogeneity by salt fractionation, gel filtration and ion-exchange chromatography. The purified isolated enzyme was free from contaminating proteins and other isoperoxidases. The molecular weight of A1 was calculated to be approximately 40 000. The major amino acid residues were glycine, serine and glutamic acid, representing approximately 48% of the protein moiety.

INTRODUCTION

Peroxidase (EC 1.11.1.7, donor: H_2O_2 oxidoreductase) is a ubiquitous enzyme distributed in the plant kingdom, in animal tissues and in microorganisms (Reed, 1975). The enzyme catalyses the peroxidatic reaction.

$$H_2O_2 + 2AH \rightarrow 2H_2O + A - A$$

Generally, plant peroxidases have a specific requirement for hydrogen peroxide and may be considered as peroxide scavengers. The peroxidatic reaction also includes the oxidation of a suitable electron donor, which may be a phenolic compound, ascorbic acid, anthocyanins and possibly thiols. Guaiacol, *o*-dianisidine, pyrogallol and 2,2'-azino-bis(3-ethylbenzthi-6-sulphonic acid) have been used to estimate peroxidase activity in foods.

Also peroxidase can catalyse catalytic, oxidatic and hydroxylation reactions (Whitaker, 1972). In a catalytic reaction, peroxidase catalyses the disproportionation of H_2O_2 into H_2O and O_2. In an oxidatic reaction peroxidase catalyses the oxidation of a number of substrates, including dihydroxyfumaric acid (DHFA) and indole acetic acid in the presence of O_2 and certain cofactors (e.g. Mn^{2+} and phenol): peroxidase may also catalyse the hydroxylation of aromatic compounds in the presence of oxygen and a H-donor.

Peroxidases are believed to be associated with deterioration in flavour, colour, texture and nutritional qualities of raw foods such as fruits and vegetables and of processed products (Haard, 1977). Since peroxidase is one of the more thermostable enzymes, it is frequently used as an index of blanching treatment and may also be used as an early indicator of darkening during storage of lychees (Huang *et al.*, 1990). Both soluble and ionically-bound forms of peroxidase isolated from various fruits and vegetables occur as discrete isoenzymes (McLellan & Robinson, 1983; Moulding *et al.*, 1987, 1989; Robinson *et al.*, 1989). It is generally accepted that the isoperoxidases not only show differences in charge when placed in an electric field, but also exhibit differences in catalytic properties, pH optima, molecular size, heat stability and regeneration properties, amino acid composition and carbohydrate content. In the present communication we report the isolation and purification of an anionic isoperoxidase from mango and its characterization in terms of molecular weight and amino acid composition.

* To whom correspondence should be addressed.

Food Chemistry 0308-8146/92/$05.00

MATERIALS AND METHODS

Extraction of peroxidase

Ripe fresh mangoes (variety *chaunsa*) were purchased from local shops in Leeds. The fruit was washed in tap water and peeled. The flesh was homogenized in ice-cooled 0·01M sodium phosphate buffer, pH 7·5, in the ratio 1 g:2 ml containing 1% (w/v) polyvinylpyrrolidone. The homogenate was filtered through a double-layer muslin cloth and centrifuged at 19 000 g for 20 min (4°C) to remove cell debris and clarify the extracts.

Isolation of isoenzyme

Ammonium sulphate precipitation

Ammonium sulphate (22·6 g 100 ml^{-1}) was added to the centrifuged ice-cooled extract at 4°C in order to achieve 40% (w/v) salt saturation. Precipitated protein was removed following centrifugation. The concentration of the ammonium sulphate in the supernatant was then increased to 90% salt saturation by adding 33·5 g

ammonium sulphate 100 ml^{-1} of the extract. The precipitate was collected by centrifugation and then dissolved in a minimum volume of 0·01M sodium phosphate buffer, pH 6·0. To lower the high viscosity of the supernatant, due to pectin, pentosanase (Novo Products Ltd), 2 μl 10 ml^{-1}, was added to the extract. After 24 h at 4°C, the precipitate was removed by centrifugation. The clarified enzyme extract was dialysed against 0·1M sodium phosphate buffer (pH 6·0) prior to gel filtration.

Gel filtration

Enzyme solution (about 10 ml, maximum protein 100 mg) was applied to a Pharmacia C16/70 (16 mm i.d. × 700 mm long) Sephadex G-100 column. Elution was with 0·1M sodium phosphate buffer, pH 6·0, at a flow rate of 30 ml h^{-1}. Fractions (5 ml) containing peroxidase activity were collected, pooled and concentrated by dialysis with a PM 10 membrane against 0·025M Tris–HCl buffer (pH 7·5).

Ion-exchange chromatography

The dialyzed enzyme solution was applied to a Pharmacia C16/40 (16 mm i.d. × 400 mm long) Q-Sepharose column that had been previously equilibrated with 0·025M Tris–HCl buffer (pH 7·5) at a flow rate of 30 ml h^{-1}. Initial peroxidase fractions (5 ml) were eluted for at least 1 hour with the start buffer followed by a linear gradient up to 0·5M NaCl.

Determination of molecular weight

The molecular weight of the purified isoenzyme was determined by gel filtration on Sephadex G-100, using bovine albumin (mol. wt. 67 000), ovalbumin (mol. wt 43 000), chymotrypsinogen A (mol. wt. 25 000) and ribonuclease A (mol. wt. 13 700) as standards.

Enzyme assay

Peroxidase activity was assayed with *o*-dianisidine (McLellan & Robinson, 1981). The reaction mixture contained 2·7 ml of 0·03% (w/v) H_2O_2 in 0·01M phosphate buffer (pH 6·0) plus 0·2 ml of the peroxidase extract. The enzymic reaction was initiated by the addition of 0·1 ml of 1% (w/v) *o*-dianisidine solution; the change in absorbance was recorded at 460 nm, at 25°C. One unit of peroxidase activity was defined as an increase of 1·0 optical density units at 460 nm min^{-1}.

Protein contents of the crude and purified peroxidase (POD) extracts were determined by the biuret (Scopes, 1982) and Bradford methods (Bradford, 1976), respectively.

Analytical isoelectric focusing (IEF)

IEF was carried out in thin-layer (0·5 mm) polyacrylamide gels containing ampholytes (pH 3·5–10·0, 2·7–5·0). Focused gels were stained for peroxidase activity by the *o*-dianisidine method (McLellan & Robinson, 1987). The gels were washed in distilled water and immersed in 0·2% (w/v) *o*-dianisidine in 80% methanol at pH 6·0. The gels were kept in the solution for at least 5 min and the enzymic reaction was initiated following addition of 0·3% (w/v) H_2O_2. Stained bands indicating peroxidase activity appeared within 30 min at room temperature and the gels were rinsed in distilled water. We have found that peroxidase protein is difficult to detect by the normally used Coomassie Blue staining procedure and, therefore, gels were stained for protein with the more sensitive silver staining method recently described by Kling *et al.* (1988).

Amino acid analysis

Hydrolysis was carried out for 24 h at 110°C in 6M HCl containing phenol (1% w/v). Derivation was with phenylisothiocyanate and the PTC-amino acids were separated by reverse-phase (C_{18} HPLC).

RESULTS AND DISCUSSION

To assess the occurrence and type of mango isoperoxidases, IEF and specific staining for peroxidase activity with the highly sensitive *0*-dianisidine reagent were carried out. For the centrifuged mango extract, four anionic and two cationic isoenzymes (Fig. 1(a)) were observed by IEF. For the pooled G-100 fraction (Fig. 1(b)) only two major anionic isoenzymes (A1 and A2) and one cationic (C2) isoenzyme were readily detected, while the concentration of the second cationic (C1) had decreased. Prabha and Patwardhan (1986) detected five bands of peroxidase activity in the extracts prepared from Badami mango pulp at the climacteric stage. van Lelyveld (1978) reported the presence of two major and three minor, and two major and five minor isoperoxidases in Haden and Sensation cultivars,

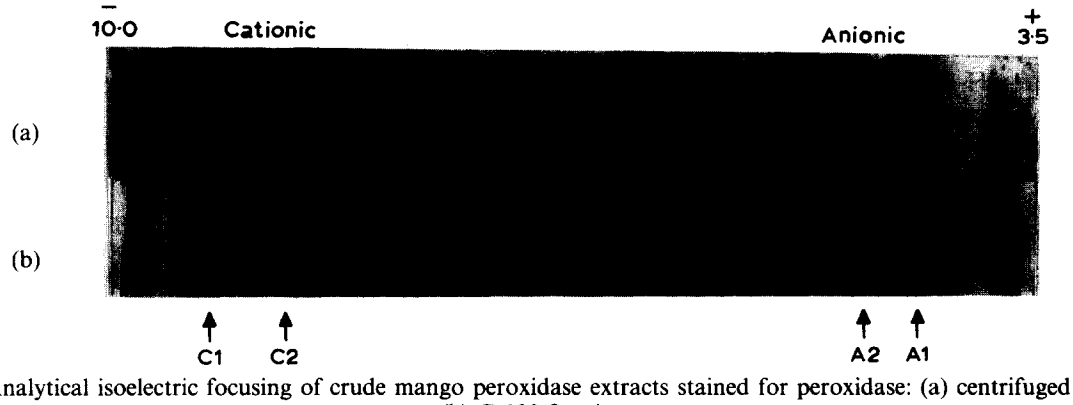

Fig. 1. Analytical isoelectric focusing of crude mango peroxidase extracts stained for peroxidase: (a) centrifuged mango extract; (b) G-100 fraction.

Table 1. Purification of soluble peroxidase isoenzyme A1 from chaunsa mango

Fraction	Vol. (ml)	POD activity[a]	Total activity	Per cent recovery	Protein (mg/ml $^{-1}$)	POD specific activity[b]	Purification factor
Crude extract (undialysed)	1000	3·5	3500	100	9·25	0·38	1
90% amm. sulphate, following pentosanase treatment	42	38·8	1630	47	37·3	1·04	2·75
Sephadex G-100	40	20	800	23	13·5	1·48	3·9
Ion-exchange rechromatographed A1 isoenzyme	20	2·9	58	1·7	0·16	18	48

[a] Peroxidase activity = change in absorbance at 460 nm min $^{-1}$ ml $^{-1}$.
[b] Specific activity of peroxidase = change in absorbance at 460 nm min $^{-1}$ mg $^{-1}$ protein.

respectively. Such small variations are compatible with those that might be expected to be due to differences in fruit variety, stage of maturity or the use of different detection methods, and it is concluded that mangoes, like many other fruits, contain a number of isoperoxides with a wide range of isoelectric points.

Isolation of the isoenzyme

Ammonium sulphate fractionation and gel filtration on Sephadex G-100 increased the purification of the enzyme by a factor of approximately four (Table 1). A summary of the results for the purification of the anionic (A1) isoenzyme is presented in Table 1. These are average results for the purification of several batches of the mango isoperoxidase, and it is noteworthy that a 48-fold purification was achieved for A1 isoperoxidases. During gel filtration the peroxidase activity was eluted from the G-100 column between the two protein peaks and fractions collected and pooled between the two arrows shown in Fig. 2. Ion-exchange chromatography of the pooled peroxidase activity obtained after gel filtration (Fig. 2, peak 2) resulted in separation of three isoperoxidases (Fig. 3). The cationic isoenzymes (Fig. 3, peak 1) were not bound to the anion-exchanger and were thus eluted with the start buffer, whereas the anionic species were eluted by an 0–0·2M NaCl gradient followed by an 0·2–0·5M NaCl gradient. Most of the peroxidase activity was concentrated in peak 3 (Fig. 3). Fractions (5 ml) containing peroxidase activity were pooled and assayed for the number and type of isoperoxidases using IEF. Peak 2 in Fig. 3 contained an anionic isoperoxidase (pI 4·79). After repeated (three times) ion-exchange chromatography of peak 3, a single band (anionic isoperoxidase, A1, pI 3·48) of peroxidase activity was observed after isoelectric focusing in a pH gradient 2·7–5·0. The protein of the isolated isoenzyme could not be stained with Coomassie Blue, due either to the small amount of protein responsible for peroxidase activity or possibly to the low sensitivity of the Coomassie Blue for peroxidases. However, a positive result was obtained with the silver staining technique for protein, which showed coincidence with the A1 band for peroxidase activity. As judged by the absence of other bands of silver staining, the purified mango A1 isoenzyme preparation was homogeneous with respect to enzymic activity and, importantly, protein: a criterion which has not always been used for establishing the purity of isoperoxidases.

Enzyme characterization

The molecular weight determined by gel filtration was about 40 000 for the purified mango A1 isoenzyme.

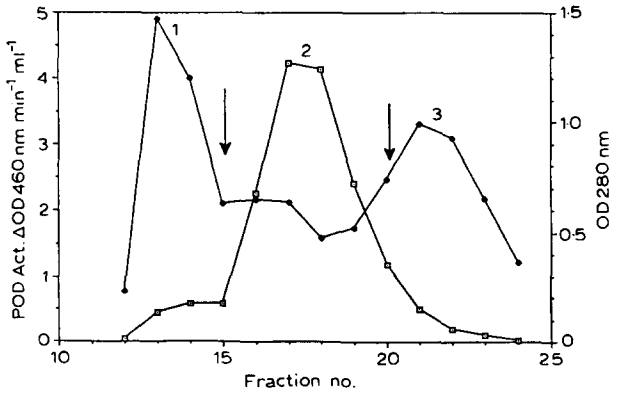

Fig 2. Gel filtration on Sephadex G-100 of mango peroxidase: ◆—◆, protein measured at 280 nm; □—□, peroxidase activity.

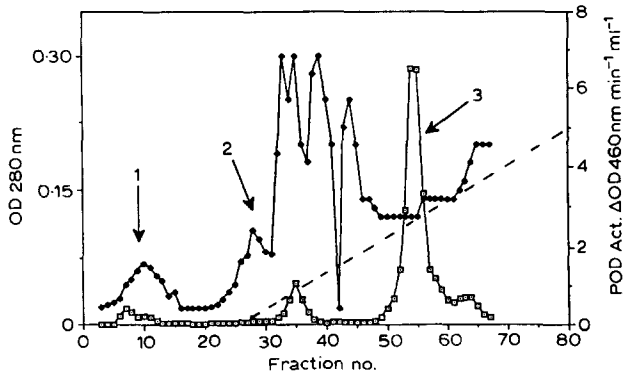

Fig. 3. Ion-exchange chromatography of mango isoperoxidase on Q-Sepharose: ◆—◆, protein measured at 298 nm; □—□, peroxidase activity; – – – –, NaCl gradient (0–0·5M).

Table 2. Amino acid composition of purified mango A1 isoenzyme

Amino acid	% mol	Amino acid	% mol
Aspartic acid	8·7	Proline	3·8
Glutamic acid	14·5	Tyrosine	2·3
Serine	16·4	Valine	4·5
Glycine	17·0	Methionine	1·2
Histidine	1·6	Isoleucine	2·2
Arginine	3·4	Leucine	5·8
Threonine	4·2	Phenylalanine	2·3
Alanine	10·4	Lysine	1·8

Note: Cysteine and Tryptophan were not determined, because of their lability.

This value is in good agreement with the molecular weight of peroxidase isoenzymes previously purified from other plant sources; for example, kiwi fruit mol. wt. 40 000–42 000 (Prestamo, 1989), palm mol. wt. 40 000 (Draetta & Ben-Shalom, 1984), and horseradish peroxidase c mol. wt. 44 000 (Welinder, 1979). However, it should be noted here that we have also determined (unpublished results) by gel filtration the molecular weights (22 000 and 27 000) of the partially purified mango cationic isoperoxidases C1 and C2 (pIs about 9·6 and 8·9, respectively). Such lower values for molecular weights of peroxidases have not previously been reported and are therefore surprising, although a microperoxidase arising from limited proteolysis of cytochrome c peroxidase has recently been reported by Pressey (1990). These results indicate that the cationic isoperoxidases might have arisen from limited proteolysis of, initially, a smaller number of isoenzymes.

Criteria of purity for amino acid analysis

Glycine, serine and glutamic acid were the major amino acids for the purified A1 isoperoxidase followed by alanine and aspartic acid. For the hydroxy amino acids, about four times as much serine as threonine was found (Table 2). These results contrast with those reported for horseradish anionic peroxidases, where aspartic acid was the major amino acid and significant amounts of threonine, serine, alanine and leucine were also found (Shannon *et al.*, 1966). However, unlike the horseradish isoperoxidases, the mango A1 isoenzyme described here has been shown by the highly sensitive silver staining technique to be homogeneous for protein. It is suggested that the substantial difference in the amino acid composition between horseradish and mango A1 isoenzyme might, in part, be accounted for by the different criteria used to assess the purity of the isoenzymes, even though the isoperoxidases have been obtained from quite different botanical sources.

ACKNOWLEDGEMENTS

Thanks are given to Professor J. B. C. Findlay at the SERC unit, Department of Biochemistry, University of Leeds, for carrying out amino acid analysis. The authors are highly indebted to the British Council for granting a PhD fellowship to Mr A. A. Khan.

REFERENCES

Bradford, H. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–54.

Draetta, I. S. & Ben-Shalom, N. (1984). Interference of carbohydrates during purification of peroxidase from palm (*Euterpe edulis Mart*). *J. Food Biochem.*, **8**, 109–21.

Haard, N. F. (1977). Physiological roles of peroxidase in post-harvest fruits and vegetables. In *Enzymes in Food and Beverage Processing*, ed. R. L. Ory and A. J. St Angelo. American Chemical Society, Symposium Series 47, Washington, DC, pp. 143–71.

Huang, S., Hart, H., Lee, H. & Whicker, L. (1990). Enzymatic and colour changes during postharvest storage of lychee fruit. *J. Food Sci.*, **55**, 1762–3.

Kling, H., Sawatzki, G. & Geis, W. (1988). Silver staining for proteins in ultrathin polyacrylamide gels: A sensitive precipitation technique. *Anal. Biochem.*, **174**, 589–92.

McLellan, K. M. & Robinson, D. S. (1981). The effect of heat on cabbage and Brussels sprout peroxidase enzymes. *Food Chemistry*, **7**, 257–66.

McLellan, K. M. & Robinson, D. S. (1983). Cabbage and Brussels sprout peroxidase isoenzymes separated by isoelectric focusing. *Phytochemistry*, **22**, 645–7.

McLellan, K. M. & Robinson, D. S. (1987). Purification and heat stability of Brussels sprout peroxidase isoenzymes. *Food Chemistry*, **23**, 305–19.

Moulding, P. H., Grant, H. F., McLellan, K. M. & Robinson, D. S. (1987). Heat stability of soluble and ionically bound peroxidase extracted from apples. *Int. J. Food Sci. Technol.*, **22**, 391–7.

Moulding, P. H., Goodfellow, J., McLellan, K. M. & Robinson, D. S. (1989). The occurrence of isoperoxides in Conference pears. *Int. J. Food Sci. Technol.*, **24**, 269–75.

Prabha, T. N. & Patwardhan, M. Y. (1986). Polyphenol oxidase (PPO) and peroxidase (POD) enzyme activities and their isoenzyme patterns in ripening fruits. *Acta Alimentaria*, **15**, 199–207.

Pressey, R. (1990). Anions activate the oxidation of indoleacetic acid by peroxidases from tomato and other sources. *Plant Physiol.*, **93**, 798–804.

Prestamo, G. (1989). Peroxidase of kiwi fruit. *J. Food Sci.* **54**, 760–2.

Reed, G. (1975). Peroxidase. In *Enzymes in Food Processing*. Academic Press, New York, pp. 243–7.

Robinson, D. S., Bretherick, M. R. & Donnelly, J. K. (1989). The heat stability and isoenzyme composition of peroxidases in Ohane grapes. *Int. J. Food Sci. Technol.*, **24**, 613–8.

Scopes, R. K. (1982). In *Protein Purification: Principles and Practice*. Springer Verlag, New York, p. 265.

Shannon, L. M., Kay, E. & Lew, J. Y. (1966). Peroxidase isoenzymes from horseradish roots: 1. Isolation and physical properties. *J. Biol. Chem.*, **241**, 2166–72.

van Lelyveld, L. J. (1978). Peroxidase activity and isoenzymes in abscised and normal mango (*Mangifera indica* L.) fruits. *Z. Pflanzenphysiol. Bd.*, **89**, 453–6.

Welinder, K. G. (1979). Amino acid sequence studies of horseradish peroxidase: Amino and carboxyl termini, cyanogen bromide and tryptic fragments, the complete sequence, and some structural characteristics of horseradish peroxidase c. *Eur. J. Biochem.*, **96**, 483–502.

Whitaker, J. R. (1972). Catalase and peroxidase. In *Principles of Enzymology for the Food Sciences*. Marcel Dekker, Inc., New York, pp. 591–600.