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Regeneration and the kinetics of peroxidase inactivation*

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The objective of this work was to investigate why non-linear first-order kinetics of inactivation are observed when high-purity horseradish peroxidase (HRP) is heated under mildly acidic conditions. On heating HRP at 70°C in acetate, pH 5.6, strong activity regeneration was found after short heating times whilst weak regeneration was observed after more extensive heating. This variation in regeneration is proposed as the major cause of the non-linear inactivation behaviour. Support for this proposal was the finding that simple first-order kinetics are followed when no regeneration occurred after heating the enzyme under neutral conditions.

Using capillary electrophoresis, the major isoenzyme was shown to reform during activity regeneration. Under neutral conditions, new protein species were formed as a result of heating but these did not revert to the original isoenzyme on cooling. © 1997 Elsevier Science Ltd

INTRODUCTION

Because of its high heat stability and ease of detection, peroxidase remains the major enzyme used to determine whether heat processing of vegetables has been sufficient. If it cannot be detected, other less stable enzymes are assumed to be inactive. Thus, a 'safety margin' is built in by completely inactivating the enzyme (Adams, 1991).

Peroxidases from a number of sources lose activity on heating following biphasic–linear first-order kinetics. Such behaviour has been attributed either to isoenzymes of different heat stabilities (Yamamoto *et al.*, 1962) or to the conversion of the native enzyme to new isoforms with enhanced stability (Clochard & Guern, 1973; Servant *et al.*, 1986). If simple first-order kinetics are assumed when modelling peroxidase inactivation in foods, then the true biphasic–linear behaviour can lead to serious underestimation of enzyme activity (Luna *et al.*, 1986). Similar consequences would also be expected if the enzyme followed non-linear first-order inactivation kinetics as found for horseradish peroxidase (HRP) under mildly acidic conditions (Adams & Ongley, 1983). A knowledge of the factors underlying such non-linear behaviour is therefore important if a satisfactory model of peroxidase inactivation in foods is to be developed.

In the present study, the effect of pH on peroxidase activity regeneration has been studied and the relationship of regeneration to the non-linear kinetics of inactivation explored.

MATERIALS AND METHODS

Type VI HRP was purchased from Sigma Chemical Company and used without further purification. Reagents were of analytical grade and were supplied by Merck or Sigma.

Heat treatments

Solutions of HRP (0.025 mM) were made up in the following buffers: 20 mM sodium phosphate (pH 5.6), 200 mM sodium acetate (pH 5.6), 10 mM sodium phosphate (pH 7.0), 20 mM sodium phosphate (pH 7.0), 10 mM morpholinopropanesulphonic acid (MOPS)–KOH buffer (pH 7.5 at 20°C; pH 7.0 at 70°C) and 20 mM MOPS–KOH buffer (pH 7.0 at 70°C). The HRP solutions were heated for the appropriate time in screw-cap borosilicate tubes at 70°C in a thermostatted bath. After rapid cooling in water, they were immediately assayed for peroxidase activity and then held for 20 h at 30°C before being assayed again.

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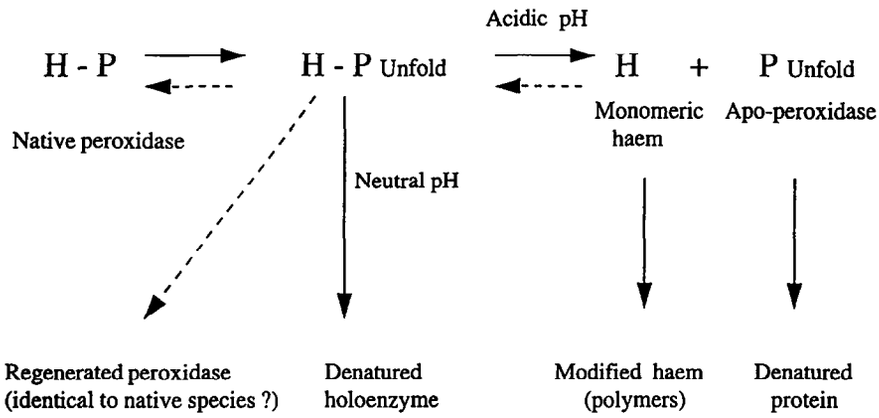

Fig. 1. pH dependence of the thermal inactivation of peroxidase (full arrows, forward reactions at high temperature; dotted arrows, back reactions on cooling).

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 PU8700 UV/VIS recording spectrophotometer. Peroxidase activity was determined from the slope of the regression line over 30 s, expressed as $A_{420\ nm}\ min^{-1}$ per 0.1 ml 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.

Capillary electrophoresis

CE was carried out using a Waters Quanta® 4000 with 820 Maxima® Workstation. A fused silica capillary of 50 μm internal diameter and 62.5 cm effective length was conditioned with 100 mM sodium hydroxide for

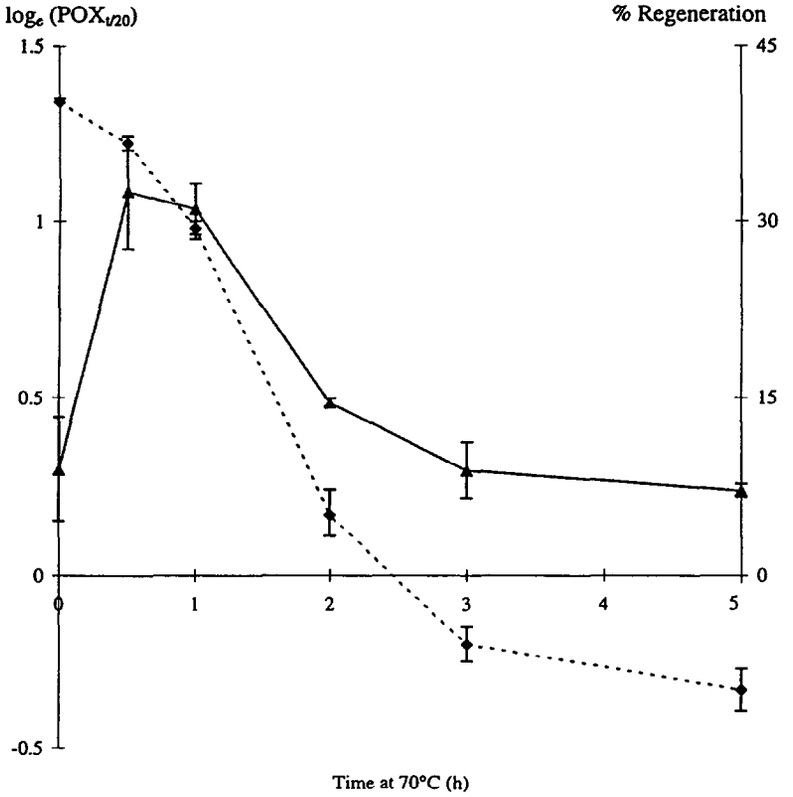

Fig. 2. Peroxidase inactivation at 70°C in 200 mM acetate buffer, pH 5.6 (◆, log$_e$ ($POX_{t/20}$); ▲, % regeneration).

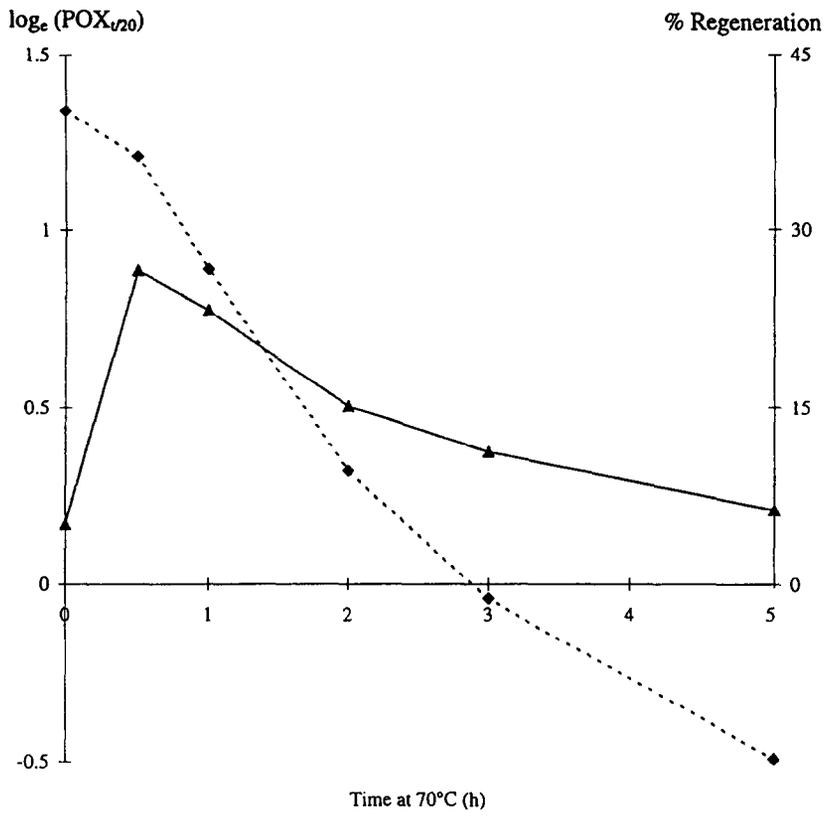

Fig. 3. Peroxidase inactivation at 70°C in 20 mM phosphate buffer, pH 5.6 (◆, log$_e$ (POX$_{t/20}$); ▲, % regeneration).

10 min and then with running buffer for 30 min. The latter comprised 200 mM phosphate buffer, pH 7.0, containing 1 M Accupure Z1-Methyl (Waters Ltd) to prevent protein binding. The sample was injected hydrostatically (50 s) and a 15 kV potential applied. Separated species were detected by UV absorption at 214 nm. Between samples, the capillary was purged for 2 min with the running buffer.

RESULTS AND DISCUSSION

As part of a study on regenerated and denatured peroxidases as potential lipid oxidation catalysts, evidence was presented for the thermal inactivation mechanism shown in Fig. 1 (Adams *et al.*, 1996). Under acidic conditions, loss of activity is considered to be initially due mainly to haem splitting from the enzyme, whilst at neutrality the haem bond to the protein is stable and activity is lost through chemical changes to the haem and through protein denaturation. Regeneration of activity after partial inactivation under mildly acidic conditions is therefore due to recombination of haem with the unfolded apoenzyme followed by refolding of the product to give native or native-like forms of peroxidase. In acetate or phosphate buffers at pH 5.6, 30% of the activity of the unheated enzyme was regenerated after 0.5–1 h at 70°C (Figs 2 and 3). After heating for 1 h at 70°C in acetate, 'cooling' and immediately adjusting the pH to 7.0 with a predetermined quantity of sodium hydroxide, the regeneration increased to 57% of the activity of the unheated enzyme (Table 1). This can be attributed to the greater stability of the haem–protein bond at neutrality (Eriksson *et al.*, 1971). After longer heat treatments, activity regeneration was limited by permanent changes to the haem and apoprotein that prevented recombination to give functional enzyme. Whilst some denaturation of the apoprotein probably took place on heating, previous studies using gel filtration have shown no significant formation of high molecular weight protein aggregates (Adams & Ongley, 1983). Haem polymerises readily under aqueous conditions, however, and circular dichroism spectroscopy has

Table 1. The effect of pH adjustment from 5.6 to 7.0 on the regeneration of peroxidase activity

Sample	POX$_{t/10}$	POX$_{t/20}$	Regeneration (%)
1	1.17, 1.05	2.09, 2.34	28.0
2	1.02, 1.07 (pH 5.6)		56.6
	1.87, 1.91 (pH 7.0)	3.21, 3.40	

Sample 1 was heated for 1 h at 70°C in acetate, pH 5.6; pH was not adjusted.
Sample 2 was heated for 1 h at 70°C in acetate, pH 5.6, and peroxidase was assayed; the pH was adjusted to 7.0 (see text) and peroxidase assayed again (15 min between pH 5.6 and pH 7.0 assays).
Regeneration (%) was calculated using means of duplicate peroxidase activities; value for sample 2 was determined using mean activity obtained prior to pH adjustment. See text for further details.

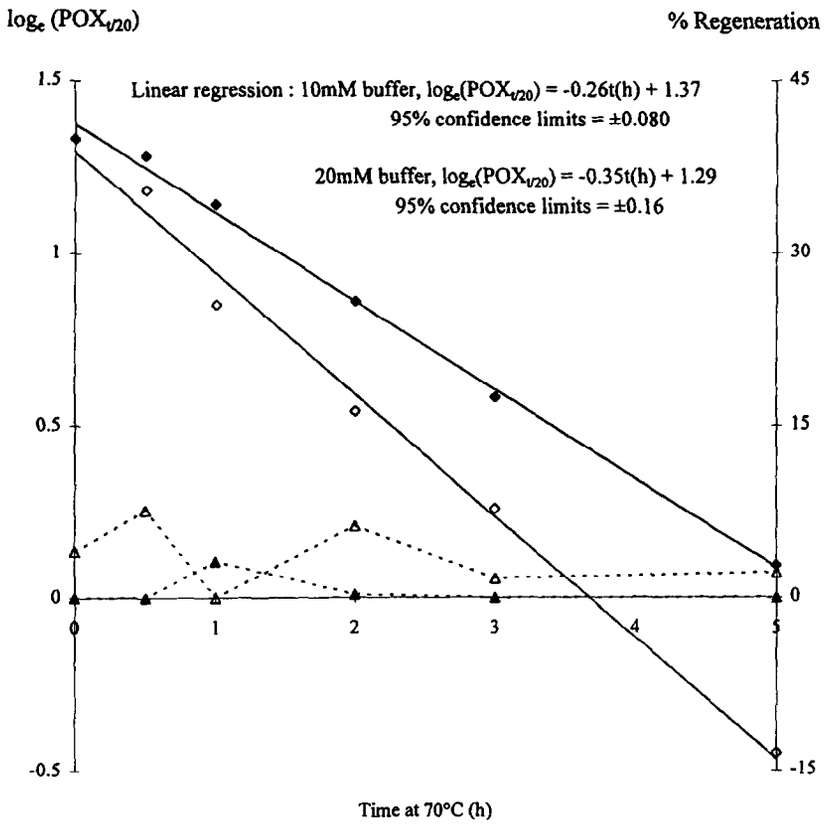

Fig. 4. Peroxidase inactivation at 70°C in phosphate buffer, pH 7.0 (◆, log$_e$ (POX$_{t/20}$), 10 mM buffer; ◇, log$_e$ (POX$_{t/20}$), 20 mM buffer; ▲, % regeneration, 10 mM buffer △, % regeneration, 20 mM buffer).

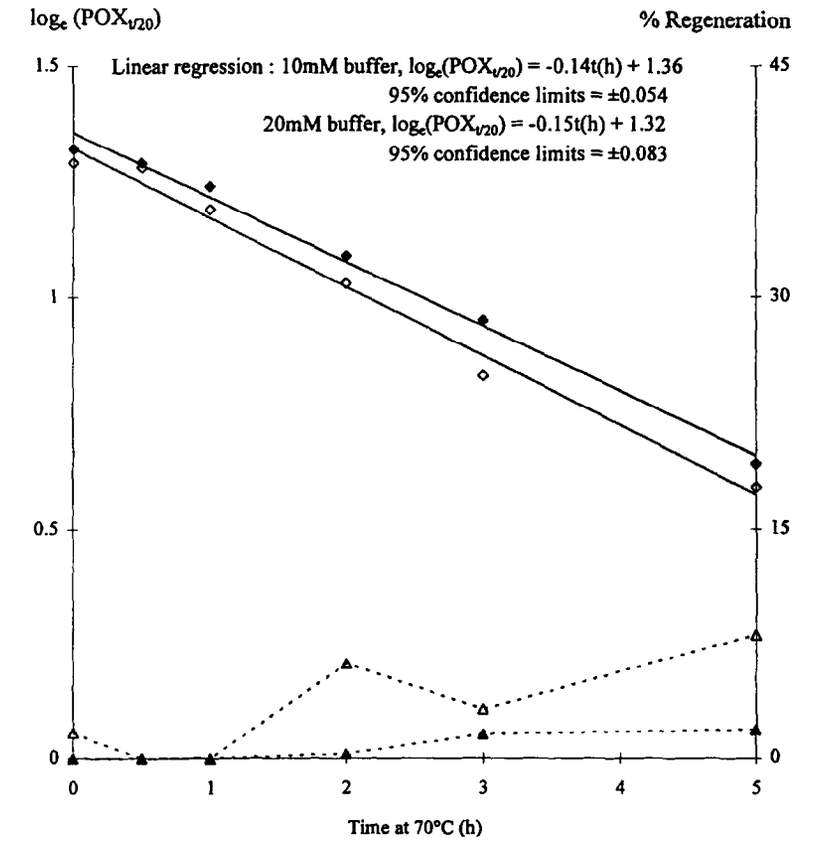

Fig. 5. Peroxidase inactivation at MOPS buffer, pH 7.0 (◆, log$_e$ (POX$_{t/20}$), 10 mM buffer; ◇, log$_e$ (POX$_{t/20}$), 20 mM buffer; ▲, % regeneration, 10 mM buffer △, % regeneration, 20 mM buffer).

strongly suggested that dimerisation occurs after the initial release of monomeric haem (Adams *et al.*, 1996). Thus, after short heating times, monomeric haem recombined with the apoenzyme to give the unfolded native or native-like holoenzyme and thereby gave relatively high activity regeneration whilst, after long heating times, the polymeric haem was unable to recombine with the apoenzyme and low activity regeneration resulted. This variation in regeneration, depending on the extent of haem polymerisation, is proposed as the major cause of the non-linear first-order kinetics of inactivation. Further support for this proposal was provided by the observation that simple first-order inactivation kinetics were followed when no regeneration occurred. Thus, after heating in phosphate or MOPS buffers at pH 7.0, the change in peroxidase activity on holding for 20 h at 30°C was generally within the experimental error of activity measurement (±5%), and linear first-order inactivation plots were obtained (Figs 4 and 5). The higher rate constants in phosphate buffer and the sensitivity of the rate constant to phosphate concentration, compared with MOPS, is considered to be due to Ca^{2+} chelation by phosphate causing destabilisation of the secondary structure of the haem pocket (Adams *et al.*, 1996).

Confirmation that the original protein species regenerated at pH 5.6, and not at pH 7.0, was obtained using CE (Fig. 6). The unheated peroxidase gave a single peak presumed to be due to isoenzyme C, the predominant isoenzyme in the preparation. On heating at 70°C in 200 mM acetate buffer, pH 5.6, an additional species was found that reverted to isoenzyme C on cooling and

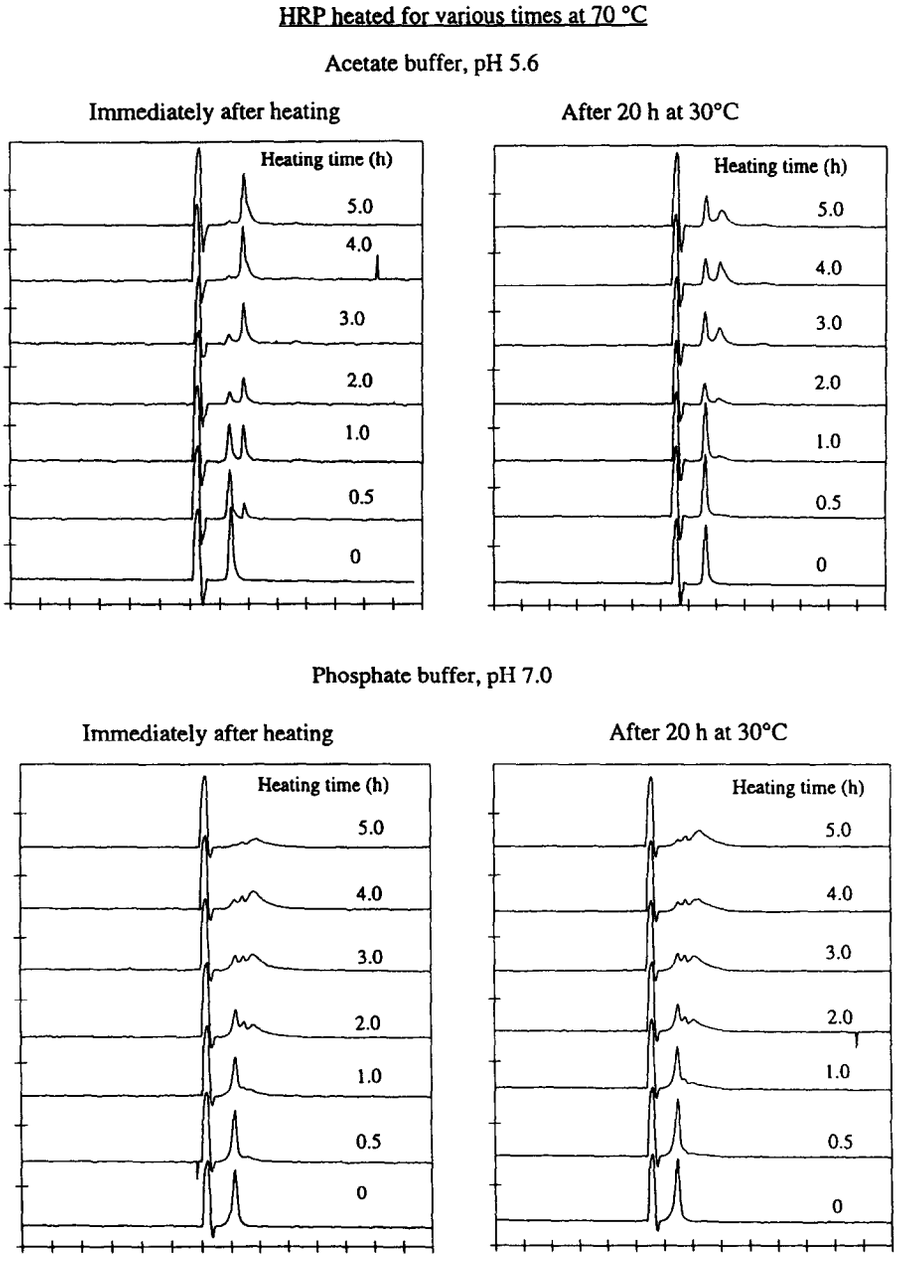

Fig. 6. Capillary electrophoresis study of peroxidase inactivation and regeneration.

then holding for 20 h at 30°C. In 20 mM phosphate at pH 7.0, several new species were formed on heating at 70°C but no reversion to the original isoenzyme was detected. Loss of isoenzyme C followed simple first-order kinetics with a rate constant of 0.35 h^{-1}, closely similar to that obtained for activity loss. This suggests that the isoenzyme is the major active species throughout the heat treatment.

In contrast to the monophasic linear first-order kinetics of inactivation of HRP at pH 7.0 found in the present study, others have observed biphasic linear kinetics at the same pH (Chang *et al.*, 1988; Hendrickx *et al.*, 1992). The data of Hendrickx *et al.* (1992) can be readily explained by the presence of heat-labile and heat-stable isoenzymes in their relatively crude enzyme preparation. However, the observations by Chang *et al.* (1988) were made using a high-purity HRP preparation of equal RZ value to that used in the present study. This suggests some compositional variation between enzyme preparations. The published electrophoretic data of Chang *et al.* (1988) does indeed indicate that their preparation contained a relatively high proportion of acidic isoenzymes. Such isoenzymes may feasibly have different heat stabilities from that of isoenzyme C. The suggestion by Clochard & Guern (1973) that biphasic linear inactivation behaviour is due to heat-induced formation of new peroxidase isoforms with enhanced thermal stability has not yet been substantiated by experiment. Previous studies, either in phosphate buffer at pH 7.0 (Delincée *et al.*, 1973) or in acetate buffer at pH 5.6 (Adams & Ongley, 1983), have shown that new, more acidic isoenzymes are formed on heating HRP at temperatures above 90°C. However, Adams & Ongley (1983) found no correlation with the non-linear kinetics at pH 5.6, suggesting that the new isoenzymes had similar heat stabilities to the native isoenzyme, perhaps through insignificant loss of secondary structure at the active site. Deamidation of asparagine and glutamine residues distant from the active site is a potential cause of the acidic isoenzyme formation (Wright, 1991).

Modelling the peroxidase inactivation process in real foods requires a knowledge of factors that affect the rate constants for each isoenzyme. As the release of haem during inactivation can influence the observed kinetics, it is important that the effect of haem from other sources is understood. In particular, haem released from catalase and the cytochrome system could contribute to the total haem concentration and thereby affect haem polymerisation reactions.

CONCLUSIONS

Evidence is presented that regeneration of peroxidase activity on cooling was the cause of non-linear first-order kinetics of thermal inactivation under mildly acidic conditions. Regeneration was not observed under neutral conditions and, as a consequence, simple first-order kinetics were found.

Confirmation that regeneration involved the reformation of the native isoenzyme was obtained using capillary electrophoresis.

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