ELSEVIER

Food Chemistry 80 (2003) 499–510

Food Chemistry

www.elsevier.com/locate/foodchem

Heat-inactivated peroxidases and the role of calcium abstraction as a cause of their enhanced lipid oxidation activity: potential effects on the flavour quality of heat-processed vegetables

J.B. Adams[a,*], H.M. Brown[a], D.A. Ledward[b], R. Turner[b]

[a]*Campden and Chorleywood Food Research Association, Chipping Campden, Gloucestershire GL55 6LD, UK*
[b]*The University of Reading, School of Food Biosciences, PO Box 226, Whiteknights, Reading RG6 6AP, UK*

Received 3 May 2002; accepted 28 June 2002

Abstract

Cationic swede and anionic turnip peroxidases were partially purified by ion-exchange and gel-filtration chromatography, respectively. Heat treatment of these enzymes and of a commercial high purity horseradish peroxidase (HRP) caused a loss of enzyme activity and a corresponding increase in linoleic acid hydroperoxide formation activity. The hydroperoxide levels in model systems increased only in the early stages of the oxidation reaction and then declined as degradation became more significant. The presence of a dialysed blend of cooked swede markedly lowered the hydroperoxide level formed. Analysis of volatile compounds formed showed that hexanal predominated in a buffer system and in a blend of cooked turnip. In dialysed blends of cooked swede, hexanol was the primary volatile compound generated. After inactivation under mild conditions in the presence of EDTA, the peroxidases showed hydroperoxide formation activity and patterns of volatile compounds from linoleic acid that were similar to those found on heat-inactivation. This suggested that calcium abstraction from the peroxidases was critical for the enhancement of lipid oxidation activity.

Keywords: Peroxidase; Calcium abstraction; Lipid oxidation; Heat-processed vegetable flavour

1. Introduction

Identification of the catalysts of lipid oxidation is important in the development of strategies for stabilising the flavour of heat processed vegetables. Vegetable lipoxygenases are heat-labile and normal thermal processing would be expected to control lipid oxidation if these enzymes were the sole catalysts. However, lipid oxidation is known to continue in the absence of active enzyme. In processed tomatoes, hexanal was formed (Schreier, Drawert, & Junker, 1977), and in blanched cauliflower linolenic acid levels fell and sensory quality diminished during frozen storage (Pizzocaro, Senesi, & Monteverdi, 1986). The causes of this non-enzymatic lipid oxidation in foods are unknown, though model system studies have shown that heat-denatured peroxidases may be involved (Eriksson & Vallentin, 1973; Adams, Harvey, & Dempsey, 1996). The available evidence suggests that the increased lipid oxidation activity was due to intact denatured peroxidase rather than haem released from the enzyme, or iron from the haem, as a result of the heat treatment (Adams & Lock, 1997). It is proposed that the heat-induced increase in lipid oxidation activity is due to a greater exposure of the lipid to the haem in the denatured enzymes. NMR studies have confirmed that structural changes occurred in heat-treated horseradish peroxidase (HRP) that could be linked with the observed enhancement of lipid oxidation activity (Adams et al., 1996).

Of the three proposed classes of enzyme within the plant peroxidase superfamily, calcium ions are an integral part of the structure of both the class II fungal lignin peroxidase (Nie & Aust, 1997) and manganese peroxidase (Sutherland, Zapanta, Tien, & Aust, 1997) and the secretory class III enzymes, such as the isozyme C of HRP (Haschke & Friedhoff, 1978) and the cationic isozyme of peanut peroxidase (Barber, Rodriquez-Maranan, Shaw, & Van Huystee, 1995). No calcium ions have been found in the class I enzymes, such as the ascorbate peroxidase of pea cytosol and the cytochrome *c* peroxidases.

* Corresponding author.

0308-8146/03/$ - see front matter
PII: S0308-8146(02)00319-9

The purpose of the present work was to determine the potential effects of heat-inactivated peroxidases on off-flavour formation in processed vegetables, and to study the role of calcium abstraction from peroxidases as a cause of the increased lipid oxidation activity that occurs on heat treatment, Swede (*Brassica napus*) and turnip (*Brassica rapa*) were chosen as they contain high levels of class III peroxidase, and because they are consumed as cooked vegetables or in the form of industrially heat-processed products.

2. Materials and methods

2.1. Materials

HRP (Type VI) was purchased from Sigma Chemical Company and used without further purification. Reagents were of analytical grade and were supplied by Merck or Sigma Chemical Co. Swedes and turnips were purchased from local stores.

2.2. Purification of swede and turnip peroxidases

2.2.1. Swede peroxidase

The cationic peroxidase from swede was extracted and purified using a method based on that of Mazza, Charles, Bouchet, Ricard, and Raynaud (1968). Peeled and chopped swede (100 g) was blended for 30 s in 200 ml of 0.1 M sodium phosphate buffer containing 1 M sodium chloride (pH 6.8). This extract was held for 18 h at 4 °C, and then centrifuged for 20 min at 32,000 *g*. Ammonium sulphate was added to the supernatant at 50% saturation to remove non-peroxidase protein, and centrifuged for 10 min at 12,000 *g*. Ammonium sulphate was added to this supernatant at 90% saturation to precipitate the peroxidase. After centrifugation for 10 min at 12,000 *g*, the pellet was dissolved in a small volume of phosphate buffer (0.1 M, pH 6.8) and dialysed against the buffer to remove residual salts. The swede peroxidase extract was applied to a column of Sephadex CM-50 (100 ml, radius 1.2 cm), pre-equilibrated with 0.1 M phosphate buffer. After elution of the anionic and neutral peroxidase isoenzymes with the 0.1 M buffer, the concentration of the eluting buffer was changed to 0.3 M to release the bound cationic isoperoxidases. Fractions were collected and those with the highest peroxidase activity were combined, dialysed against 0.1 M phosphate, and concentrated using solid polyethylene glycol. The swede peroxidase concentrate was applied to a column of Sephadex G-100 (120 ml, radius 1.1 cm), pre-equilibrated with 0.1 M phosphate buffer (pH 6.8). After elution with phosphate, fractions with the highest peroxidase were combined and concentrated using polyethylene glycol. The concentrate was held at 4 °C prior to use.

2.2.2. Turnip peroxidase

The anionic peroxidase from turnip was extracted and purified using a method based on that of Agostini, Medina, Milrad de Forchetti, and Tigier (1997). Peeled and chopped turnip (100 g) was blended for 30 s in 200 ml of 0.1 M sodium acetate buffer (pH 4.0) and centrifuged for 15 min at 14,000 *g*. Ammonium sulphate was added to the supernatant at 40% saturation to remove the non-peroxidase protein, and then centrifuged for 10 min at 12,000 *g*. Ammonium sulphate was added to this supernatant at 90% saturation to precipitate the peroxidase. After centrifugation for 10 min at 12,000 *g*, the pellet was dissolved in a small volume of 0.1 M phosphate (pH 6.8) and the solution was then dialysed against the buffer to lower the level of acetate ion.

The turnip peroxidase extract was applied to a column of Sephadex G- 100 (120 ml, radius 1.1 cm), pre-equilibrated with 0.1 M phosphate buffer (pH 6.8). Fractions were collected and those with the highest peroxidase activity were combined and concentrated by ultrafiltration using a Minitan UF unit. The concentrate was held at 4 °C prior to use.

2.3. Peroxidase assay

Peroxidase assays were performed using a substrate solution that contained 0.057 ml of 30% hydrogen peroxide and 0.056 ml of liquid guaiacol in 100 ml of 0.1 M acetate buffer, pH 5.6. The peroxidase activity was determined by adding 0.1 ml of sample, appropriately diluted, to 3.0 ml of substrate solution equilibrated at 30° C for 5 min. The rate of increase in absorbance at 420 nm, due to the formation of brown guaiacol oxidation products, was measured using a single beam (ThermoSpectronic PU8755) or double beam (Perkin Elmer, Lambda Bio 20) spectrophotometer. The activity was calculated from the slope of a linear segment of at least 30 s duration, expressed as A 420 nm/min/0.1 ml of undiluted sample.

2.4. Protein assay

Protein determination was carried out using the commercial form of the Coomassie Blue dye-binding method of Bradford (1976) as recommended by the suppliers (Bio-Rad).

2.5. RZ value determination

The purity of peroxidase preparations was determined by measuring the ratio of the haem absorbance (at 403nm) to the protein absorbance (at 280 nm). This ratio is denoted the RZ value.

2.6. Lipid oxidation assay

Linoleic acid (99% pure, 0.125 ml) was mixed with Tween 20 (0.125 ml) and NaOH (1 M, 0.325 ml) to give

a clear solution. Borate buffer (0.1 M, pH 9.0, 25 ml) was then added in small aliquots, followed by distilled water (25 ml). The pH of the solution was adjusted to 7.0 with concentrated HCl added dropwise. This stock solution was stable for approximately 1 week under chill conditions. In order to test samples for lipid oxidation activity, the stock solution was diluted 50-fold in citrate-phosphate buffer (0.1 M, pH 7.0).

The determination of the lipid oxidation activity of inactivated peroxidases involved measurement of (1) the linoleic acid hydroperoxide formation rate (2) the levels of hydroperoxides and (3) the levels of volatile compounds. All three methods used the diluted linoleic acid solution in citrate-phosphate buffer. The methods involving measurement of hydroperoxide levels and volatile compounds were also employed when dilution was carried out using blended cooked swede suspensions that had been dialysed prior to use. The lipid oxidation activity of the sample was determined in triplicate for each method.

2.6.1. Hydroperoxide formation rate

Sample (0.1 ml) was added to diluted linoleic acid solution (3.0 ml), pre-equilibrated at 30 °C. The rate of increase in absorbance at 234 nm, due to hydroperoxide formation, was then measured from the slope of a linear segment of at least 30 s duration. This was expressed as A 234 nm/min/0.1 ml of sample.

2.6.2. Hydroperoxide level

Stock linoleic acid solution (0.5 ml) was added to aliquots of citrate-phosphate buffer (7 ml), pre-equilibrated at 30 °C in 20 ml sealed glass bottles. Heat-inactivated peroxidase (0.1 ml) was added, and after times up to 20 h, the mixtures were extracted with iso-octane (7 ml) (Gardner, 1975). The absorption spectrum of the iso-octane extract was measured from 200 to 300 nm and the hydroperoxide level determined from the peak maximum at 234 nm, after subtraction of baseline absorbance. Buffer controls were run alongside the inactivated peroxidase samples.

Similar experiments were carried out using extracts of cooked swede as a medium for the linoleic acid. Peeled and chopped swede (100 g) was heated for 5 min in boiling water, drained and then blended with citrate-phosphate buffer (200 ml). The heat-inactivated peroxidase was added to this mixture without further treatment, and also to another portion of the mixture that had been thoroughly dialysed.

2.6.3. Volatile compound formation

Stock linoleic acid solution (0.5 ml) was added to citrate-phosphate buffer (7 ml, pH 7.0) (or blended cooked swede/turnip suspension in citrate-phosphate) in a 40 ml septum-sealed vial. Inactivated peroxidase (0.1 ml of 1 mg/ml HRP in citrate-phosphate buffer, or 0.3 ml of swede/turnip peroxidase extracts, heated for 10 min at 100 °C or 14 days at 30 °C in the presence of 0.01 M EDTA), or an appropriate volume of buffer (control) was added to the linoleic acid. The vial was placed in a water bath for 24 h at 30 °C and then the volatile compounds in the headspace above the sample were trapped using solid phase micro-extraction (SPME) with a Supelco fibre assembly and injection device (60 μm Carboxen-PDMS coating). This was inserted (unexposed) into the vial and then exposed to the headspace for 30 min. Immediately on removal from the vial, the trapped compounds were desorbed for 3 min at 40 °C and cryoscopically focused in the injector of a Hewlett Packard 5890 Series II gas chromatograph, pre-loaded with 1,2-dichlorobenzene (0.1 μl) as an internal standard. The compounds were then chromatographed using a fused silica capillary column (CP-Sil8, 60 m×0.25 mm I.D., 0.25 μm film thickness) in the GC oven which was temperature-programmed at 4 °C/min, to reach 220 °C and held at this temperature for 5 min. Prior to chromatography of the trapped compounds, a 6–24 carbon alkane mixture was run to determine the linear retention index (LRI) of the column. This was repeated after the chromatography to check for changes in column behaviour.

After passing through the column, the volatile compounds were introduced into a Hewlett Packard 5972 mass spectrometer for detection by electronic ionisation. Identification was performed using a G1034C Chemstation and a standard MSM data analysis program. Quantification was carried out assuming that the internal standard and the separated compounds had equal response factors. The compounds derived from linoleic acid were determined for each of three identically treated septum-sealed vials and those detected at greater than 1 ng per sample were recorded.

2.7. Heat- and EDTA-inactivation of peroxidases

The peroxidases were thermally inactivated by heating for 10 min at 100 °C in citrate-phosphate buffer (0.1 M, pH 7.0). The peroxidases were treated with EDTA by holding for 14 days at 30 °C in 0.1 M citrate-phosphate buffer containing 0.01 M EDTA (pH 7.0). Control samples, in citrate-phosphate without EDTA, were held under the same conditions. Both the heat- and the EDTA-treated samples were dialysed against the citrate-phosphate buffer containing no EDTA.

3. Results and discussion

3.1. Vegetable peroxidase purification

Purification of the swede cationic peroxidase by ammonium sulphate fractionation and ion-exchange

chromatography yielded a preparation with an RZ value of approximately 1.5. The purity of the preparation was not improved using gel chromatography, due to the presence of non-peroxidase proteins with similar chromatographic behaviour to the swede peroxidase (Turner, 2001). The RZ value was low compared with that of the highest grade of commercial HRP, the cationic Type VI with RZ~3.0. Purification of the turnip anionic peroxidase, using ammonium sulphate fractionation and gel chromatography, also gave a preparation with a low RZ value, suggesting the presence of non-peroxidase proteins with similar molecular weights to that of the enzyme.

3.2. Hydroperoxide formation activity

The hydroperoxide formation activity of all three peroxidases (swede, turnip and horseradish) increased on heating at 100 °C as the enzyme activity decreased (Figs. 1–3). After around 4 min heating, and before all of the peroxidase had been inactivated, the hydroperoxide formation activity became constant, suggesting that the heat-treated peroxidases were causing the breakdown of the hydroperoxides as well as their formation. Involvement in the hydroperoxide breakdown mechanism has been confirmed by showing that volatile compounds are produced on addition of heat-inactivated HRP to preformed linoleic acid hydroperoxides (Turner, 2001).

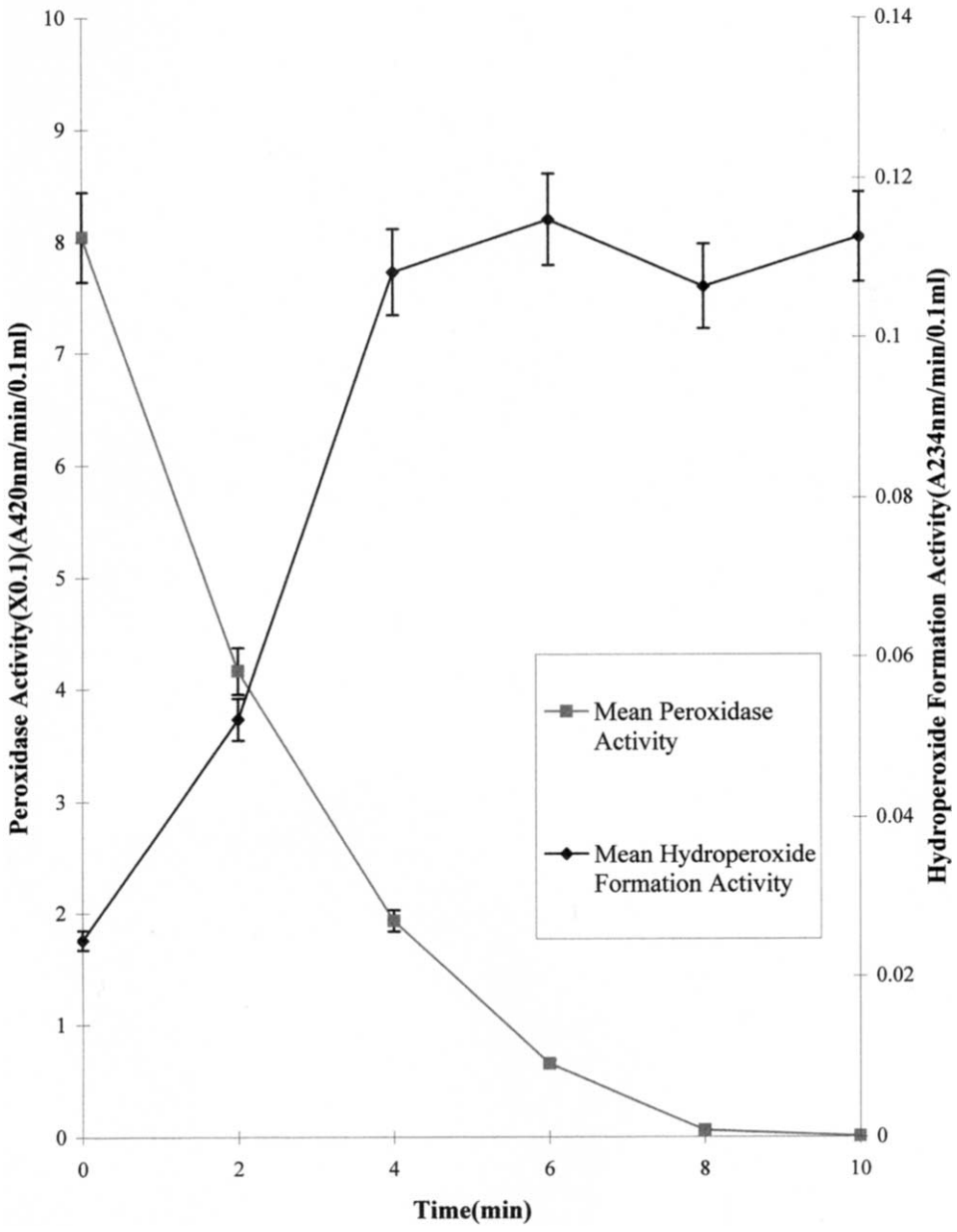

Fig. 1. Effect of heating time at 100 °C on the enzyme and hydroperoxide formation activities of HRP.

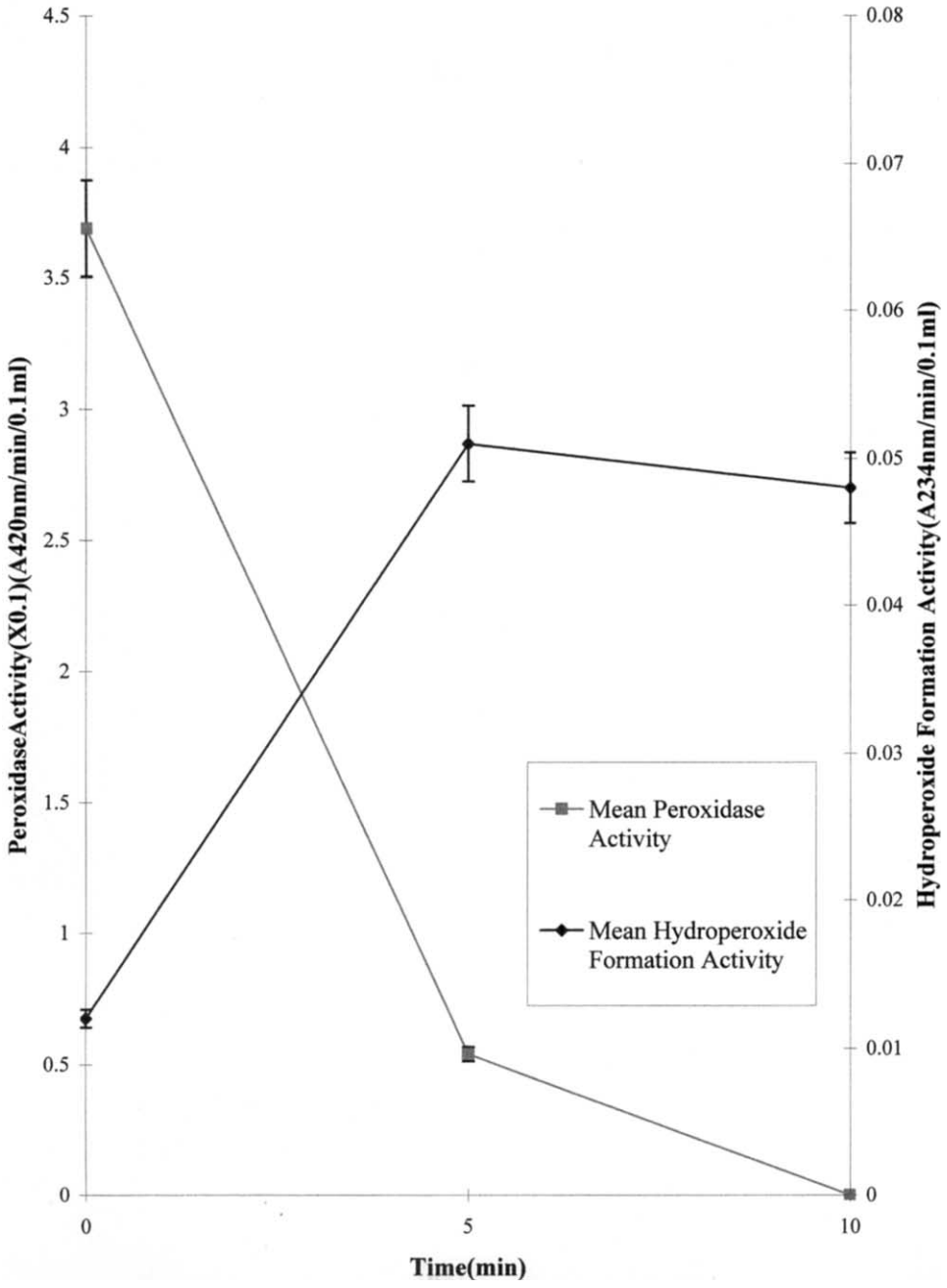

Fig. 2. Effect of heating time at 100 °C on the enzyme and hydroperoxide formation activities of swede peroxidase.

The similar increases in the hydroperoxide formation activities of each peroxidase indicated that the non-peroxidase proteins did not have a significant influence on the hydroperoxide-forming activity of the heated swede and turnip peroxidases. This was further confirmed by means of experiments with the swede and turnip preparations containing different amounts of non-peroxidase protein but identical peroxidase activities (Turner, 2001). These have shown that the hydroperoxide formation activity depended only on the peroxidase level.

EDTA treatment, under non-denaturing conditions, caused a similar rate of activity loss for all three peroxidases, with complete inactivation taking about 14 days at 30 °C (Turner, 2001). The untreated peroxidases lost little or no activity during this time. These results are in agreement with the effect of EDTA on peanut peroxidase under non-denaturing conditions (50 mM EDTA, pH 7.0, 2 days at 4 °C) where the loss of enzyme-bound calcium caused 50% inactivation (Barber et al., 1995). The hydroperoxide formation activities of the heat- and EDTA-treated enzymes were similar (Table 1), suggesting that calcium abstraction on heating had a significant influence on the enhancement in lipid oxidation activity. The peroxidases of class II and

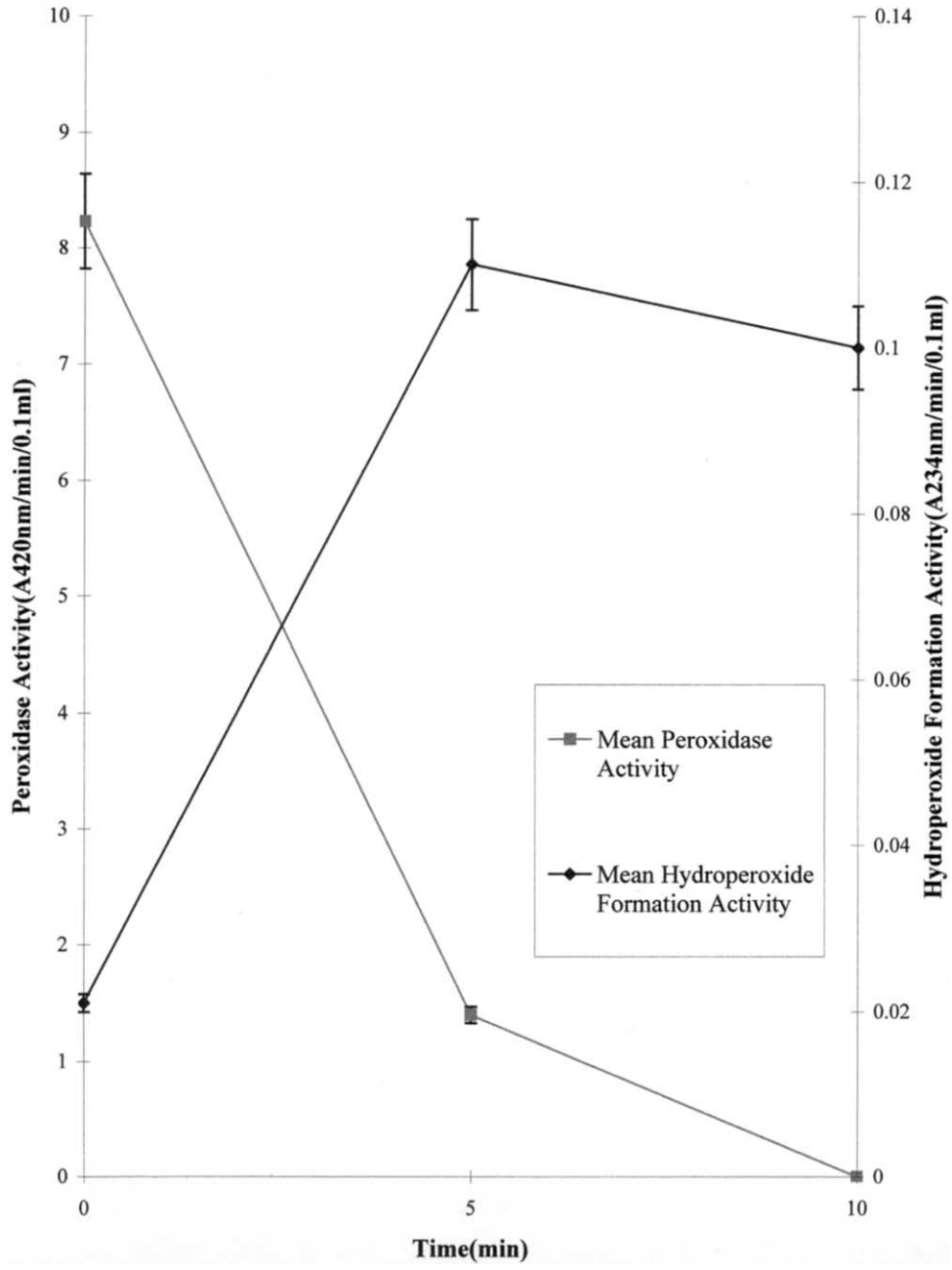

Fig. 3. Effect of heating time at 100 °C on the enzyme and hydroperoxide formation activities of turnip peroxidase.

Table 1
A comparison of the hydroperoxide formation activities of the heat- and EDTA-inactivated peroxidases

Vegetable source of peroxidase	Hydroperoxide formation activity: (A234 nm/min/0.1 ml)	
	Heat treatment	EDTA treatment
Horseradish	0.092 (0.004)	0.087 (0.005)
Swede	0.043 (0.003)	0.041 (0.004)
Turnip	0.066 (0.002)	0.063 (0.003)

Hydroperoxide formation activity: mean result of three replicate experiments with standard deviation in parentheses.

III contain two calcium-binding sites, one proximal and the other distal to the haem, which have important stabilising effects on the native enzymes. It has been suggested that removal of the proximal calcium in HRP (under denaturing conditions) significantly perturbs the proximal cavity near the histidine ligand with only minor effects at the distal histidine (Howes, Feis, Raimandi, Indiani, & Smulevich, 2001). In calcium-free peroxidases, however, molecular dynamics calculations have shown that it is the distal histidine that moves away from the iron and that this is the main cause of reduced enzyme activity (Banci, Carloni, Diaz, &

Savellini, 1996). As the distal histidine, together with an arginine residue, causes polarity in the haem cavity of the native enzyme, this relatively large movement could allow other, more hydrophobic residues, such as phenylalanine, to be dominant and thereby increase the hydrophobicity of the haem environment in the calcium-depleted peroxidase. Pahari, Patel, and Behere (1995) have provided evidence that calcium removal caused an increased separation of tryptophan from haem in a hydrophobic crevice, concluding that this led to a more hydrophilic region being formed around the tryptophan. This may be consistent with the distal histidine moving away from the haem. It can therefore be speculated that the hydroperoxide formation reaction is favoured by the following effects that occur as a result of calcium abstraction:

1. The movement of the distal histidine away from the haem that increases lipid access to a more hydrophobic haem environment.
2. The formation of an amphiphilic region at a distance from the haem that could be the site of the subsequent breakdown of the relatively polar hydroperoxides.

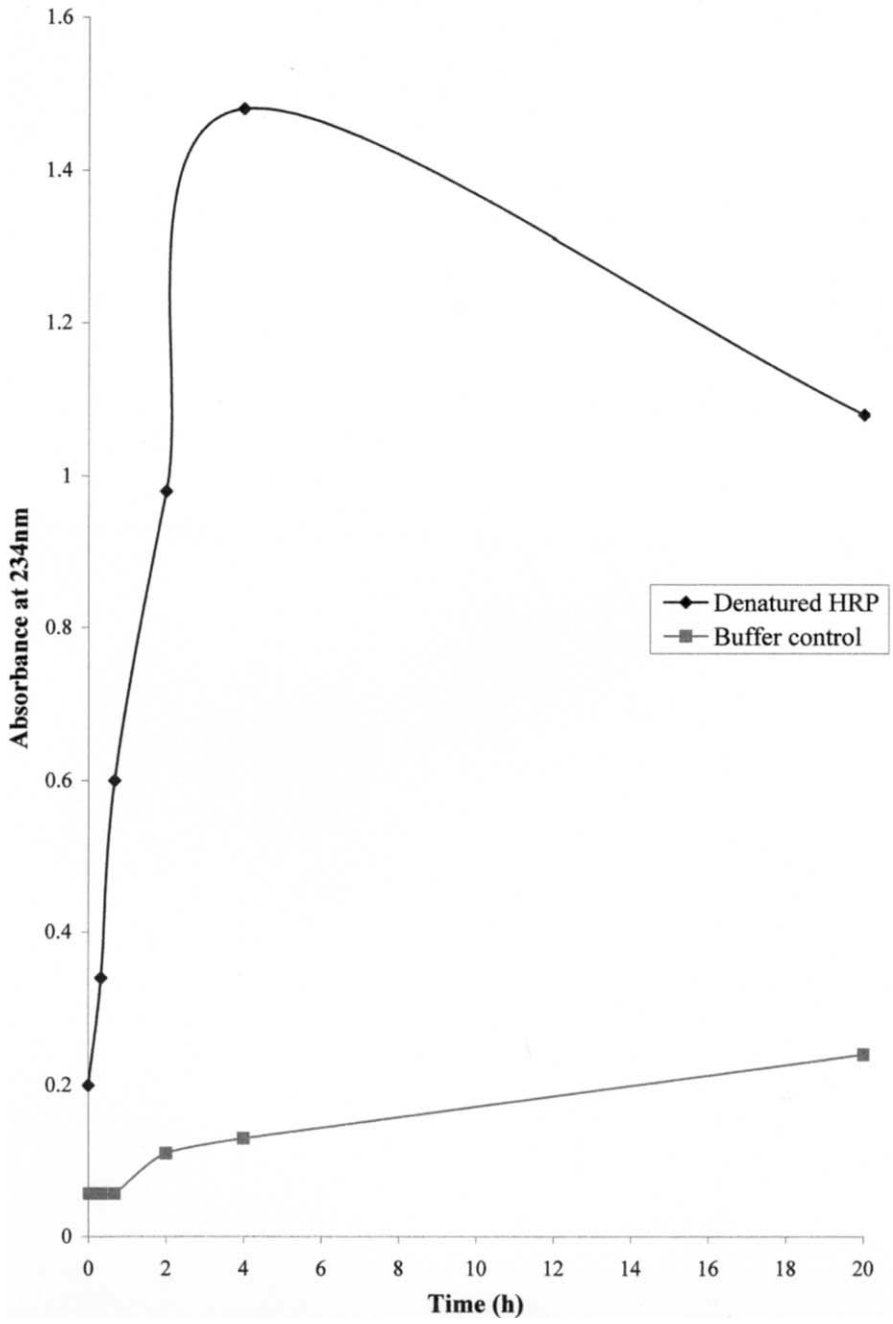

Fig. 4. Linoleic acid hydroperoxide formation in buffer catalysed by heat-denatured HRP.

The removal of calcium ions is known to have a significant effect on the spin-state of the iron that is dependent on the source of the peroxidase. Sutherland et al. (1997) have provided evidence that thermal inactivation of the manganese peroxidase from the white-rot fungus, *Phanerochaete chrysosporium*, caused a loss of calcium from the enzyme and this resulted in the distal histidine being bound as a sixth ligand, converting the iron from high-spin to low-spin. This was in contrast to the behaviour of cationic peanut peroxidase and HRP in that both remained predominantly high-spin. It was proposed that the calcium was only loosely bound in the manganese peroxidase and did not contain the disulphide bridge, adjacent to the calcium-binding site, of the peanut enzyme. The latter proposal has been supported by addition of a single disulphide bond in the distal region of manganese peroxidase that maintained a pentacoordinate, high-spin iron under destabilising conditions (Reading & Aust, 2001). Thus, it can be hypothesised that the haem in the heat- or EDTA-inactivated

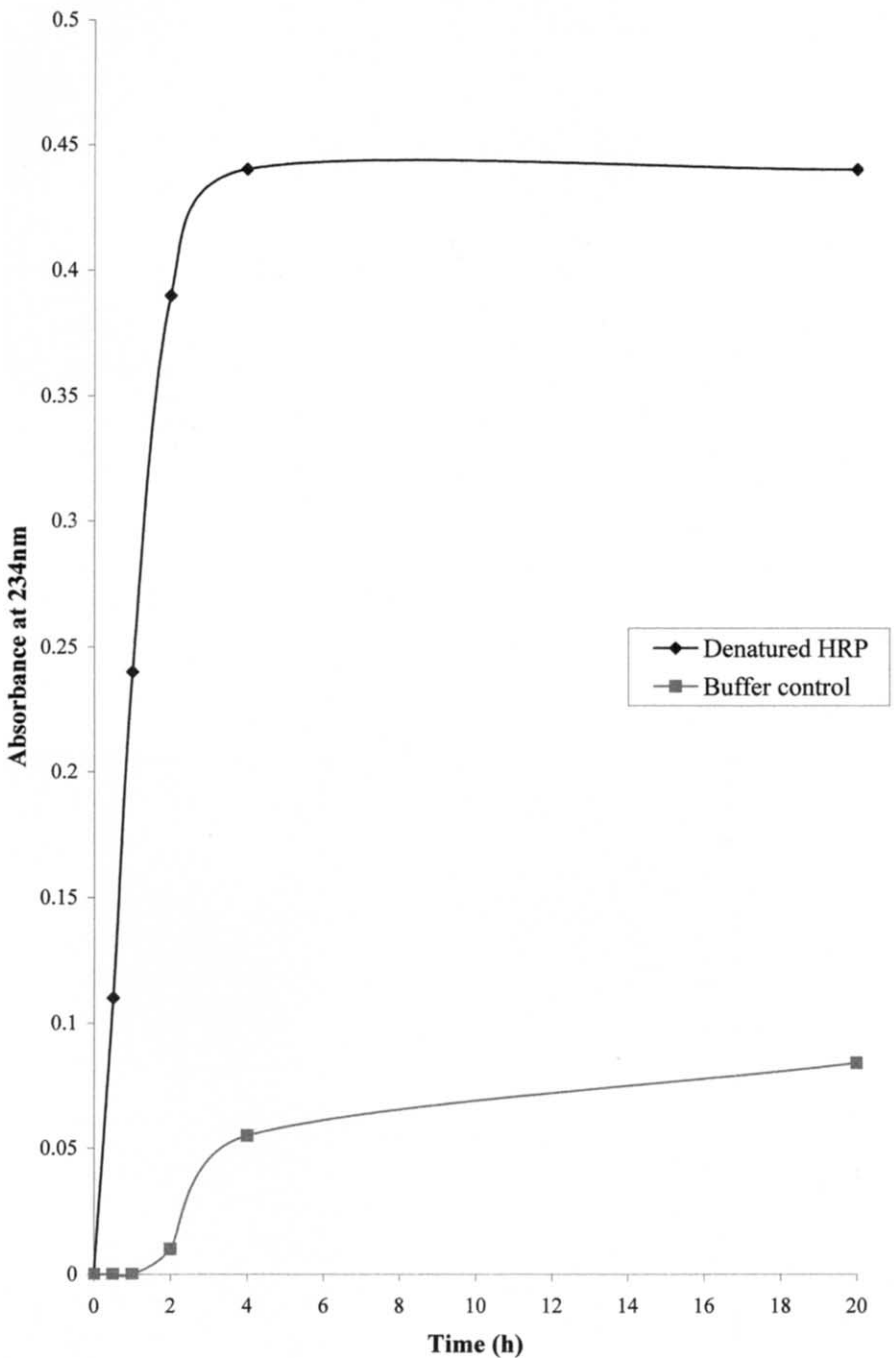

Fig. 5. Linoleic acid hydroperoxide formation in a dialysed cooked swede mixture catalysed by heat-denatured HRP.

peroxidases contains predominantly pentacoordinate, high-spin iron that enhances the formation of free radicals in the hydrogen atom abstraction step of the lipid oxidation reaction mechanism.

3.3. Hydroperoxide levels formed

Iso-octane extraction and UV absorption measurements showed that hydroperoxides were formed from linoleic acid in the presence of heat-denatured horseradish and swede peroxidases (Figs. 4–6). The highest level of hydroperoxides, in buffer containing heat-denatured HRP, represented approximately 10% conversion of linoleic acid to the hydroperoxide, assuming that the linoleic acid hydroperoxide molar extinction coefficient at 234 nm was in the region of 25,000 litre/mole/cm (Gibian & Vandenberg; 1987). Hydroperoxide formation evidently took precedence only in the early stages of the oxidation reaction and then the levels declined as degradation became more significant.

The presence of a dialysed blend of cooked swede markedly lowered the hydroperoxide level formed, possibly due to reduction of the ferric form of the denatured peroxidases by small amounts of reducing agents, such as

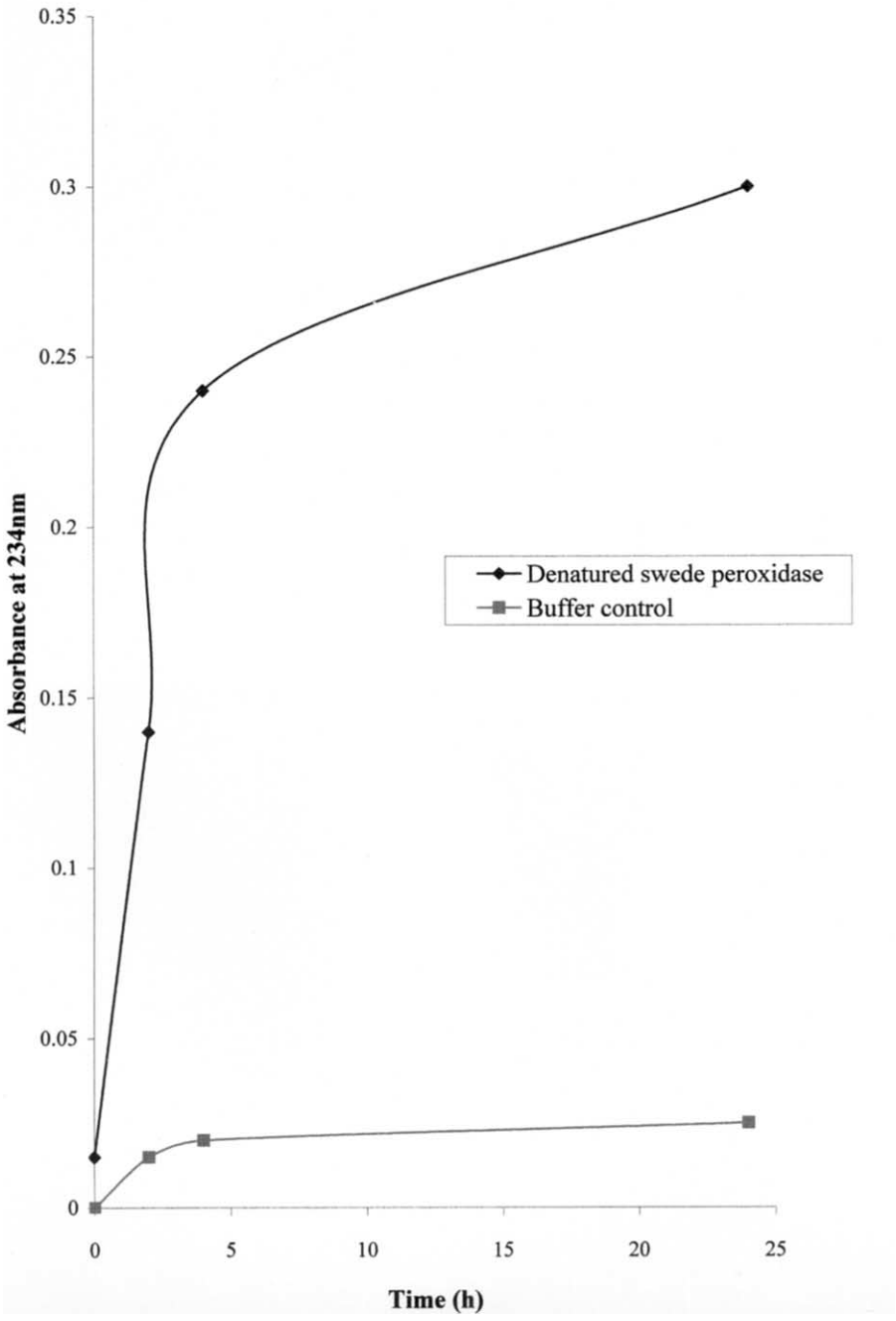

Fig. 6. Linoleic acid hydroperoxide formation in a dialysed cooked swede mixture catalysed by heat-denatured swede peroxidase.

ascorbic acid and glutathione, left behind after the dialysis procedure. Glutathione can also react directly with linoleic acid hydroperoxide (Zamora et al., 1989).

3.4. Volatile compound formation and potential flavour changes

Each heat-inactivated peroxidase enhanced the amount of volatile compounds formed from linoleic acid in citrate-phosphate buffer (pH 7.0) over 24 h at 30 °C (Table 2). Hexanal was the primary volatile formed in all cases. A similar result was obtained using heat-treated HRP in morpholinosulphonic acid buffer at pH 7.0, suggesting that the buffer type was not an important factor in the catalysis of the lipid oxidation (Turner, 2001). Under the prevailing aqueous conditions, the hexanal could have formed via an ionic mechanism that would have been expected to give rise mainly to hexanal from the 13-hydroperoxide of linoleic acid, and to 2-nonenal from the 9-hydroperoxide (Belitz & Grosch, 1999). This mechanism involves acid-base catalysis, which may therefore occur in the amphiphilic region of the inactivated peroxidase, putatively formed on calcium loss. The absence of 2-nonenal could have been due to the preferential formation of the 13-hydroperoxide under aqueous conditions (Chan & Newby, 1980), and to the high autoxidation rate of the 2-alkenal.

Whilst the flavour changes in linoleic acid model systems, caused by the presence of heat-inactivated peroxidases, would probably be dominated by the amount of hexanal formed because of its low flavour threshold, the flavour changes in real foods would be much more complex. This is evident from the results obtained using cooked swede and turnip (Table 3). Dialysis of the cooked swede blend was shown to be essential before any of the linoleic acid-derived volatile compounds was detected (Turner, 2001). Even after dialysis, hexanol was found at higher levels than hexanal, confirming the incomplete removal of reducing agents from the blend suggested by the relatively low hydroperoxide levels. The conversion of some of the hexanal to hexanol could have a significant influence on the flavour of the cooked swede because of the different flavour character of the alcohol and its relatively high flavour threshold. In normally cooked swede that contains an appreciable proportion of the reducing agents of the raw vegetable, it is expected that the hexanal would be completely reduced and the hexanol produced would make only a small contribution to the final flavour. Dialysis was unnecessary in the case of the cooked turnip blend and the lack of detectable hexanol suggests that the level of reducing agents was minimal. The level of hexanal formed under these conditions was approximately five times higher than the flavour threshold level in water and the aldehyde could therefore contribute to the overall flavour of cooked turnip.

Both heat- and EDTA-inactivated peroxidases led to similar volatile compounds from linoleic acid, with hexanal the major compound formed in all cases (Tables 4 and 5). The similar behaviours of the heat- and EDTA-treated enzymes suggest that calcium abstraction was a major cause of the enhancement in hydroperoxide degradation activity that occurred on heating. The movement of the distal histidine away from the haem iron could have caused the formation of an amphiphilic region in the inactivated peroxidases where the hydroperoxides can bind and then break down.

The variation in the susceptibility of peroxidases to calcium loss during the heat treatment of processed foods could have a significant bearing on their flavour in storage. The compositional factors that control this loss are unknown at present although evidence has recently been presented that, under certain circumstances, it is dependent on the glycosylation patterns of the peroxidase isoforms (Wright & English, 2001).

Peroxidases are not the only haem-containing proteins to show enhanced lipid oxidation activity after heat treatment. Catalases are also widely distributed in vegetables and the bovine enzyme has been shown to behave similarly to peroxidase (Eriksson, Olsson, &

Table 2
The influence of heat-inactivated peroxidases on the formation of volatile compounds from linoleic acid in buffer: potential flavour effects

Volatile compound	Volatile mass (ng)						Potential flavour
	Inactivated HRP		Inactivated swede peroxidase		Inactivated turnip peroxidase		
	Present	Omitted	Present	Omitted	Present	Omitted	
Hexanal	178 (31.9)	32.2 (9.0)	66.9 (14.2)	34.2 (9.5)	118 (13.1)	48.1 (5.9)	Cut grass
2-Heptenal(E)	40.1 (8.8)	6.4 (1.9)	<1	<1	42.6 (4.5)	7.5 (1.3)	Green/putty
l-Octen-3-ol	3.6 (0.9)	<1	1.9 (0.3)	<1	1.2 (0.1)	<1	Mushroom
4-Octen-3-one	15.6 (1.5)	<1	3.5 (1.5)	<1	6.4 (1.2)	2.1 (1.6)	Metallic/mushroom
2-Decenal	<1	<1	<1	<1	5.4 (1.9)	<1	Stale/paint-like/rancid
2,4-Decadienal (E,E)	1.9 (0.6)	<1	4.3 (1.0)	<1	<1	<1	Frying odour
2-Pentylfuran	<1	<1	13.8 (1.6)	2.7 (0.6)	<1	<1	Butter/green bean

Volatile mass: mean result of three replicate experiments with standard deviation in parentheses. Potential flavour: commonly accepted aroma of the pure compound at a concentration in excess of its recognition threshold.

Table 3
The influence of heat-inactivated peroxidases on the formation of volatile compounds from linoleic acid in cooked swede and turnip: potential flavour effects

Volatile compound	Volatile mass (ng)						Potential flavour
	Inactivated HRP in blended cooked swede		Inactivated swede peroxidase in blended cooked swede		Inactivated turnip peroxidase in blended cooked turnip		
	Present	Omitted	Present	Omitted	Present	Omitted	
Hexanol	88.1 (7.1)	18.5 (2.6)	68.2 (8.2)	12.4 (2.6)	<1	<1	Green leafy
Hexanal	11.8 (0.3)	2.1 (0.1)	9.2 (2.3)	1.2 (0.4)	81.9 (15.2)	18.2 (5.1)	Cut grass
2-Heptenal	<1	<1	<1	<1	12.6 (4.2)	4.7 (2.2)	Green/putty
1-Octen-3-ol	3.1 (0.8)	<1	2.2 (0.3)	<1	2.4 (0.7)	0.4 (0.7)	Mushroom
4-Octen-3-one	5.4 (1.2)	1.2 (0.2)	3.6 (2.1)	<1	2.5 (0.6)	1.5 (0.4)	Metallic/mushroom
2-Pentylfuran	28.1 (5.1)	5.7 (0.2)	21.4 (1.1)	4.3 (0.2)	19.4 (4.5)	5.0 (2.3)	Butter/green bean

Volatile mass: mean result of three replicate experiments with standard deviation in parentheses. Potential flavour: commonly accepted aroma of the pure compound at a concentration in excess of its recognition threshold. The blended, cooked swede was exhaustively dialysed prior to use whilst the blended cooked turnip was not dialysed.

Table 4
A comparison of the effects of heat- and EDTA-inactivated HRP on the volatile compounds produced from linoleic acid in buffer

Volatile compound	Volatile mass (ng)		
	Heat treatment	EDTA treatment	Buffer control
Hexanal	182 (7.1)	169 (10.3)	33.1 (4.1)
2-Heptenal(E)	35.0 (1.6)	38.4 (6.8)	9.3 (0.9)
1-Octen-3-ol	3.5 (0.9)	3.3 (0.4)	1.0 (0.1)
2-Octenal(E)	7.1 (5.3)	4.7 (1.3)	1.5 (0.6)
4-Octen-3-one	15.1 (1.7)	18.1 (0.5)	2.6 (0.7)

Volatile mass: mean result of three replicate experiments with standard deviation in parentheses.

Table 5
A comparison of the effects of heat- and EDTA-inactivated peroxidases on the hexanal produced from linoleic acid

Vegetable source of peroxidase	Hexanal ratio	
	Heat treatment	EDTA treatment
Horseradish	5.5	5.1
Swede	3.5	3.4
Turnip	5.7	5.5

Hexanal Ratio = Hexanal level in sample/Hexanal level in buffer control (mean of three determinations).

Svensson, 1971). As one of the main purposes of heat-treating vegetables is to inactivate the peroxidases and catalases, it is evident that the inactivated enzymes produced could lead to residual lipid oxidation activity with potential effects on flavour quality during further processing and storage.

4. Conclusions

Heat-inactivated horseradish, swede and turnip peroxidases catalysed the oxidation of linoleic acid to hydroperoxides and to volatile compounds, both in buffer systems and in dialysed blends of cooked vegetables.

Hexanal was formed in the greatest amount in buffer systems and in cooked turnip blend, whilst hexanol was the volatile compound formed at the highest level in cooked swede blend. This indicated the presence of a reducing agent in the cooked swede that could significantly diminish the impact of lipid oxidation on flavour.

Peroxidases, inactivated under mild conditions in the presence of EDTA, formed linoleic acid hydroperoxides at similar rates and generated similar levels of volatile compounds to heat-inactivated peroxidases. This suggested that abstraction of calcium from the peroxidase during the heat treatment was probably critical for the enhancement of lipid oxidation activity.

References

Adams, J. B., Harvey, A., & Dempsey, C. E. (1996). Regenerated and denatured peroxidase as potential lipid oxidation catalysts. *Food Chemistry*, *57*, 505–514.

Adams, J. B., & Lock, S. J. (1997). Electrospray mass spectrometric study of haem changes during peroxidase denaturation. *Food Chemistry*, *58*, 173–175.

Agostini, E., Medina, M. I., Milrad de Forchetti, S. R., & Tigier, A. M. (1997). Properties of two anionic peroxidase isoenzymes from turnip (*Brassica napus*) roots. *Journal of Agricultural and Food Chemistry*, *45*, 596–598.

Banci, L., Carloni, P., Diaz, A., & Savellini, G. G. (1996). Molecular dynamics calculations on peroxidases: the effect of calcium ions on protein structure. *Journal of Biological Inorganic Chemistry*, *1*, 264–272.

Barber, K. R., Rodriguez-Maranon, M. J., Shaw, G. S., & Van Huystee, R. B. (1995). Structural influence of calcium on the heme cavity of cationic peanut peroxidase as determined by 1H-NMR spectroscopy. *European Journal of Biochemistry*, *232*, 825–833.

Belitz, H.-D., & Grosch, W. (1999). *Food chemistry* (2nd ed., pp. 152–236). Berlin, Heidelberg: Springer-Verlag.

Bradford, M. N. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. *Analytical Biochemistry*, *72*, 248–254.

Chan, H. W.-S., & Newby, V. K. (1980). Haemoprotein- and transition metal ion-catalysed oxidation of linoleic acid: selectivity of the position of oxygenation. *Biochimica et Biophysica Acta*, *617*, 353–362.

Eriksson, C. E., Olsson, P. A., & Svensson, S. G. (1971). Denatured hemoproteins as catalysts in lipid oxidation. *Journal of the American Oil Chemists' Society*, *48*, 442–447.

Eriksson, C. E., & Vallentin, K. (1973). Thermal activation of peroxidase as a lipid oxidation catalyst. *Journal of the American Oil Chemists' Society*, *50*, 264–268.

Gardner, H. W. (1975). Isolation of a pure isomer of linoleic acid hydroperoxide. *Lipids*, *11*, 248–252.

Gibian, M. J., & Vandenberg, P. (1987). Product yield in oxygenation of linoleate by soybean lipoxygenase: the value of the molar extinction coefficient in the spectrophotometric assay. *Analytical Biochemistry*, *163*, 343–349.

Haschke, R. H., & Friedhoff, J. M. (1978). Calcium-related properties of horseradish peroxidase. *Biochemical & Biophysical Research Communications*, *80*, 1039–1042.

Howes, B. D., Feis, A., Raimondi, L., Indiani, C., & Smulevich, G. (2001). The critical role of the proximal calcium ion in the structural properties of horseradish peroxidase. *Journal of Biological Chemistry*, *276*, 40704–40711.

Mazza, G., Charles, C., Bouchet, M., Ricard, J., & Raynaud, J. (1968). Isolement, purification et proprietes physico-chimiques des peroxydases de navet. *Biochimica et Biophysica Acta*, *167*, 89–98.

Nie, G., & Aust, S. D. (1997). Effect of calcium on the reversible thermal inactivation of lignin peroxidase. *Archives of Biochemistry & Biophysics*, *337*, 225–231.

Pahari, D., Patel, A. B., & Behere, D. V. (1995). Spectroscopic studies on calcium depleted horseradish peroxidase: observation of tryptophan sensitised bound terbium(III) fluorescence. *Journal of Inorganic Biochemistry*, *60*, 245–255.

Pizzocaro, F., Senesi, E., & Monteverdi, R. (1986). Lipids stability in frozen cauliflower in relation to heat treatments. *Industrie Alimentari*, *25*, 372–377, 380.

Reading, N. S., & Aust, S. D. (2001). Role of disulfide bonds in the stability of recombinant manganese peroxidase. *Biochemistry*, *40*, 8161–8168.

Schreier, P., Drawert, F., & Junker, A. (1977). Quantitative composition of natural and technologically modified plant aromas. IV. Enzymic and thermal reaction products formed during processing of tomatoes. *Zeitschrift für Lebensmittel- Untersuchung und- Forschung*, *165*, 23–27.

Sutherland, G. R. J., Zapanta, L. S., Tien, M., & Aust, S. D. (1997). Role of calcium in maintaining the heme environment of manganese peroxidase. *Biochemistry*, *36*, 3654–3662.

Turner, R. (2001). *Lipid oxidation by denatured haemproteins in heat-processed vegetables*. PhD thesis, University of Reading, School of Biosciences.

Wright, P. J., & English, A. M. (2001). Buffer-anion-dependent Ca^{2+} leaching from horseradish peroxidase at low pH. *Journal of Biological Inorganic Chemistry*, *6*, 348–358.

Zamora, R., Millan, F., Hidalgo, F. J., Alaiz, M., Maza, M. P., Olias, J. M., & Vioque, E. (1989). Interaction between the peptide glutathione and linoleic acid hydroperoxide. *Die Nahrung*, *33*, 283–288.