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PURIFICATION AND THERMOSTABILITY OF ISOPEROXIDASE FROM ORANGES

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Key Word Index—*Citrus sinenis*; Rutaceae; oranges; purification; thermostability; isoperoxidase.

Abstract—Soluble and ionically bound peroxidase were extracted from oranges (*citrus sinenis* (L.) Osbeck cultivar Large Valencia, Small Sweet and Navel. Cationic and anionic isoperoxidase were obtained by Rotofor and ion-exchange chromatography. The pI for the soluble isoenzymes (4.5 to 9.0) was measured using a surface electrode and the M_r (22 k to 44 k) was estimated by gel-filtration. The purified isoperoxidases (C_1 and C_2) were more heat stable than the peroxidase present in the crude extract. The inactivation of orange peroxidase activity in a mixture or individually in a purified state, was found to be non-linear with heating time. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Peroxidase (EC 1.11.1.7) are part of a large group of enzymes known collectively as oxidoreductases [1, 2]. Extensive studies describe the action of peroxidase on substances which yield bright colours on oxidation, but peroxidase can promote a large variety of reactions and therefore can exhibit a degree of versatility unsurpassed by any other enzyme. The quality of extracted citrus juices depends on enzymic reactions that occur not only in the fruit during the development period, but also in the juice during processing. The formation of off-flavours in canned fruit and vegetables has been associated with residual peroxidase activity following processing [3]. Peroxidase can contribute to deteriorating changes in flavour, texture, colour and nutrition in improperly processed fruits and vegetables [4]. In orange, the level of peroxidase in the juice is associated with loss of flavour quality [5]. The heat inactivation of peroxidase is non-linear, a large decrease in activity is observed during the initial stages of a given heating process, but the rate of inactivation then changes to a much slower process [6, 7]. Renaturation of peroxidase following heat inactivation has also been reported for some vegetable and fruit extracts, e.g. kohlrabi [8], Brussels sprouts and cabbage [9], grapes [10, 11], avocados [12], apples [13], pears [14], kiwifruit [15], mango [16].

The HTST (High Temperature Short Time) treatments commonly used commercially in fruit and vegetable processing are less effective for irreversible peroxidase inactivation than the traditional, more prolonged methods [17]. 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 [18]. In plant extracts, peroxidase activity has been found in both soluble and bound states which differ with respect to heat stability and regeneration properties.

Commercial fruit juice production includes some albedo and peel of the fruit in the juice, which will make a large contribution to the total peroxidase activity. In the present research, we report a study of the consideration of the heat stability of orange juice, albedo and peel crude extracts and thermal stability of orange isoperoxidases as individual purified isoperoxidases.

RESULTS AND DISCUSSION

The crude extracts obtained from the three cultivars (Large Valencia from Brazil—CLV, Navel from Spain—CN and Small Sweet from Spain—CSS) were assayed by isoelectric focusing (IEF) and both anionic and cationic isoperoxidase were present in the soluble fractions from juice and flavedo. The fraction from the albedo contained only anionic isoenzymes and the fraction from the peel contained more isoenzymes than the other fractions (Fig. 1). Similar numbers of peroxidase isoenzymes were found in the cultivar CLV, CN, CSS, and the POD activity in the different fractions of that cultivar decreased in the following order flavedo soluble peroxidase (FSP), albedo soluble peroxidase (ASP), juice soluble peroxidase (JSP).

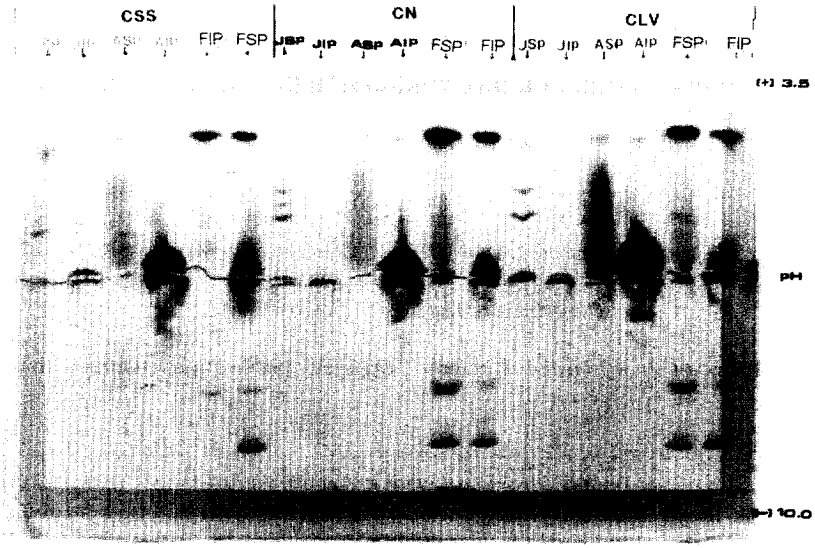

Fig. 1. IEF patterns of orange extract obtained on polyacrylamide gels (stained for peroxidase activity with *o*-dianisidine). (CLV—Cultivar Large Valencia, CN—Cultivar Navel and CSS—Cultivar Small Sweet). JSP—Juice solube peroxidase, JIP—Juice ionically bound peroxidase, ASP—Albedo soluble peroxidase, AIP—Albedo ionically bound peroxidase, FSP—flavedo soluble peroxidase and FIP—flavedo ionically bound peroxidase.

The enzymic activity in the crude extracts for different fractions of the three cultivars are shown in Table 1.

From the results shown in Table 1 it can be seen that the greatest peroxidase activity was detected in the peel fractions, probably due to the number of isoenzymes, which is highest in that fraction. The ripeness of the fruit can also be a factor for that, once the peroxidase enzymes are involved in physiologic processes. Also it has been suggested that the number of peroxidase isoenzymes from the same kind of fruit may differ, depending on ecological and environmental differences, as well as differences in variety, and also differences in stage of maturity and detection techniques [19]. The relationship between the ripeness and peroxidase activity indicates the involvement of peroxidase in post-harvest physiology, which is in agreement with the reported studies on citrus fruits [20].

Numerous studies have suggested that isoenzymes may be artifacts which arise during the course of purification. However, in the present work peroxidase isoenzymes from orange are unlikely to be artifacts, as these isoenzymes were identified before purification steps using the Rotofor and flat bed IEF, and their quantity did not seem to change during purification. In order to investigate the effect of heat on individual orange peroxidases, isoperoxidases were separated by

Table 1. Peroxidase activity in crude extracts from three oranges cultivars*

	Cultivar					
	CLV		CN		CSS	
Samples	ΔOD	Sd ±	ΔOD	Sd ±	ΔOD	Sd ±
Juice soluble peroxidase (JSP)	1.27	0.02	0.88	0.03	0.47	0.02
Juice ionically bound peroxidase (JIP)	0.11	0.01	0.10	0.01	0.25	0.03
Albedo soluble peroxidase (ASP)	2.20	0.02	0.38	0.01	0.48	0.02
Albedo ionically bound peroxidase (AIP)	0.36	0.02	0.10	0.02	0.10	0.02
Flavedo soluble peroxidase (FSP)	10.20	0.01	7.60	0.00	4.70	0.01
Flavedo ionically bound peroxidase (FIP)	8.40	0.00	2.18	0.01	1.53	0.00

* ($n = 3$), n = number of measurements, CLV—Cultivar Large Valencia, CN—Cultivar Navel, CSS—Cultivar Small Sweet, Sd—standard deviation, ΔOD—change in A (460 nm/min.ml)

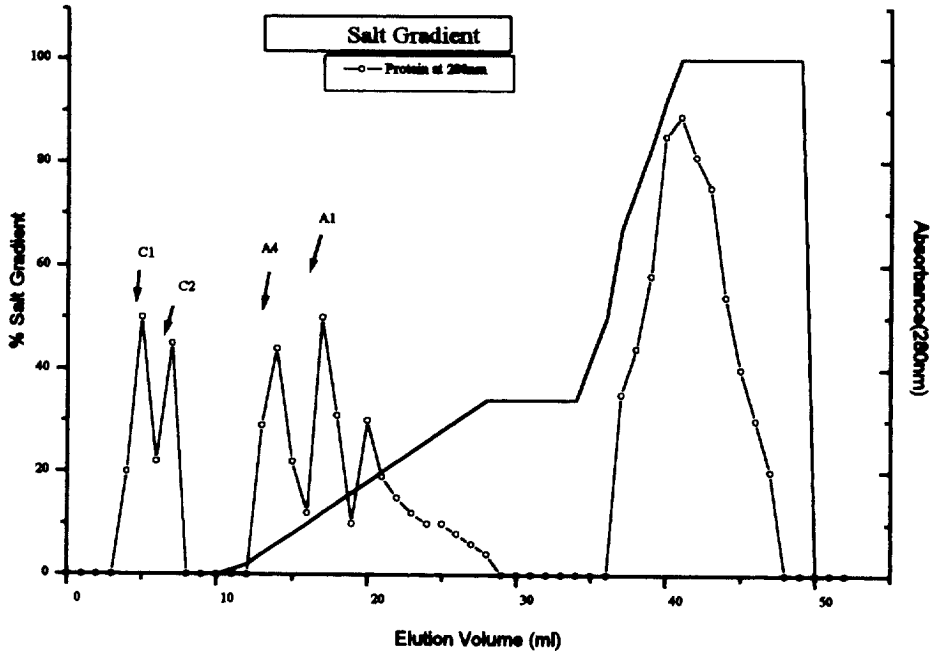

Fig. 2. Anionic-exchange chromatography of the fractions contained the cationic isoenzymes (C_1 and C_2) and anionic isoenzymes (A_1 and A_4) on FPLC (mono Q column).

a series of chromatographic methods. The fractions collected after preparative isoelectric focusing (PIF), were applied for gel filtration on Sephacryl S-100 HR and then on ion-exchange chromatography on FPLC, resulting in a separation of distinct peaks (Fig. 2). The distribution of a number of isoperoxidases in the peaks, was determined by isoelectric focusing (IEF).

The A_2, A_3 and A_7 were isolated by PIF and gel filtration on Sephacryl S-100HR and Sephadex G-50 were used to eliminate contaminants. This step was carried out after the first series of PIF and gel filtration series. In Fig. 3 are shown the results of PIF for the isolated isoenzymes, and in Fig. 4 the protein silver stain for the same fractions.

The pI for the isoenzymes was measured using a surface electrode. In addition, a narrow strip of focused gel was cut into 5 mm pieces parallel with the electrode strips. Each piece was soaked in 2 ml of water for 2 h before pH of each result was measured. The M_r of the purified isoenzymes was estimated following the Pharmacia gel filtration calibration kit instruction manual by using gel-filtration (Sephacryl S-100 HR, a crossed linked dextran). The eqn of the calibration curve of the standards M_r used for the determination of M_rs of purified isoenzymes was $Y = 2.3452–0.4533X$, where $Y = \text{Kav}$ elution parameter and $X = \log(M_r)$, the elution parameter is $(\text{Ve}-\text{Vo})/(\text{Vt}-\text{Vo})$. The equation above was obtained by regression analysis of the data (Table 2).

The values obtained for M_rs of the anionic isoenzymes were constant with the M_r of peroxidase from our sources. For example, kiwifruit M_r 40k–42 k [15], papaya fruit M_r 41 k–54 k [21], peanuts M_r 40 k–42 k [22], and horseradish peroxidase C M_r 44 k [23].

Table 2. pIs and M_rs of purified orange ispperoxidases

Isoperoxidase	pI	M_r k
A_1 (JSP)	4.5	44
A_2 (JSP)	5.0	22
A_3 (JSP)	5.3	30
A_4 (JSP)	5.6	26
C_1 (JSP)	9.0	26
C_2 (JSP)	8.0	43
A_1 (ASP)	4.5	44
A_1 (FSP)	4.5	44
A_4 (FSP)	5.6	26
C_1 (FSP)	9.0	24
C_2 (FSP)	8.0	40

A—anionic isoenzymes; C—cationic isoenzymes

The M_rs of purified orange cationic isoenzymes determined by the gel filtration technique, were found to be similar to those enzymes in mango, where the cationic isoperoxidase had a M_r value less than 30 k [18]. However two anionic isoperoxidase identified in this work were shown to have M_r less 30 k which is low for peroxidase generally.

Inactivation plots for peroxidase activity present in crude soluble extracts from orange juice, albedo and peel are shown in Fig. 5. For all three extracts the loss of peroxidase activity was non-linear with heating time. The non-linearity for heat inactivation in the crude extracts, may be due to the presence of a number of isoperoxidases with differing thermostabilities.

The peroxidase activity and the number of isoenzymes in the flavedo fraction were higher than in

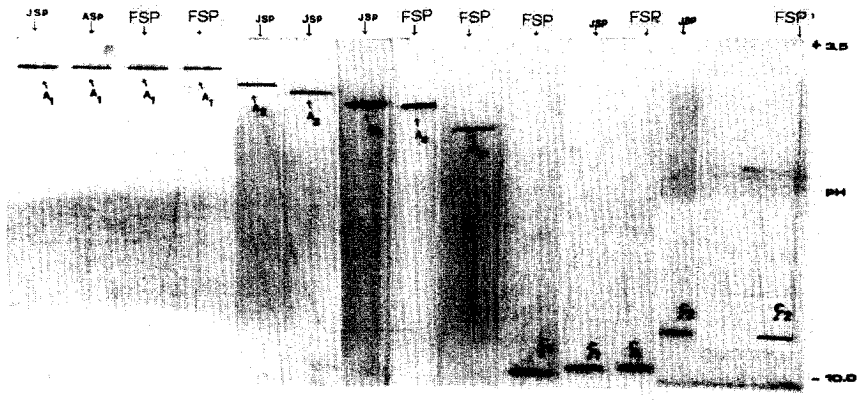

Fig. 3. Isoelectric focusing of the purified oranges peroxidases obtained from the soluble fractions of the juice (JSP), albedo (ASP) and flavedo (FSP), stained for peroxidase activity with *o*-dianisidine.

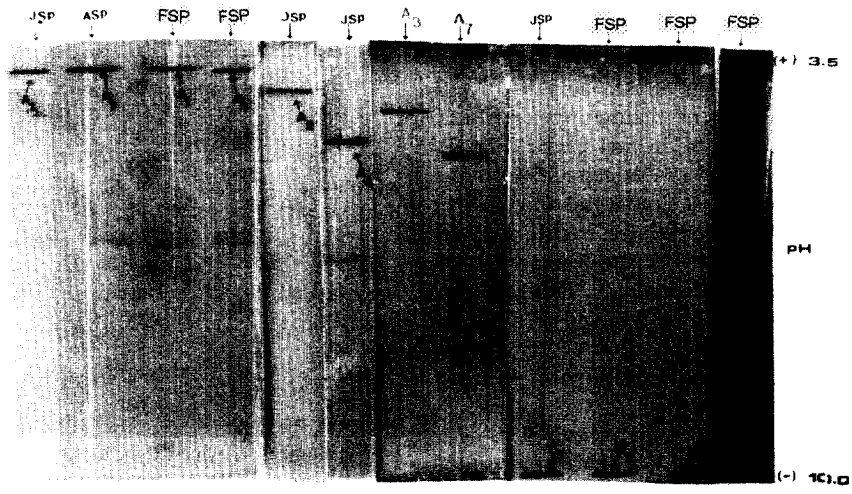

Fig. 4. Protein silver stain of purified orange isoperoxidases.

the other fractions (JSP and ASP), and the higher heat stability of the soluble peroxidase from the flavedo fraction, could be due to the presence of isoenzymes with different degrees of heat stability which do not appear in the other fractions. In mango crude extracts the initial rapid loss of enzymic activity may be due to the inactivation of heat labile isoperoxidases, and the further smaller losses may be due to the presence of more thermostable isoperoxidase [16].

The purified isoperoxidases obtained from orange juice (A_1, A_4, C_1 and C_2) lost about 70%, 80% and 15% of their original activity when exposed to 70° for 50 s (Figs 6 and 7) compared with a loss of about 85% when present as a mixture in the soluble crude extract.

The A_1 (ASP) and A_1 (FSP) (Fig. 6) showed similarity with the A_1 isoperoxidase obtained from the juice fraction when heated at 70°. After heating at 70° purified C_1 and C_2 (obtained from the fraction JSP and FSP) isoperoxidase were shown to possess greater thermostability (Fig. 7), when compared with the purified anionic isoenzymes. This indicated that the cationic isoenzymes were responsible for the higher POD stability.

The enzymic activity of purified orange iso-

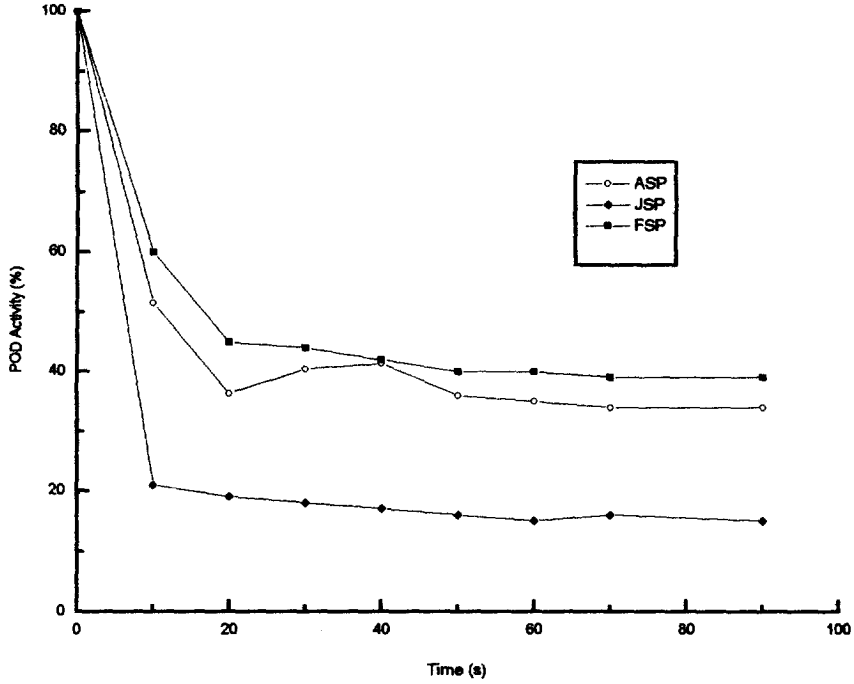

Fig. 5. Heat inactivation (at 70°) for the crude extracts of orange peroxidase, JSP—Juice soluble peroxidase, ASP—Albedo soluble peroxidase and FSP—Flavedo soluble peroxidase (cultivar Large Valencia).

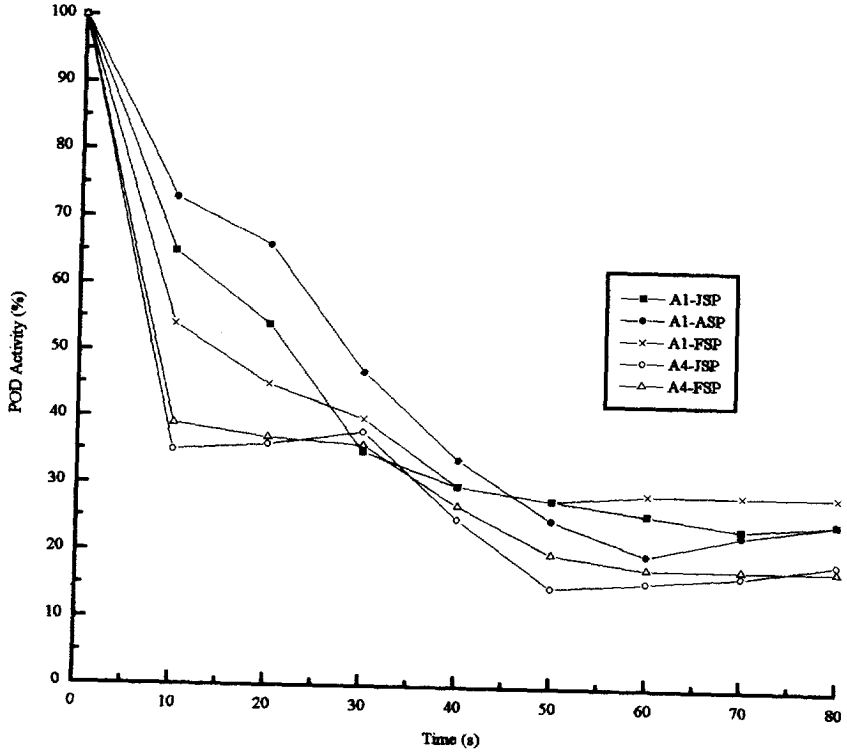

Fig. 6. Isoenzyme activity (% of original) versus time in sec (at 70°).

peroxidases did not regenerate substantially during a 30 min period after heat treatment. The isoperoxidases (A_1, A_4, C_1 and C_2) showed about 7–10% regain in enzymic activity at 30° when held for 40 min.

The β-secondary structure in protein is considered the most stable when there is a high content of hydroxy amino acids [24]. The higher heat stability of the purified mango A_1 isoenzyme was attributed to a

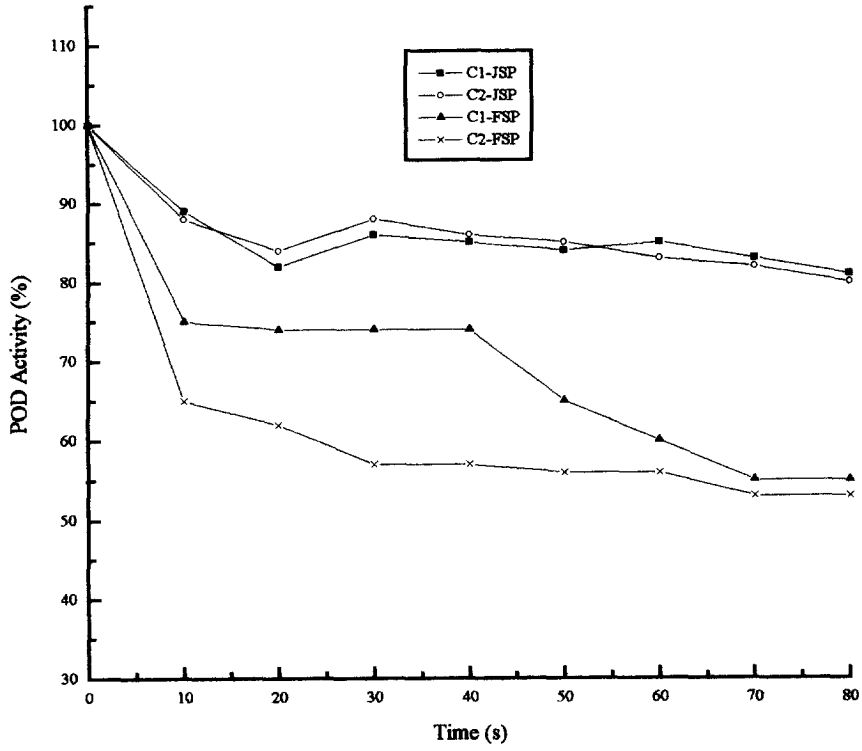

Fig. 7. Isoenzyme activity (% of original) versus time in sec (at 70°).

greater proportion of hydroxy amino acids [16]. The non-linear inactivation of a single purified isoenzyme from oranges may happen because of microheterogeneity due to the presence of variable amounts of covalently bound neutral carbohydrate which will not affect the isoelectric point but might affect the thermostability. The possible effect of covalently bound carbohydrates on thermostability of peroxidase has not yet been proven.

EXPERIMENTAL

Material

Fresh ripe oranges (*Citrus sinenis* (L.) Osbeck) cultivars Large Valencia, Small Sweet and Navel were purchased in a local supermarket. Sephacryl S-100 HR, Sephadex G-75, Ampholine Carrier Ampholyte and all other materials and equipment for analytical isoelectric focusing and FPLC was used as supplied by LKB Instruments Ltd. Rotofor (preparative isoelectric focusing) and silver stain kit were used as supplied by BioRad. *o*-Dianisidine was obtained from Koch Light; Bovine serum albumin was from Sigma and Pentosanese from Novo Products. All other Chemicals where available in Analar grade, were obtained from BDH.

Sample preparation

Orange fruit were washed in tap H_2O and peeled. The juice sacs were carefully separated from the albedo, seeds and central placenta or core, 500 g sample of the juice sacs were then homogenized in 500 ml of 0.1 M Na-P_i buffer at pH 6 using a liquidizer. 1% (w/v) insoluble polyvinylpolypyrrolidone was added to the extract buffer to improve the stability of the enzyme by removing phenolic compounds [18]. The homogenized suspension was filtered through a double layer of muslin cloth and the resultant filtrate was centrifuged at 17,000 *g* for 20 min at 4°. The supernatant was collected and stored at −18° and designated juice soluble peroxidase (JSP). For extraction of the soluble peroxidase from albedo and from flavedo, first the albedo was carefully separated from the endocarp and flavedo. Then 10 g of albedo and also 10 g of flavedo were cut into small pieces, and each sample was homogenized for 1 min in 100 ml of 0.1 M Na-P_i buffer at pH 6 containing 1% (v/v) of pentosanase, using a Waring blender. Then the same process was used for filtration and centrifugation to obtain the soluble fraction from the juice; two fractions were obtained and were designated albedo soluble peroxidase (ASP) and flavedo soluble peroxidase (FSP) respectively. The preparation of the fractions with ionically bound peroxidase forms, was carried out from each residue remaining after the extraction

of the soluble fractions. The residue was washed twice to remove any traces of soluble peroxidase activity, by resuspending in 100 ml of 0.1 M Na-P_i buffer pH 6. Each suspension was centrifuged at 17,000 *g* and the supernatant was discarded. Then the washed residue was resuspended in 100 ml 1 M NaCl in Tris/HCl buffer (pH 7.5), the suspension centrifuged at 17,000 *g* for 20 min at 4° and the supernatant fluids collected and stored at −18°. Those fractions were designated 'juice ionically bound peroxidase' (JIP), 'albedo ionically bound peroxidase' (AIP) and 'flavedo ionically bound peroxidase' (FIP).

Analysis

Peroxidase activity was assayed with the ortodianisidine method at pH 6 at 25° [25]. The reaction mixture contained 2.7 ml of 0.03% H_2O_2 in 0.1 M Na-P_i buffer at pH 6 and 0.2 ml of the peroxidase extract. The enzymatic reaction was initiated by the addition of 0.1 ml, 1% (w/v) *o*-dianisidine and the initial change in *A* was recorded at 460 nm at 25° using a Pye Unicam SP 8-200 UV/VIS spectrophotometer for a period of 1 min. Each sample was assayed in triplicate.

Preparative isoelectric focusing (PIF)

All extracts were dialysed overnight in deionised H_2O, to remove the excess of small M_r compounds, with changes of deionised H_2O each 2 h for a period of 10 h, prior to analysis in Rotofor. PIF was carried out using a Rotofor unit equipped with a BioRad Multitemp thermostatic circulator. following that, the pH gradient was measured in the 20 fractions originated from Rotofor and then applied to gel filtration.

Gel filtration

The enzyme soln (10 ml) collected after gel filtration in Sephacryl S-100 HR (column 16 mm i.d. × 70 cm, eluent 0.1 M Na-P_i at pH 6, flow rate 30 ml h^{-1}) was dialysed against 12.5 mM Tris/HCl buffer (pH 7.5) overnight and then applied to a FPLC-LCC500 (Mono-Q column 5/5). Fractions were eluted with buffer (0.025 M Tris/HCl buffer pH 7.5) up to 0.5 M NaCl. Isoelectric focusing (IEF) was carried out using an Ultrophor Electrofocusing unit equipped with an LKB Multi-Temp thermostatic circulator. The pH gradient in the gel was measured by surface electrode. The focused gels were stained for peroxidase activity with *o*-dianisidine [26].

Heat inactivation studies

Micro tubes (1 cm length and 1 mm inside diam.) were filled with 50 μl of POD samples using a syringe. The tubes were sealed at both ends, and plunged in a water bath at 70° for a period of 10, 20, 30, 40, 60, 80 and 120 s. Once the required heating time was reached the micro tubes were immediately transferred in triplicate to a water bath at 0° and the activity of each sample was then measured.

Total protein content of the crude and purified extracts was estimated by dye binding [27].

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