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Regenerated and denatured peroxidase as potential lipid oxidation catalysts

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The aim of this study was to determine the relative lipid oxidation activities of regenerated and denatured peroxidase using horseradish peroxidase (HRP) as a model. Poor correlation between peroxidase activity regeneration and enhancement of lipid oxidation activity suggested that the regenerated enzyme did not possess increased potential to catalyse lipid oxidation. Heat denatured peroxidase, not containing any regenerated enzyme, was shown to have a greater lipid oxidation activity than the native enzyme. The denatured peroxidase comprised three haemoproteins with similar isoelectric points that were significantly lower than that of the major native isoenzyme. Two of the proteins, present in relatively minor amounts, possessed most of the lipid oxidation activity.

Evidence for the release of haem on heating HRP under conditions that led to regeneration of activity of the cooled enzyme was provided by circular dichroism (CD) spectroscopy. The CD study also suggested that irreversible polymerisation of haem occurred on heating, which may have restricted subsequent activity regeneration. The freed haem appeared to have only a low lipid oxidation activity compared with the denatured holoenzyme.

Nuclear magnetic resonance (NMR) spectroscopy detected 'structural regeneration' of the haem pocket under conditions that did not lead to regeneration of functional enzyme. 'Structurally regenerated' forms could potentially enhance lipid oxidation on storage of heat processed foods. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

The reappearance of peroxidase activity in vegetables that have no measurable activity immediately after heat processes such as blanching or HTST sterilisation has concerned food manufacturers for a great many years. The major concern is that the regenerated enzyme, whilst having an intact active site, may have other properties that are different to the native enzyme. In particular, it may have a more open structure that allows greater lipid access to the active site and thereby enhances lipid oxidation activity that can lead to deterioration of product quality during storage. This idea arose as a result of the association between regenerated peroxidase activity and off-flavour formation believed to be due to lipid oxidation. For example, 'viny' off-flavours have been linked with peroxidase regeneration in HTST sterilised canned peas (Guyer & Holmquist, 1954). This has led to increased process severity to denature the enzyme to a point from which no regeneration can occur. In some vegetables, such as green beans, the extra time required to inactivate peroxidase permanently is excessive and the loss in product quality is serious (Resende *et al.*, 1969).

An alternative explanation for the enhanced lipid oxidation in food products being caused by regenerated peroxidase is that the heat denatured enzyme could accelerate lipid oxidation. Eriksson *et al.* (1970) have demonstrated the potential for this using heated peroxidase and catalase. They proposed that the lipid oxidation activity was due to a greater exposure of the substrate to the haem in the denatured active site, or to a shift towards a higher spin state of the ferric ion. No comparison of the lipid oxidation activity of denatured haemoproteins with that of regenerated enzyme has been carried out, however, and so the present study set out to determine which species could potentially catalyse lipid oxidation in foods.

MATERIALS AND METHODS

Type VI horseradish peroxidase (HRP) 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.

UV/VIS measurements

Solutions of HRP (0.025 mM), either in 200 mM acetate buffer pH 5.6, in 20 mM phosphate buffer pH 5.6, in 20 mM phosphate pH 7.0, or in 200 mM phosphate pH 7.0, were heated for up to 5 h in screw-cap borosilicate tubes at 70 °C (±0.2 °C) in a thermostatted bath. After rapid cooling in water, the solutions were immediately assayed for peroxidase activity and the absorption spectra measured. Repeat peroxidase assays and spectral measurements were carried out after holding samples for 20 h at 30 °C. Lipid oxidation activity, protein and haem levels were also determined at this time.

Peroxidase assay

Peroxidase assays were performed using a substrate solution that contained 5 mM hydrogen peroxide and 5 mM guaiacol in 200 mM acetate buffer, pH 5.6. The peroxidase activity was determined by adding 0.1 ml of sample, appropriately diluted, to 2.5 ml of substrate solution at 30 °C. The rate of increase in absorbance at 420 nm due to the formation of brown guaiacol oxidation products was measured with a Unicam PU 8700 UV/VIS recording spectrophotometer. Peroxidase activity was determined from the slope of the regression line over 30 s, expressed as $A_{420\ nm}$ min^{-1} 0.1 ml^{-1} of undiluted sample.

Percentage regeneration was defined as $(POX_{t/20} - POX_{t/0}) \times 100/POX_{0/0}$ where $POX_{t/20}$ is peroxidase activity of sample heated for t (h) at 70 °C and held for 20 h at 30 °C, $POX_{t/0}$ is peroxidase activity of sample heated for t (h) at 70 °C and assayed immediately, and $POX_{0/0}$ is peroxidase activity of unheated sample assayed immediately.

Lipid oxidation assay

Lipid oxidation assays were carried out using 0.16 mM linoleic acid (99% pure) in 200 mM acetate buffer, pH 5.6, containing 0.005% Tween 20. The lipid oxidation activity was determined by adding 0.1 ml of sample to 2.5 ml of the linoleic acid solution at 30 °C. The rate of increase in absorbance at 234 nm due to the formation of hydroperoxides containing the conjugated diene chromophore was measured using the PU 8700 spectrophotometer. Lipid oxidation activity was determined from the slope of the regression line over 30 s expressed as $A_{234\ nm}$ min^{-1} 0.1 ml^{-1}.

Protein assay

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

Haem assay

Haem was assayed by mixing 0.5 ml of sample with 0.5 ml of absolute ethanol and 0.02 ml of concentrated hydrochloric acid. The absorbance of the haem–ethanol complex at 398 nm was measured (Maehly & Åkeson, 1958).

Circular dichroism (CD) spectroscopy

CD spectra were obtained on a Jobin Yvon CD6 spectra-polarimeter. Samples of HRP were prepared in buffer as described in the previous sections at concentrations between 0.025 and 0.25 mM and placed in CD cuvettes with 1, 2 or 10 mm pathlength, depending on the protein concentration and spectral region scanned. The temperature in the cuvette holder was held constant at 70 °C (±0.2 °C) with a circulating waterbath. Raw data from the average of three scans were imported into Sigmaplot (Jandel Scientific) and the ellipticities converted to molecular ellipticities using a molecular weight for HRP of 44 000.

Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were obtained on the JEOL alpha 500 MHz NMR spectrometer of the Bristol University Centre for Molecular Recognition. Samples of HRP at a concentration of 25 mg ml^{-1} (0.6 mM) were made in buffer in ^2H_2O and centrifuged in a microcentrifuge to remove insoluble material before placing in glass NMR tubes. The pH values are those directly measured with a hydrogen electrode calibrated with aqueous pH buffers; i.e. no correction for the deuterium isotope effect was made. One-dimensional NMR spectra were accumulated over a spectral width of 60 000 Hz (120 ppm) into 16K real data points using a relaxation delay of 150 ms. The residual H^2HO signal was suppressed where necessary by gated irradiation during the relaxation delay. The temperature was maintained at 70 °C with a thermostatted airflow. Each spectrum is the sum of 1024 free induction decays which were Fourier-transformed after multiplication with an exponential factor equivalent to a line-broadening of 20 Hz. Each spectrum took 7 min to acquire. Chemical shifts are relative to the residual H^2HO signal at 4.7 ppm (40 °C). The assignments of the ring current shifted signals are taken from Shiro *et al.* (1986) and Thanabal *et al.* (1987).

Isoelectric focusing (IEF)

IEF was carried out using an LKB 440 ml column following the manufacturer's instructions. HRP, 50 mg in 50 ml of 20 mM sodium phosphate buffer, pH 7.0, was heated for 5 h at 70 °C and then held for 20 h at 30 °C prior to layering in the sucrose/ampholine gradient. After focusing for 16 h at 4 °C, the column was emptied and fractions were collected. Sucrose and ampholines were removed from combined fractions by dialysis and the sample was concentrated against solid polyethylene glycol (average molecular weight 20 000). The sample was prepared for NMR spectroscopy by replacing water with ^2H_2O using repeated ultrafiltration in a Sartorius Ultrasart Cell 10. Sodium phosphate buffer (20 mM, pH (^2H_2O) 7.0) was employed and a final sample volume of 1 ml gave a suitable protein concentration.

Similar IEF experiments were carried out after heating HRP for 0.5 h and 5 h at 70 °C in 200 mM acetate

buffer, pH 5.6. Only 10 mg of HRP in 10 ml of buffer was used for the 5 h heat treatment. No samples were prepared for NMR spectroscopy in either case.

RESULTS AND DISCUSSION

Factors affecting thermally induced lipid oxidation activity

Earlier work with HRP had shown that new isoenzymes were only formed at temperatures greater than 90 °C (Delincée *et al.*, 1973; Adams & Ongley, 1983). All heat treatments in the present study were therefore carried out at 70 °C in order to reduce the number of species formed and thereby simplify data interpretation. The consequence of heating at this temperature was that long heating times were necessary to inactivate the peroxidase.

It is generally accepted that regeneration of peroxidase activity involves recombination of haem and apoprotein (cleaved apart during thermal inactivation) to form the active enzyme (Robinson, 1991). However, inactivation is not a simple reversible reaction. Haem readily polymerises under aqueous conditions leading to oxygen-bridged haem dimers and possibly other higher molecular weight polymers (White, 1978). In the present study, CD spectroscopy has shown that the band of positive ellipticity at 410 nm due to the asymmetrically bound haem is almost completely lost after 30 min at 70 °C in acetate buffer, pH 5.6 (Fig. 1). However, a high recovery of the band was found on holding the heated

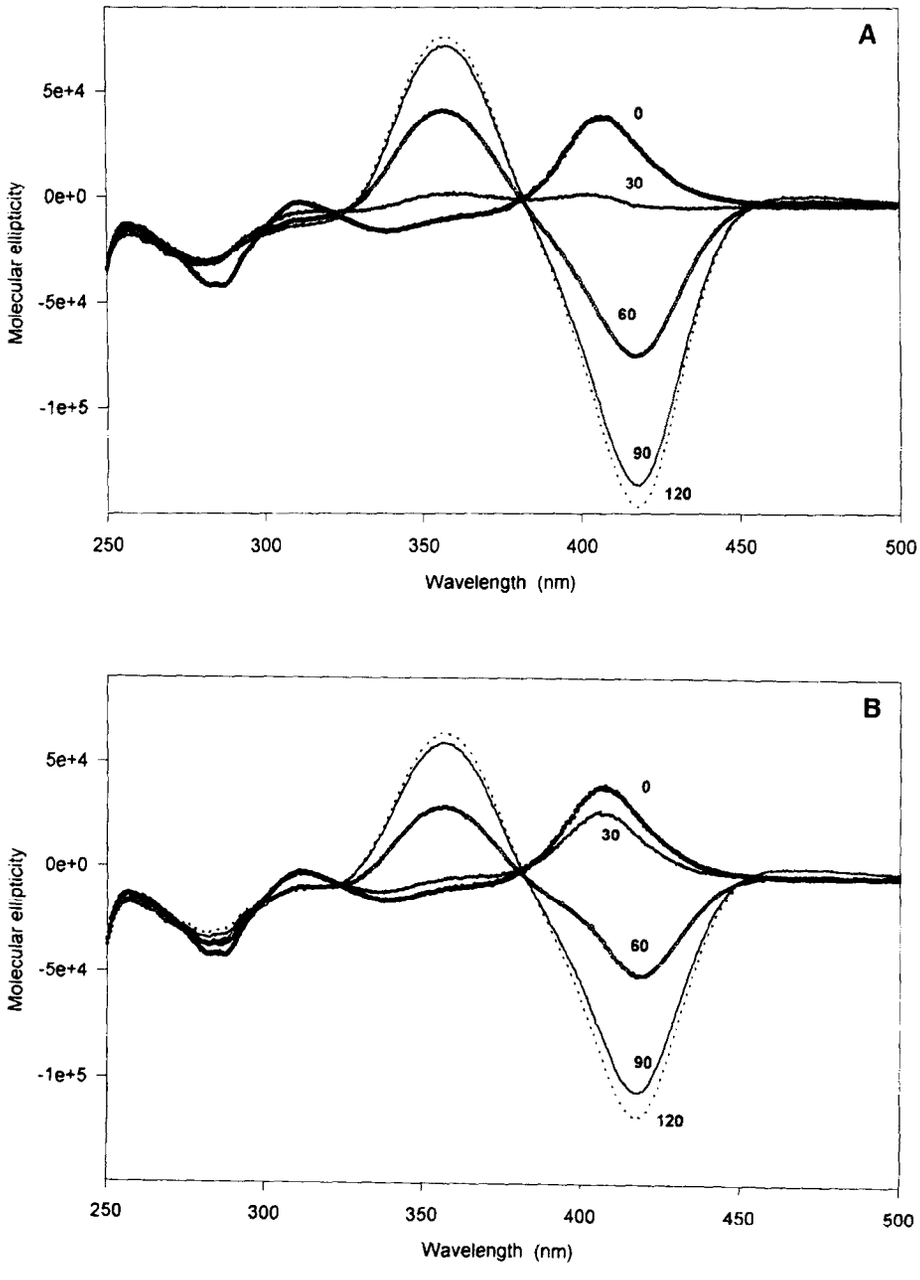

Fig. 1. CD spectra of HRP (0.125 mM) after heating at 70 °C in 200 mM sodium acetate buffer, pH 5.6: (A) immediately after heating for the times indicated (min); (B) the same samples after incubation at 30 °C for 24 h.

sample for 24 h at 30 °C. At longer heating times, a band with positive ellipticity around 360 nm developed with a second band with negative ellipticity at about 420 nm. Similar bands have been attributed to interactions between haem molecules (Blauer *et al.*, 1993).

At higher pH values around neutral, Eriksson *et al.* (1971) used UV/VIS spectroscopy to show that heating HRP did not lead to a hypsochromic shift of the Soret absorption at 403 nm, suggesting that haem is not cleaved from the enzyme under these conditions. This was confirmed in the present study by the lack of haem dimer bands in the CD spectrum on heating HRP in phosphate at pH 7.0, suggesting that the haem–iron coordination bond to the proximal histidine nitrogen in the enzyme active site is stable at around pH 7 (Fig. 2). This is also strongly indicated by the fact that regeneration of peroxidase activity occurs more readily under neutral or slightly alkaline conditions than in acidic media (Lu & Whitaker, 1974). The loss of haem ellipticity in this case is presumably due to disruption (opening) of the haem binding site on Ca^{2+} chelation, with the resulting loss of induced haem asymmetry.

On the basis of the above evidence, a mechanism of peroxidase inactivation is proposed involving five haem-containing species that could potentially contribute to thermally induced lipid oxidation activity (Fig. 3). These species are regenerated enzyme, unfolded native enzyme, monomeric haem, haem modified mainly by polymerisation, and denatured holoenzyme. In acetate buffer at pH 5.6, peroxidase activity regeneration occurred to the greatest extent after relatively mild heat treatments (0.5 h at 70 °C), falling off after longer heating times (Fig. 4). Similar results were found in phosphate buffer, pH 5.6 (Fig. 5). Regeneration probably reached a maximum after short heating times due to the high levels of both unchanged haem monomer and unfolded apoenzyme which formed during the early stages of inactivation. On cooling, these species could

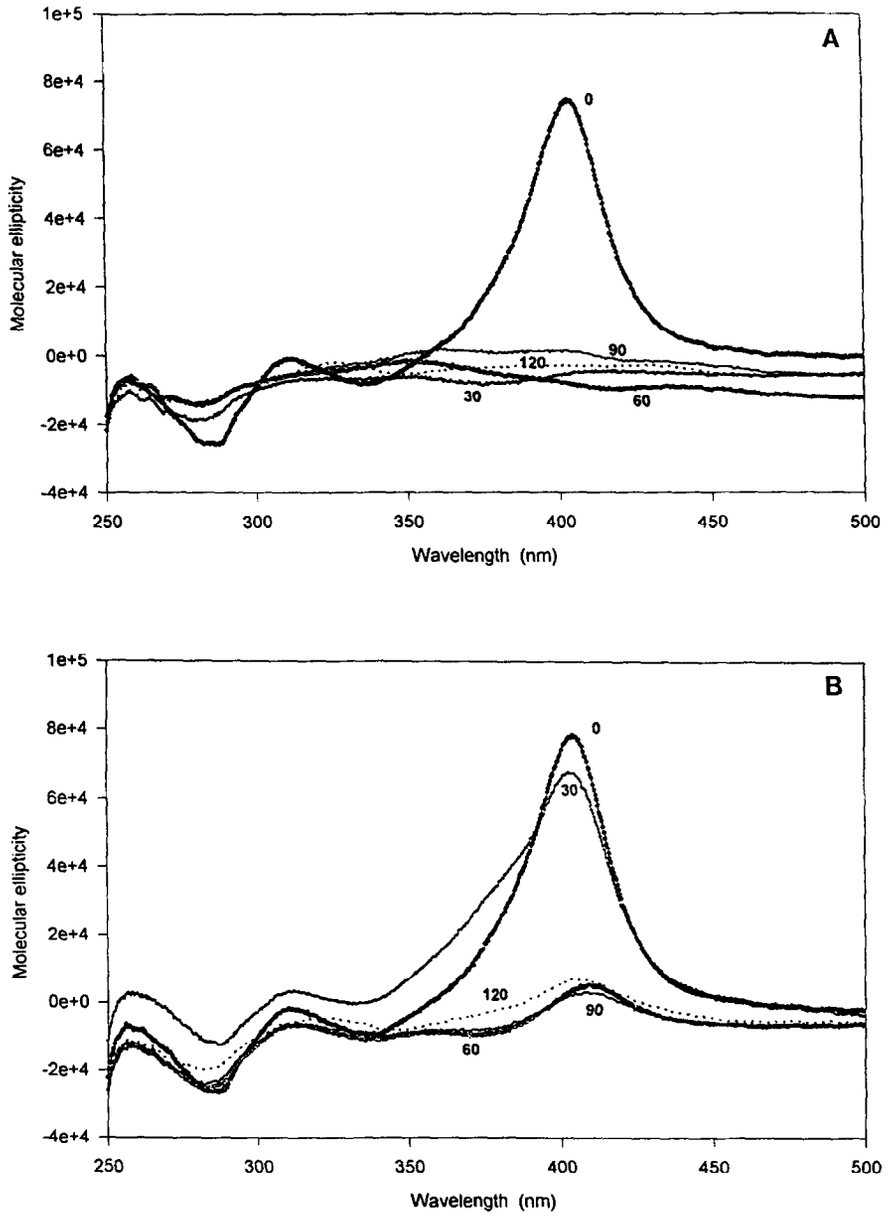

Fig. 2. CD spectra of HRP (0.125 mM) after heating at 70 °C in 200 mM sodium phosphate buffer, pH 7.0: (A) immediately after heating for the times indicated (min); (B) the same samples after incubation at 30 °C for 48 h.

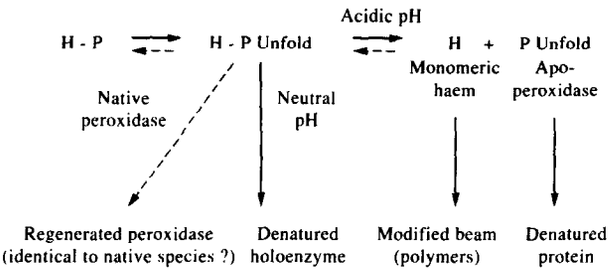

Fig. 3. pH dependence of the thermal inactivation of peroxidase. Solid arrow, forward reactions at high temperature; dashed arrow, back reactions on cooling.

have recombined and then re-folded to produce native or native-like forms of peroxidase (Fig. 3). After longer heating times, haem polymerisation and permanent denaturation of the apo-peroxidase could have prevented re-formation of the native enzyme. In contrast to enzyme activity regeneration, lipid oxidation activity increased rapidly on heating for short times and then remained constant. In phosphate buffer, pH 7.0, no regeneration was observed after heating whereas lipid oxidation activity increased rapidly to levels similar to those found in acetate, pH 5.6, and then slowly decreased at longer heating times (Fig. 6). The lack of correlation between peroxidase activity regeneration and lipid oxidation activity strongly suggests that regenerated peroxidase was not the main cause of the thermally induced increase in lipid oxidation activity.

The breakdown products of peroxidase shown in Fig. 3 are all likely to be present during the inactivation process, particularly whilst the enzyme is being heat-treated. On cooling, however, the back reactions may lead to complete disappearance of some species, thereby reducing the number available to catalyse lipid oxidation. The unfolded native peroxidase ($H\text{-}P_{Unfold}$) was probably

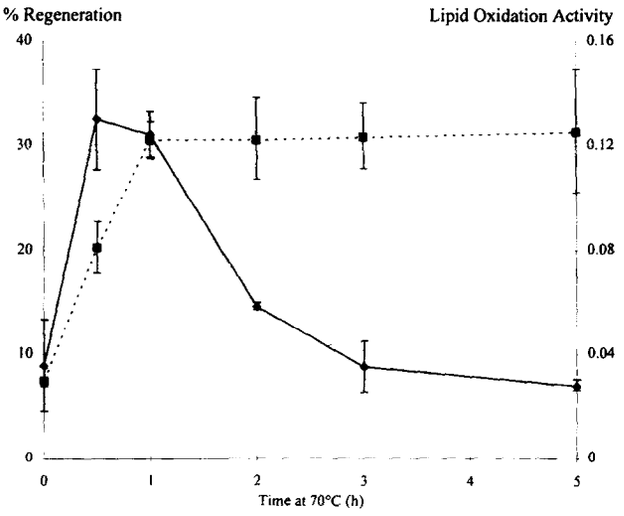

Fig. 4. Relationship between peroxidase regeneration and lipid oxidation activity after heating HRP at 70 °C in acetate buffer, pH 5.6, for various times. ◆, % regeneration; ■, lipid oxidation activity.

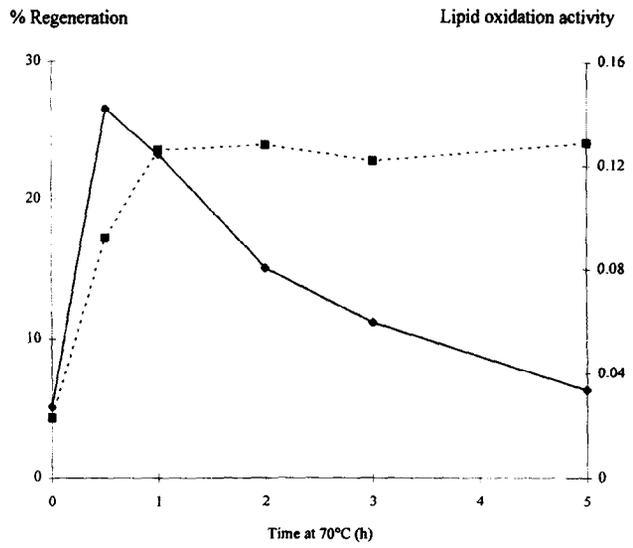

Fig. 5. Relationship between peroxidase regeneration and lipid oxidation activity after heating HRP at 70 °C in phosphate buffer, pH 5.6, for various times. ◆, % regeneration; ■, lipid oxidation activity.

absent, at least at pH 7.0, as re-folding of the unfolded enzyme would have been expected on cooling and this would have led to activity regeneration. The absence of regeneration at pH 7.0 suggests that the unfolded peroxidase did not make a significant contribution to lipid oxidation activity. Free haem was also unlikely to be present in significant amounts; at pH 7.0, only a low level would be expected because of the stability of the haem–histidine bond (Eriksson *et al.*, 1971), and at pH 5.6 a low level would be expected due to polymerisation reactions at high temperature and recombination of monomers with apoenzyme on cooling. The haem polymers appeared not to have a direct influence on lipid oxidation activity as their time course of formation, determined by CD spectroscopy, was not similar to the

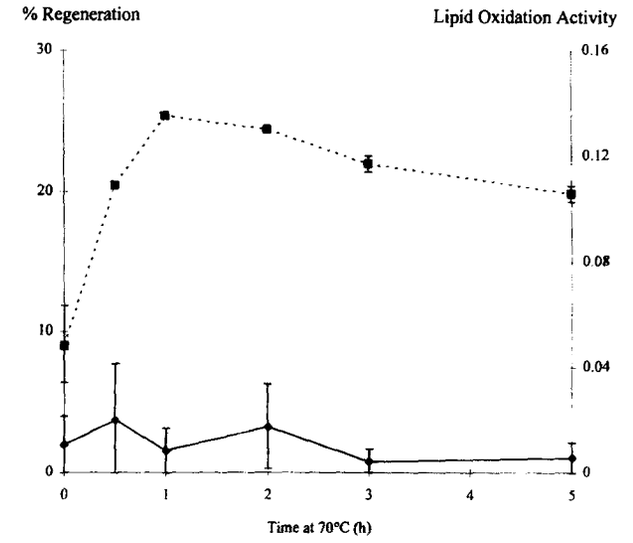

Fig. 6. Relationship between peroxidase regeneration and lipid oxidation activity after heating HRP at 70 °C in phosphate buffer, pH 7.0, for various times. ◆, % regeneration; ■, lipid oxidation activity.

rate of formation of the lipid oxidation catalysts. Such arguments suggest that the most effective lipid oxidation catalyst contained haem attached to the protein and was probably therefore a denatured form of the holoenzyme.

Characterisation of regenerated and denatured peroxidase

Proton NMR has proved invaluable for studying the haem environment in native peroxidases (Veitch & Williams, 1990; Sette *et al.*, 1993). The ring current shifted proton signals from the haem methyl and α-protons, and from the side-chain methylene protons of the proximal and distal histidines, have enabled a characteristic signature of the integrity of the haem pocket to be obtained. The hyperfine shifted resonances of the haem and histidine protons disappeared on heating at 70 °C. Cooling to 30 °C after heating in phosphate at pH 7.0 led to an immediate partial recovery of the haem pocket resonances followed by further changes during holding (Fig. 7). The slow recovery of the 74 ppm resonance of native HRP and concomitant loss of signal at 68 ppm is indicative of re-acquisition of Ca^{2+} by the protein (Shiro *et al.*, 1986). Confirmation of this slow recovery was obtained using CD spectroscopy (Fig. 2). As no regeneration of peroxidase activity took place under these conditions, it is surmised that 'structural regeneration' has occurred without recovery of functional enzyme.

HRP contains two bound Ca^{2+} which are important for both the activity and thermostability of the protein (Haschke & Friedhoff, 1978), although NMR has suggested that only one is essential for maintaining protein structure in the vicinity of the haem (Ogawa *et al.*, 1979). Shiro *et al.* (1986) have shown that removal of all Ca^{2+} from HRP drastically alters the protein structure on the distal haem side and reduces the basicity of the distal histidine. It is therefore possible to speculate that the Ca^{2+} determines the stability of the secondary structure on the distal haem side and, once it has been removed, the structure irreversibly unfolds, leading to an increase in the effective volume of the haem pocket. This increase would allow larger molecules to enter, such as lipid hydroperoxides, which can then interact with the haem and break down to volatile compounds that could potentially influence food flavour (Grosch, 1982). Removal of an Ca^{2+} also causes thermal spin mixing between ferric high and low spin states which contributes to reduced enzyme activity (Shiro *et al.*, 1986). Thus, in addition to the loss of protein structure, the loss of the high spin state of the native enzyme may also influence lipid oxidation activity when Ca^{2+} is removed.

In contrast to the behaviour in phosphate buffer at pH 7.0, no recovery of the haem-linked resonances was detected after heating in acetate buffer at pH 5.6 (Fig. 8). This suggests that, at pH 5.6, the haem cleavage and polymerisation reactions predominated at the HRP concentrations required for NMR measurements, thereby preventing 'structural regeneration'.

In order to distinguish the haem pocket resonances due to denatured peroxidase from those associated with residual native enzyme, separation of the denatured and native forms was carried out by IEF. The major native

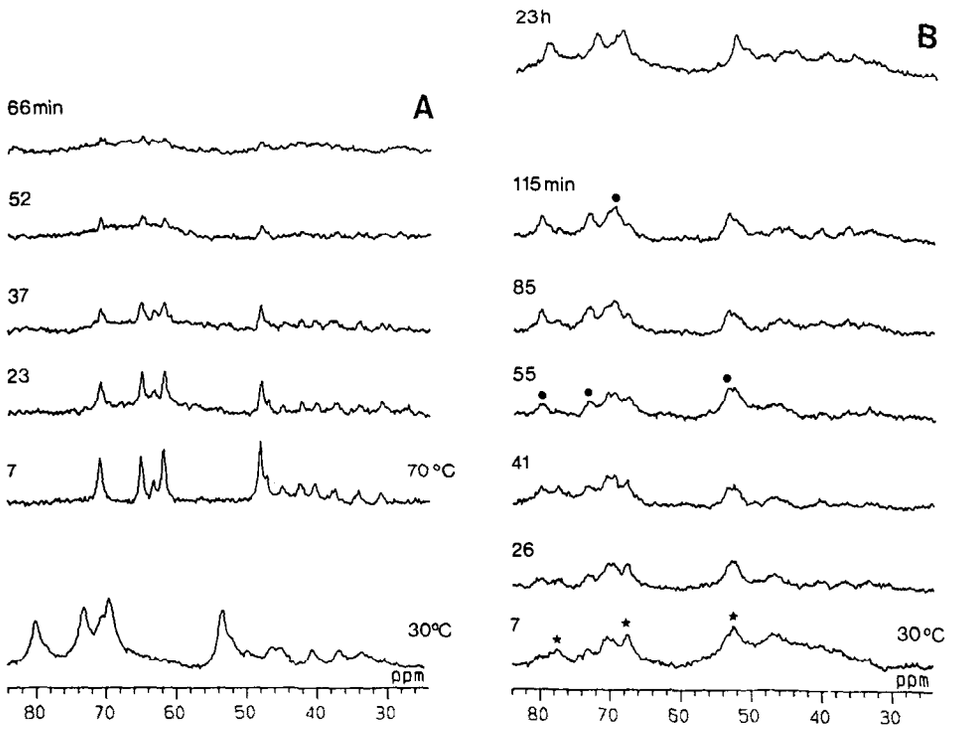

Fig. 7. Downfield region of ^{1}H-NMR spectra of HRP (0.6 mM) after heating at 70 °C in 20 mM sodium phosphate buffer, pH 7.0: (A) at 30 °C followed by increasing times at 70 °C; (B) after cooling and holding for increasing times at 30 °C. ⋆, signals characteristic of Ca^{2+}-free HRP; ●, signals characteristic of native HRP.

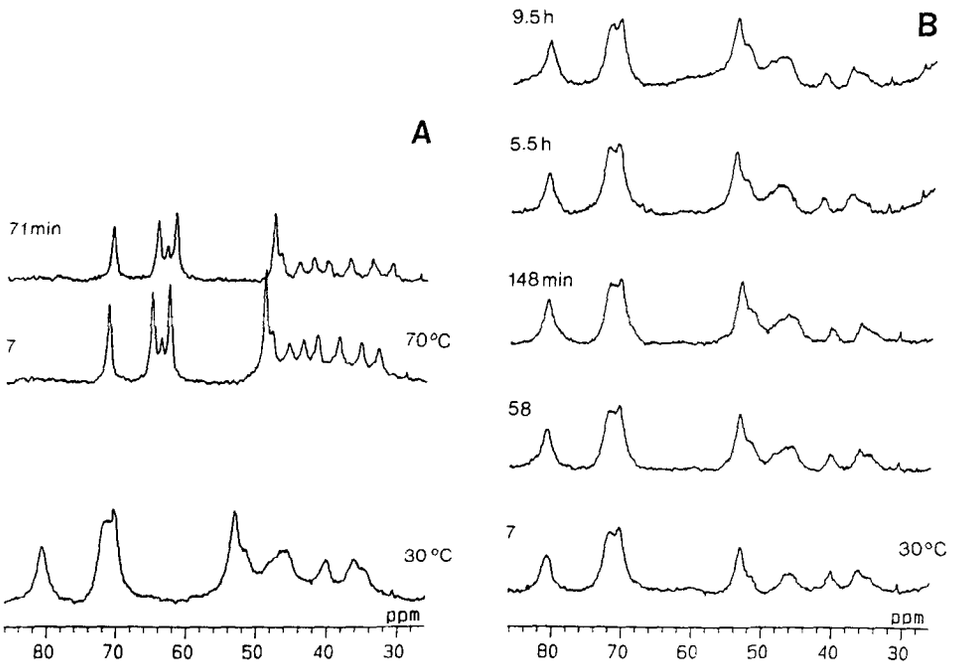

Fig. 8. Downfield region of ^{1}H-NMR spectra of HRP (0.6 mM) after heating at 70 °C in sodium acetate buffer, pH 5.6: (A) at 30 °C followed by increasing times at 70 °C; (B) after cooling and holding for increasing times at 30 °C.

isoenzyme (isoenzyme C), focusing with an isoelectric point (pI) of 9.0 (Welinder, 1979), is well resolved from two minor isoenzymes with pI values of 7.3 and 6.1. As isoenzyme C represents some 90–95% of the total enzyme activity, further purification has been considered to be unnecessary for NMR studies of native HRP (Thanabal *et al.*, 1988).

After a long heat process (5 h at 70 °C) in phosphate buffer at pH 7.0, three tightly clustered brown bands of denatured peroxidase were observed on the IEF column. One major band of haemoprotein was eluted having a pI around 5 that did not correspond with a band of lipid oxidation activity (Fig. 9). On each side of this, a band of lipid oxidation activity was detected (pI 4.7 and 5.5). This suggests that the central band containing the majority of the haemoprotem had little lipid oxidation activity whilst relatively minor amounts of haemoprotein corresponded with high levels of lipid oxidation activity. NMR spectra on a combined haemoprotein fraction were typical of denatured protein. No hyperfine shifted haem or histidine resonances were detected, implying that any 'structurally regenerated' forms were present only at low levels. Evidently, the majority of the haem had been chemically modified whilst still attached to the protein during the long heat treatment. Confirmation of this was obtained from UV/VIS spectrophotometry, which showed that the band of denatured HRP had a broad absorption maximum at about 400 nm, in contrast to the relatively sharp absorption at 403 nm of the unheated enzyme. Determination of haem by the ethanolic HCl assay also suggested that chemical changes had taken place, as a broad maximum at around 370 nm had replaced the sharp haem absorption at 398 nm observed in unheated HRP (Fig. 10). The removal of iron from the haem by chelation with the phosphate buffer, leaving the protoporphyrin IX hydrophobically bound in the haem pocket, appears unlikely as the spectrum of the protoporphyrin IX–apo-HRP complex is known to have a strong absorption at 401 nm (Jullian *et al.*, 1989).

The fact that the denatured species focused with pI 5 compared with the native enzyme pI 9.0 suggests that heating has caused some previously bound acidic

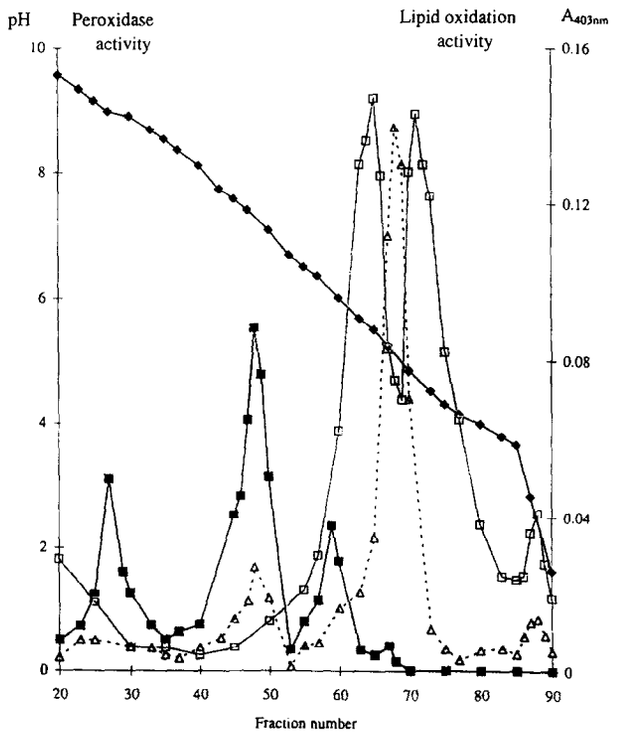

Fig. 9. Isoelectric focusing of peroxidase heated for 5 h at 70 °C in phosphate buffer, pH 7.0. ◆, pH; ■, peroxidase activity; □, lipid oxidation; △, $A_{403\ nm}$.

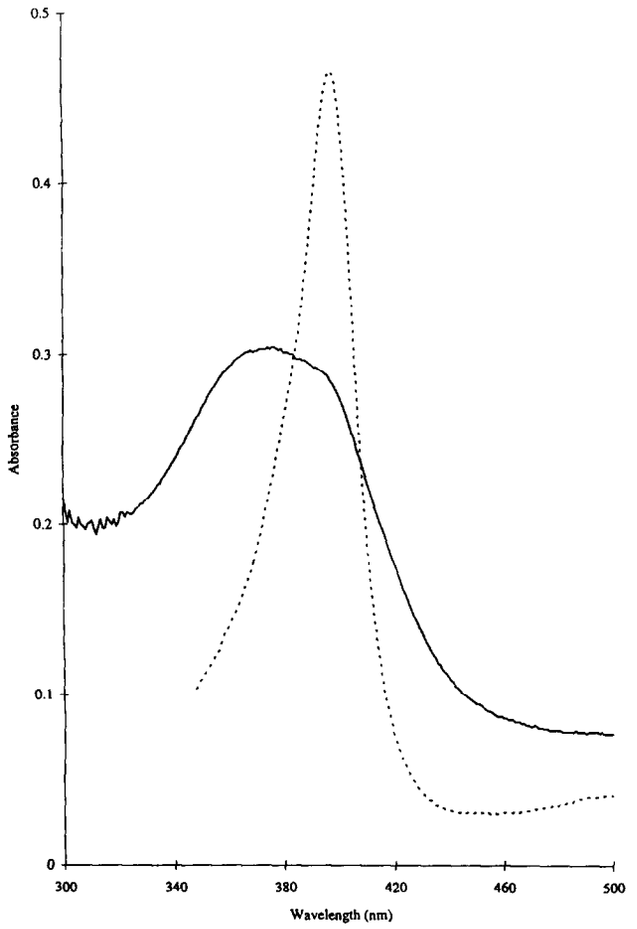

Fig. 10. UV/VIS spectra of native and denatured HRP in ethanolic HCl. – – – –, native enzyme; ———, denatured enzyme.

groups to be revealed. This could be due to chelation of enzyme-bound Ca^{2+} by the phosphate buffer. A similar effect evidently occurs during the acidified butanone preparation of apo-HRP as the latter has a pI of 6.8 (Welinder, 1979). The lower pI of the denatured species compared with apo-HRP implies that part of the increased acidity is associated with the haem.

The small band of lipid oxidation activity focusing with a pI of ≃2.5 was feasibly due to modified haem cleaved from the denatured peroxidase during the long heat process (5 h at 70 °C). The lack of reaction to Coomassie Blue reagent confirmed that protein was absent from this band. After similar heat treatment in acetate buffer at pH 5.6, a much larger protein-free band was observed, focusing with a pI of ≃3.5 (Fig. 11). Following the mechanism of Fig. 3, this band could plausibly contain a mixture of modified haems such as the oxygen-bridged dimers discussed previously. Despite the high 'haem' levels in this band, however, the lipid oxidation activity was low in comparison with that of a single band of denatured peroxidase observed with pI 5.5.

A short heat process (0.5 h at 70 °C) in acetate buffer, pH 5.6, to attain maximum regeneration of peroxidase activity (30% of the unheated enzyme activity), gave a major band on isoelectric focusing that corresponded with the predominant isoenzyme in the unheated HRP (Fig. 12). No enhancement of lipid oxidation activity was detected in this band, however, implying that regenerated peroxidase was similar to unheated peroxidase in having only a low level of lipid oxidation activity. A broad band of lipid oxidation activity was found with

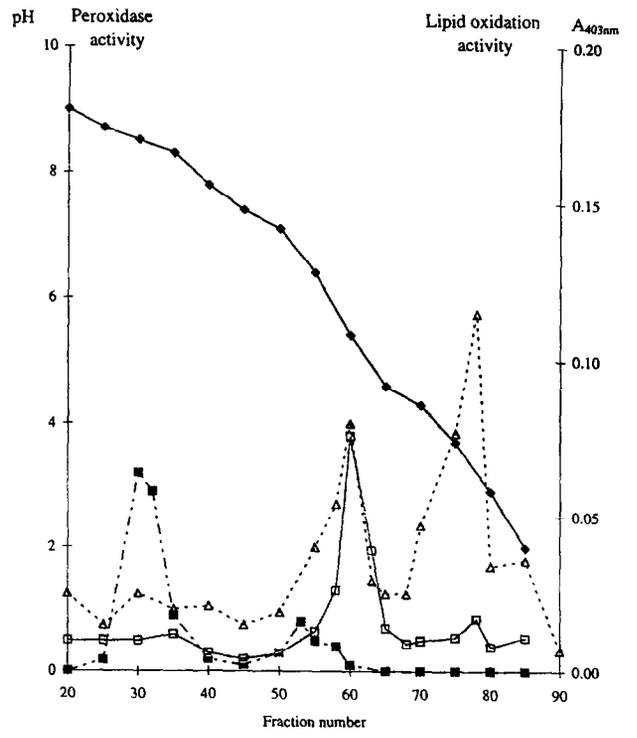

Fig. 11. Isoelectric focusing of peroxidase heated for 5 h at 70 °C in acetate buffer, pH 5.6. ◆, pH; ■, peroxidase activity; □, lipid oxidation activity; △, $A_{403\ nm}$.

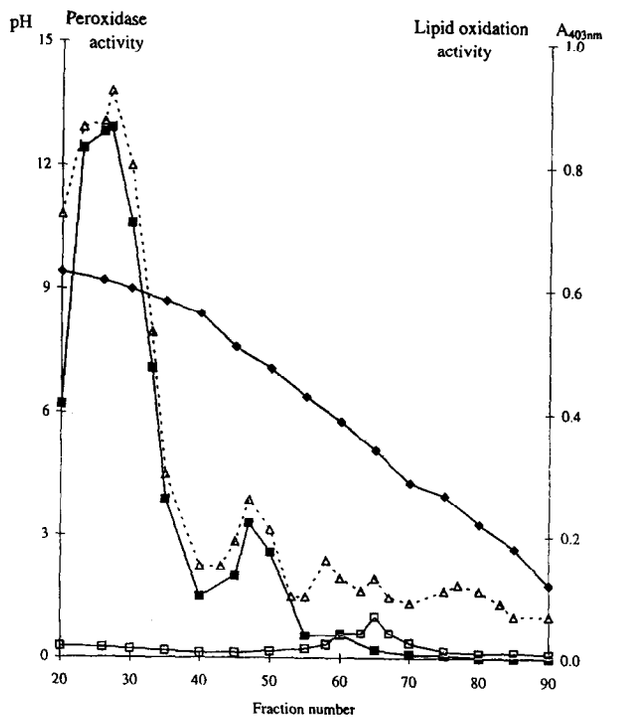

Fig. 12. Isoelectric focusing of peroxidase heated for 0.5 h at 70 °C in acetate buffer, pH 5.6. ◆, pH; ■, peroxidase activity; □, lipid oxidation activity; △, $A_{403\ nm}$.

a major peak at pI 5.1, indicating that some denatured enzyme species were formed in acetate (pH 5.6) with similar properties to those formed in phosphate (pH 7.0). As only trace amounts of lipid oxidation activity were detected at pI 2–3, corresponding to the protein-free 'haem' band, it is proposed that the denatured haemoprotein is the major lipid oxidation catalyst formed on heating at pH 5.6 as well as at pH 7.0.

Further work is evidently required to demonstrate that the findings on HRP have relevance for the quality of real foods. It is known that other peroxidases with somewhat different structures at the haem active site undergo similar changes to HRP on heating. For example, in lactoperoxidase, a milk enzyme, the haem is modified by sulphur insertion at the 8-CH_3, and a covalent disulphide bond is formed with a polypeptide cysteine side-chain (Nichol *et al.*, 1987). Nevertheless, heating lactoperoxidase in phosphate buffer at pH 6.5 increased its lipid oxidation activity by a similar amount to HRP heated under the same conditions (Eriksson, 1970). Thus, the covalently bound haem in lactoperoxidase had a similar effect to the haem bound non-covalently in HRP. Peroxidases are not the only group of enzymes that show enhanced lipid oxidation activity on heating. Other haem-containing enzymes, such as catalase, which is widely distributed in plants and animals, also show the effect when heated (Eriksson *et al.*, 1970). Increased lipid oxidation activity in foods is also known to occur as a result of heating (Pizzocaro *et al.*, 1986). However, the link between denatured haemoproteins and lipid oxidation in foods during storage remains to be explored.

CONCLUSIONS

Thermally induced enhancement of the lipid oxidation activity of HRP was associated with denatured forms of the enzyme rather than regenerated, active peroxidase. Haem cleaved from the peroxidase active site on extended heating at pH 5.6 was unable to recombine with the apoenzyme and regenerate activity because of polymerisation. The freed haem appeared to have only a low lipid oxidation activity compared with the denatured holoenzyme.

'Structural regeneration' of the haem pocket occurred after heating under conditions that did not lead to regeneration of functional peroxidase. Such 'structurally regenerated' forms of the enzyme may enhance lipid oxidation in heat-processed foods during storage.

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