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The thermostability of purified mango isoperoxidases

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Small amounts of purified mango (var. *Chaunsa*) anionic and cationic isoperoxidases have been obtained by ion-exchange chromatography. It has been shown that peroxidase activity present in crude extracts of mango pulp is less stable to heat than the enzymic activity of highly purified individual mango isoperoxidases. For the purified isoperoxidases heat-inactivation is still non-linear. It is suggested that this may be due to microheterogeneity in covalently bound oligosaccharide residues at the molecular level. Isoperoxidase activity did not regenerate after heat treatment of crude mango extracts or purified isoenzymes.

INTRODUCTION

Peroxidase (EC 1.11.1.7) is a member of a large group of enzymes, the oxidoreductases, which are widely distributed in nature. Peroxidases are not only known to be involved in enzymic browning by acting directly on phenols (Sawahata & Neal, 1982) but they also catalyse the discoloration of anthocyanins or other plant pigments, e.g. carthamin (Kanehira & Saito, 1990).

Peroxidases are recognised as being one of the most heat-stable enzymes present in higher plants. Peroxidases react with H_2O_2 to produce compound I, the oxidised Fe(V) form which can then oxidise a wide range of hydrogen-donor molecules (Khan & Robinson, 1992). These reactions are undesirable and may contribute to deteriorative changes in flavour, texture, colour and nutritive value both in raw foods such as fruits and vegetables and in processed products (Burnette, 1977). Most vegetables and fruits which are preserved by canning, freezing or even dehydration, are subjected to a blanching treatment to inactivate endogenous enzyme systems. It has been generally accepted that if peroxidases, which are regarded as the more thermostable enzymes, are inactivated by heat, then it is unlikely that other enzyme systems will remain active. For this reason, peroxidase activity of plant foods is widely used as an index of blanching. Other enzymes, e.g. polyphenol oxidase, lipoxygenase and catalase, have not been considered appropriate as blanching indices. Halpin *et al.* (1989) have recently reiterated that peroxidase will continue to serve in this capacity.

Inactivation of peroxidase activity in plant materials is generally found to be a non-linear process against heating time, which is thought to be due to the presence of separate isoperoxidases with different heat stabilities (Yamamoto *et al.*, 1962; McLellan & Robinson, 1987; Moulding *et al.*, 1987), although in some instances non-linear heat-inactivation plots have also been observed for highly purified isoperoxidases. A number of research workers have also reported partial regeneration of peroxidase activity following heat-inactivation and this indicates that the enzyme can undergo reversible inactivation (Lu & Whitaker, 1974; McLellan & Robinson, 1981). A correlation between the extent of inactivation of peroxidase activity and loss of quality in foods has been reported for some vegetables, e.g. corn-on-the-cob (Lee & Hammes, 1979; Garrote *et al.*, 1987), green beans (Zoueil & Esselen, 1959), peas (Pinsent, 1962).

In plant extracts peroxidase activity has been found in both soluble and bound states which differ with respect to heat stability and regeneration properties. The ionically bound peroxidases in orange were found to be more heat-stable than the soluble peroxidases (McLellan & Robinson, 1984), which contrasts with the findings for apple peroxidases (Moulding *et al.*, 1987) where the soluble peroxidase extracts contained the most heat-stable peroxidase activity. McLellan & Robinson (1981) observed that the heat-sensitive ionically bound peroxidases in Brussels sprouts possessed a greater ability to regenerate. This communication reports a study of the thermal stability of mango isoperoxidases in mixtures and where possible as individual purified isoperoxidases.

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MATERIALS AND METHODS

Fresh ripe mangoes (*Mangifera indica* L. var. *Chaunsa*) were purchased from Leeds local shops. Sephadex G100, Q-Sepharose, SP-Trisacryl, Ampholine Carrier Ampholyte and all other materials and equipment for analytical isoelectric focusing in thin-layers of polyacrylamide were used as supplied by LKB Instruments Ltd, Selsdon, South Croydon, Surrey. *o*-Dianisidine was obtained from Koch Light Laboratories Ltd, Colnbrook, Bucks.; solid ammonium sulphate and bovine serum albumin from Sigma Chemical Co., Poole, Dorset. All other chemicals where available in Analar grade, were obtained from BDH Chemicals Ltd, Poole, Dorset.

Extraction of soluble peroxidase activity

The mango pulp soluble peroxidase activity was extracted according to the method described by Khan & Robinson (1992).

Extraction of ionically bound peroxidase activity

The residue remaining after the extraction of mango soluble peroxidase activity was washed by resuspending and centrifuging in ice-cold sodium phosphate buffer (0·01 M, pH 6·0). The washing procedure was repeated twice in order to remove any further traces of pulp soluble peroxidase. For extraction of the ionically bound enzymic activity the residue was resuspended in 0·8 M NaCl, in 0·01 M sodium phosphate buffer, pH 6·0 at a ratio of 1:3 and centrifuged at 19 000*g* for 20 min (4°C). This supernatant was designated the mango pulp ionically bound peroxidase fraction.

Enzyme assay

Peroxidase activity was assayed with the *o*-dianisidine method at pH 6·0 at 25°C (McLellan & Robinson, 1981). Absorbance changes were recorded at 460 nm using a Pye Unicam SP8 200 UV Spectrophotometer.

Analytical isoelectric focusing in thin-layer polyacrylamide gels

All samples were dialysed against 0·01 M sodium phosphate buffer at pH 6·0 prior to analysis. Isoelectric focusing (IEF) was carried out using an Ultrophor Electrofocusing unit equipped with an LKB Multi-Temp thermostatic circulator. Following IEF, the pH gradient in the gel was measured by removing a section 0·5 cm wide across the whole width of the gel and cut into further sections. The focused gels were stained for peroxidase activity with *o*-dianisidine (McLellan & Robinson, 1987).

Heat-inactivation

Heat-inactivation studies of peroxidase activity were carried out in triplicate, on dialysed extracts in 0·01 M sodium phosphate buffer (pH 6·0) in Pyrex test tubes (15 × 1·6 cm) as described by McLellan & Robinson (1987). Following heat treatment and regeneration, the tubes were immediately immersed in ice-cold water to minimise any further change in enzymic activity. For crude mango extracts, 0·25 ml aliquots were subjected to heat treatment and, out of that, 0·2 ml was used for assay of the residual and any regained peroxidase activity when held at 30°C for 30 min. For extracts of purified isoperoxidases, where quantities were limited an exact volume of 0·2 ml was subjected to heat-inactivation and regeneration tests. Residual and regenerated enzymic activity was assayed with *o*-dianisidine.

Isolation of isoperoxidases

The purification procedure for the single mango anionic isoenzyme Al has been reported (Khan & Robinson, 1992). For the purification of other anionic and cationic isoperoxidases, the soluble peroxidase extract was partially purified by salt fractionation and gel filtration on Sephadex G-100 (Khan & Robinson, 1992) and then subjected to ion-exchange chromatography to obtain separate preparations of both anionic and cationic isoperoxidases.

Anion-exchange chromatography

The enzyme solution (10 ml) collected after gel filtration was dialysed against 0·025 M Tris/HCl buffer (pH 7·5) and was applied to a Pharmacia C16/40 (16 mm i.d. × 400 mm long) Q-Sepharose column previously equilibrated with the same buffer at a flow rate of 30 ml/h. Fractions were eluted for at least 1 h with the start buffer followed by a linear gradient up to 0·5 M NaCl. For the fractions (5 ml) which contained peroxidase activity the distribution of isoperoxidases was determined by isoelectric focusing.

Cation-exchange chromatography

The fractions (5 ml) containing peroxidase activity eluted with the start buffer from the Q-Sepharose column were concentrated to approximately 10 ml using an Amicon concentrator with a PM 10 membrane (MW cut-off 10 000) and then dialysed against 0·025 M MES buffer (pH 6·0). This (10 ml) was applied to a Pharmacia C16/40 (16 mm i.d. × 400 mm long) SP-Trisacryl column previously equilibrated with 0·025 M MES buffer (pH 6·0) at a flow rate of 30 ml/h. Fractions (5 ml) were eluted for at least 1 h with the start buffer and then by a linear gradient up to 0·2 M NaCl.

Protein determination

Total protein content of the crude and purified extracts was estimated according to the Biuret method (Scopes, 1982) and Bradford (1976) respectively.

pH Optima

The optimum pH for peroxidase activity was determined with the *o*-dianisidine assay method (McLellan & Robinson, 1981) at different pH values (pH 3·0–6·0: citrate phosphate buffer, pH 6·0–8·0: sodium phosphate buffer).

RESULTS AND DISCUSSION

Previously we have shown (Khan & Robinson, 1992) by isoelectric focusing that both anionic and cationic isoperoxidases were present in the soluble fractions extracted from mango. The mango pulp ionically-bound peroxidase fraction examined here has been shown to contain three cationic isoperoxidases (approximate pI 8·8, 9·0 and 9·7) in addition to the anionic isoenzymes. The presence of anionic isoperoxidases in the ionically-bound peroxidase fraction was not due to contamination by soluble peroxidases, as these isoenzymes had been removed by repeated washing of the residue, although it is possible that ionic-binding of soluble isoperoxidases to cellular debris may have occurred during homogenisation of the mango extract as suggested by Schloss *et al.* (1987). The optimum pH for assay of enzymic activity using *o*-dianisidine for the crude preparation of mango soluble and ionically-bound peroxidases was 5·0 and 4·5–5·0 respectively.

Ionically-bound peroxidase activity

$CaCl_2$ (0·4 M) and NaCl (0·8 M) were the most effective salts for release of the ionically-bound isoperoxidases. Only small amounts of mango peroxidase activity were released with $MgCl_2$. These results are similar to those reported by Haard (1973) for the release of banana ionically-bound isoperoxidases and by Silva *et al.* (1990) for papaya isoperoxidases. The distribution pattern of cationic and anionic isoperoxidases in the ionically-bound extract released from the resuspended first centrifugal pellet was not related to the type of salt used for extraction.

Purification of isoperoxidase

In order to investigate the effect of heat on individual mango isoperoxidases, these were separated by a series of chromatographic methods. After gel filtration (on G-100 Sephadex) of the crude mango peroxidase extract, anion-exchange chromatography on Q-Sepharose resulted in the separation of three distinct peaks of peroxidase activity (Khan & Robinson, 1992). The distribution of a number of isoperoxidases in the three peaks, as determined by isoelectric focusing, is shown in the flow diagram (Fig. 1). The A2 isoenzyme (peak 2) was not strongly bound to Q-Sepharose and this isoperoxidase was eluted from the column with very little contamination by other isoperoxidases. Cationic peroxidases (C1 and C2) in peak 1, which were not bound to the anion-exchanger, were fractionated further by cation-exchange chromatography on SP-Trisacryl (Fig. 2). The C1 isoenzyme was eluted from the column towards the middle of the salt gradient, whereas the C2 isoenzyme eluted at the start of the gradient (Fig. 2).

The smaller recovery of the C1 isoenzyme was

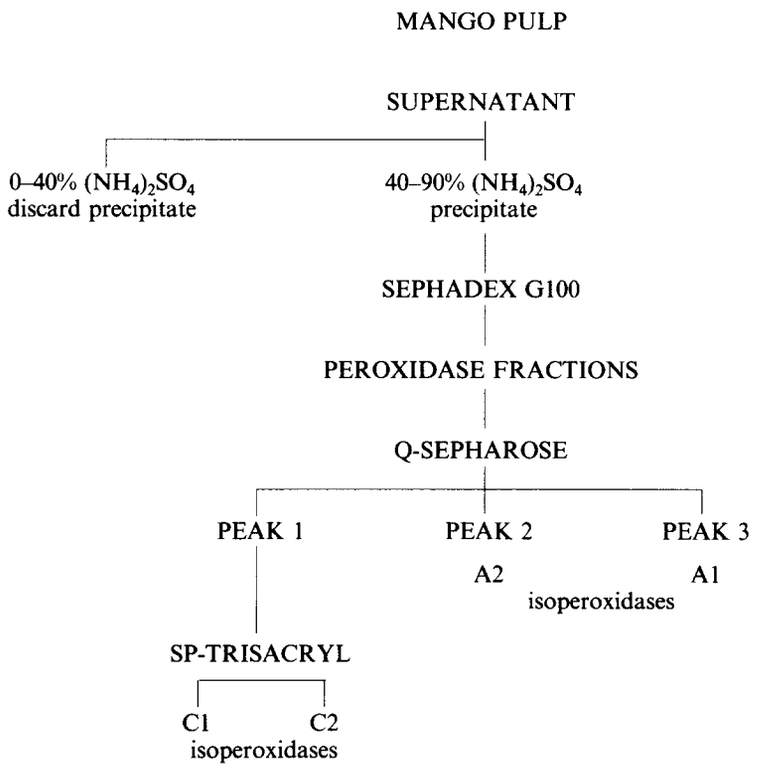

Fig. 1. Flow diagram for the purification of mango isoperoxidase.

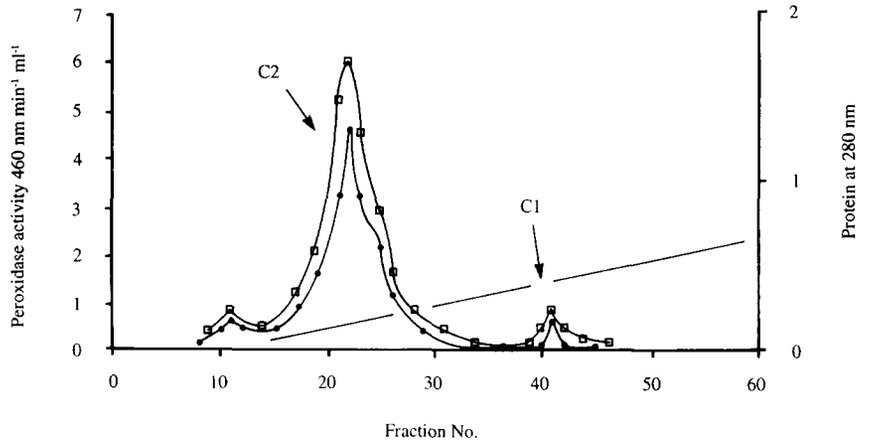

Fig. 2. Cation-exchange chromatography on SP-trisacryl of mango cationic isoperoxidases. □, peroxidase; ○, protein.

attributed to the lower contribution of this isoenzyme in the extract (Table 1). Isoelectric focusing showed that, for the isoperoxidase preparations (A1, A2, C1 and C2), only one band of peroxidase activity was present for each purified isoenzyme (Fig. 3). Although each isoenzyme preparation is homogeneous with respect to enzymic activity, staining with the highly sensitive silver technique for protein showed that the A2 and C2 preparations contained very small amounts of a number of proteins with similar pI values (Fig. 3). For the C1 isoperoxidase, silver staining for proteins was not observed, due to insufficient sample and therefore a claim for purity of the isoenzyme preparation cannot be made. The optimum pH for assay of enzymic activity using *o*-dianisidine as substrate for the purified A2 and C2 isoperoxidases was 5·0 and between 4·7 and 6·25 for isoperoxidases A1 and C1 respectively.

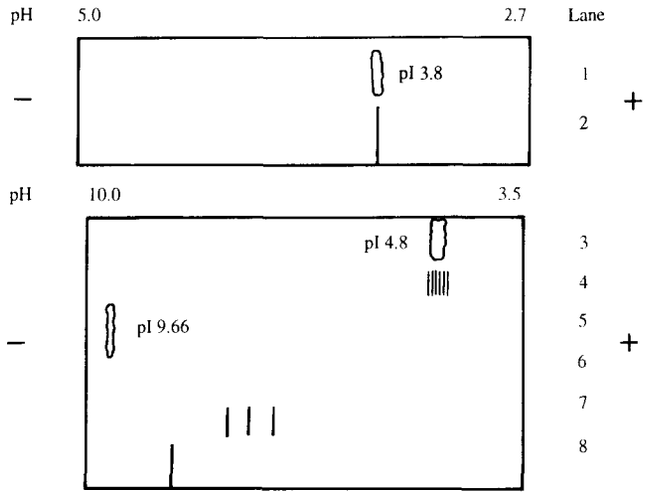

Fig. 3. Isoelectric focusing of purified mango peroxidase isoenzymes: Lane 1, peroxidase activity stain of the purified A1 isoenzyme; Lane 2, protein silver stain of the purified A1 isoenzyme; Lane 3, peroxidase activity stain of the purified A2 isoenzyme; Lane 4, protein silver stain of the purified A2 isoenzyme; Lane 5, peroxidase activity stain of the purified C1 isoenzyme; Lane 6, protein silver stain of the purified C1 isoenzyme; Lane 7, protein silver stain of the purified C2 isoenzyme; Lane 8, peroxidase activity stain of the purified C2 isoenzyme.

Heat-inactivation and regeneration of peroxidase activity

Crude extracts

Inactivation plots for peroxidase activity present in both crude soluble and ionically bound extracts from mango pulp are shown in Fig. 4. For both extracts the loss of peroxidase activity was found to be non-linear with heating time. The exact cause of the non-linear plots for heat-inactivation is not known, but it has been suggested to be due to the presence of a number of peroxidases with differing thermostabilities. It seems likely that the initial rapid loss of enzymic activity may be due to the inactivation of heat-labile isoperoxidases, and the further smaller losses of activity, in this case for mango peroxidase activity extending up to 10 min at 80°C, may be due to the presence of more thermostable isoperoxidases.

The ionically-bound peroxidase activity was more heat-stable than the soluble peroxidase activity during the initial stages of the heat treatment (Fig. 4). These results, which show a greater thermostability for mango ionically-bound peroxidases, are similar to those obtained by McLellan & Robinson (1984) for the fraction of ionically-bound peroxidases from orange juice, which were also more heat-stable than the soluble peroxidase fraction. However, for pear, both the soluble and the ionically bound peroxidase activity were heat-labile with only approximately 2% of the original enzymic activity remaining after 2 min at 80°C (Moulding *et al.*, 1989). Tests for regeneration of peroxidase activity after heat-treatment did not reveal any substantial regain in enzymic activity for the crude extracts of soluble and ionically-bound mango peroxidase extracts.

The purified isoperoxidases (A1, A2, C1 and C2) lost approximately 20% of their enzymic activity when held at 60°C for 2 min as compared to a loss of approximately 60% when present as a mixture in the soluble crude extract (Fig. 4). After heating at 80°C, purified A1 and C2 isoperoxidases were shown to possess greater thermostabilities (Fig. 5(a)). For isoperoxidase A1, approximately 40% of enzymic activity was retained after 2–8 min at 80°C. For the cationic isoperoxidases,

Table 1. Purification of soluble peroxidase isoenzymes from *Chaunsa* mango

Fraction	Vol. (ml)	OD460 (nm/min/ml)	Total act.	Recovery (%)	Protein (mg/ml)	Sp. act.[a] (OD)	Purification factor
Crude extract (undialysed)	1000	3·5	3500	100	9·25	0·38	1
After ion-exchange rechromatogaphy							
A1 isoenzyme	20	2·9	58	1·7	0·16	18	47
A2 isoenzyme	10	0·78	7·8	0·22	0·24	3·25	9
C1 isoenzyme	10	0·13	1·3	0·04	0·156	8·3	21
C2 isoenzyme	10	2·54	25·4	0·73	0·095	27	71

[a] Specific activity is optical density changes at 460 nm/min/mg of protein.

the C2 preparation was more thermostable than the C1 preparation after 2 min at 80°C (Fig. 5(b)), although after 10 min at 80°C the enzymic activity for C2 was reduced to approximately 22% of the original value. The inactivation of peroxidase activity for the purified isoenzymes was non-linear with heating time. The significantly higher thermostability of the peroxidase activity for the purified isoenzymes, as opposed to the enzymic activity present in the crude extracts, might indicate that other substances present in the crude mango soluble extracts may have enhanced deactivation of enzymic activity during heating.

The non-linear inactivation of peroxidase activity in a mixture of isoenzymes has generally been proposed to be due to the presence of multiple isoenzymes with differing heat stabilities. However, such an explanation for the non-linear inactivation of peroxidase activity with heating time for a single purified isoperoxidase seems to be inappropriate. However, the purified mango isoperoxidases may still have possessed micro-heterogeneity, due to the presence of variable amounts of covalently-bound neutral carbohydrate which will not affect the isoelectric point but might affect the thermostability: indeed Silva *et al.* (1990) have recently claimed that the carbohydrate moieties of horseradish peroxidase exert a stabilising effect on the enzyme molecule.

In proteins, B-secondary structure is considered the most stable when there is a high content of hydroxy amino acids (Tamura & Morita, 1975). Anionic horseradish peroxidase isoenzymes have been shown to have higher contents of the hydroxy amino acids, serine and threonine, than horseradish peroxidase cationic isoenzymes (Shannon *et al.*, 1966). The mango A1 isoenzyme has been shown to contain substantial amounts of serine (Khan & Robinson, 1992). Therefore, the higher heat stability of the purified mango A1 isoperoxidase may have been due to a greater proportion of hydroxy amino acids.

The enzymic activity of purified mango isoperoxidases did not regenerate substantially during a 30 min period after heat treatment. The isoperoxidases (A1, A2 and C2) showed approximately 10–13% regain in enzymic activity at 30°C when held for 30 min. The

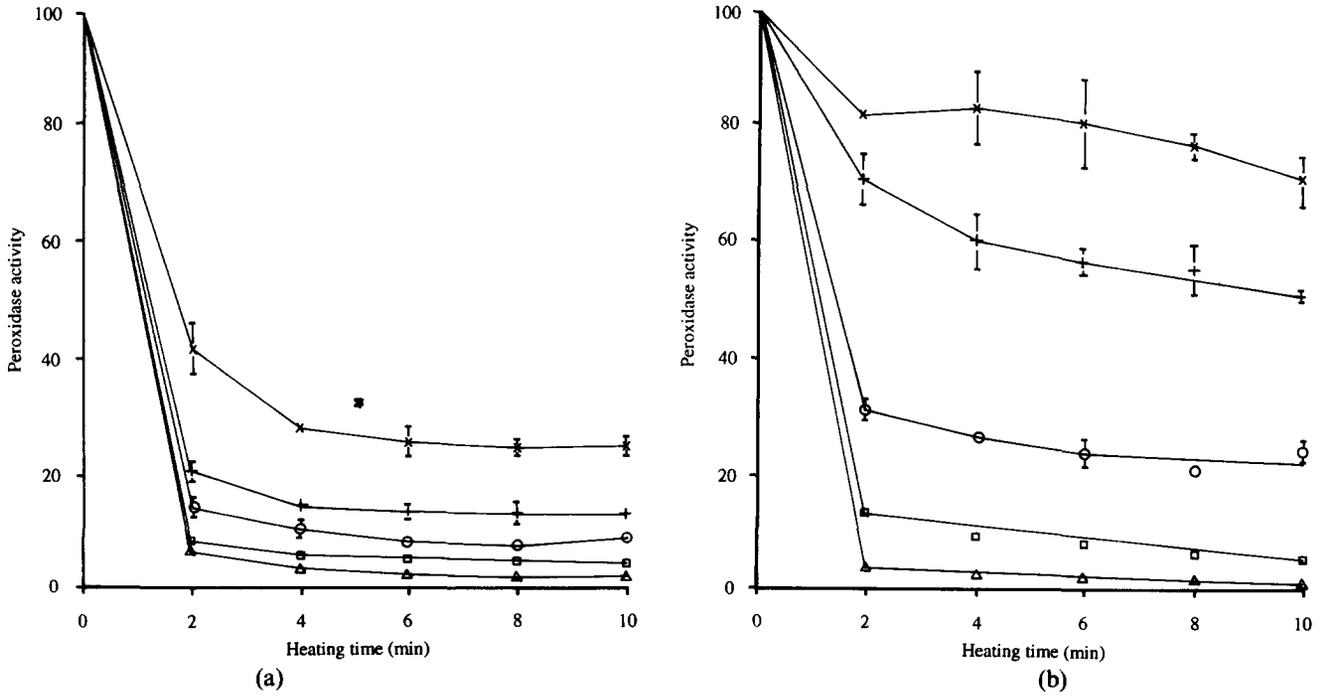

Fig. 4. Heat-inactivation of peroxidase activity in: (a) crude soluble mango extracts; (b) ionically-bound mango extracts (×, 60°C; +, 65°C; ○, 70°C; □, 75°C; Δ, 80°C).

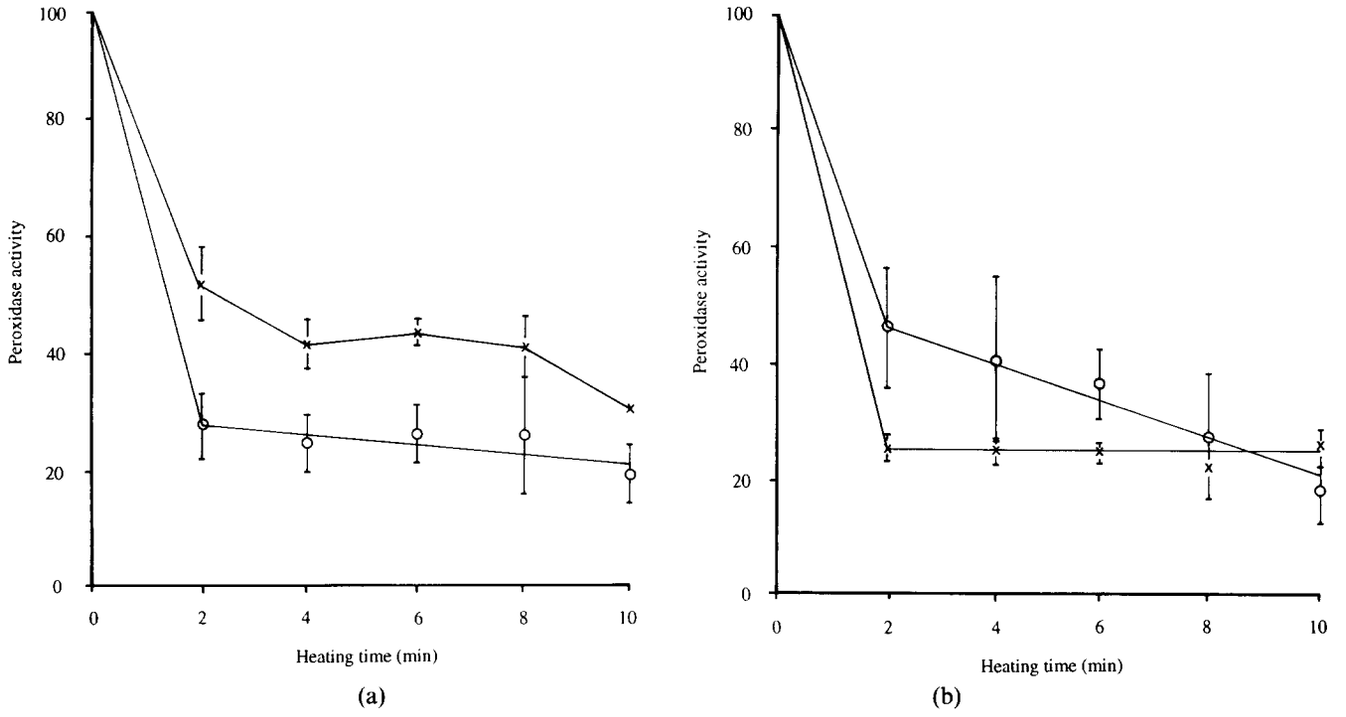

Fig. 5. Heat-inactivation at 80°C of purified: (a) anionic isoperoxidases A1 and A2 (×—×, isoperoxidase A1; ○—○, isoperoxidase A2); (b) cationic isoperoxidases C1 and C2 (×—×, isoperoxidase C1; ○—○, isoperoxidase C2).

cationic C1 isoperoxidase did not show any regeneration of enzymic activity after heat treatment at 30°C. Tamura & Morita (1975) observed that a cationic isoenzyme extracted from Japanese radish was less stable and it showed less regeneration following inactivation than an anionic isoenzyme from the same plant source. They proposed that the cationic apo-isoenzymes aggregated into inactive polymers which could not reassociate with haem, whereas they suggested that anionic apo-isoenzymes may have the ability to aggregate into active polymers due to their high carbohydrate content. However, peroxidases are enzymes with very high enzymic activity and it is difficult to isolate sufficient mass of enzyme for analysis, as is the case here with mango isoperoxidases, and therefore any possible effect of covalently bound carbohydrate on thermostability has not yet been proven.

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