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# The Heat Stability of Purified Spring Cabbage Peroxidase Isoenzymes

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*ABSTRACT*

*Two peroxidase isoenzymes, purified from extracts of Spring cabbage, have been shown to possess different heat-stabilities. The anionic isoenzyme had an isoelectric point of 3·7 and was relatively heat stable, while the cationic isoenzyme, pI 9·9, was more readily inactivated by heat. Neither isoenzyme showed regeneration of peroxidase activity following heat inactivation.*

## INTRODUCTION

Plant peroxidases have been implicated in the quality deterioration of stored fruits and vegetables. Peroxidase is often used as an indicator of the effectiveness of vegetable blanching procedures due to its heat stability. However, any residual enzyme activity may cause adverse changes in flavour and texture (Haard, 1977). The occurrence of a large number of potential H-donor substrates for peroxidase action has made the identification of a specific reaction causing quality deterioration difficult, although correlations have been reported between peroxidase activity and loss of quality during storage. The occurrence of peroxidase in fruits and vegetables has been reviewed by Vamos-Vigyazo (1981).

Heat inactivation of peroxidase activity from a number of vegetable sources has been claimed to be a biphasic process. Furthermore, partial



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regeneration of peroxidase activity following heat inactivation has been reported. The inactivation and regeneration of peroxidase activity in extracts of Spring cabbage was reported by McLellan & Robinson (1981). The Spring cabbage extracts have also been shown, using isoelectric focusing in polyacrylamide gels, to contain several peroxidase isoenzymes which could be detected using an *o*-dianisidine staining method (McLellan & Robinson, 1983). It was proposed that such multiplicity of isoenzyme forms might explain the biphasic nature of inactivation, since some isoenzymes—perhaps present in smaller quantities—may possess greater heat stability. Recently, the heat stability of isolated isoenzymes from cauliflower (Lee & Pennesi, 1984; Lee *et al.*, 1984) and asparagus (Powers *et al.*, 1984) has been investigated. The isolated cauliflower peroxidase isoenzymes were reported to show different heat stabilities (Lee & Pennesi, 1984).

Peroxidase activity can be considered as occurring in two fractions in the plant cell: a soluble fraction, and a bound fraction held by ionic interactions to cell wall constituents. The bound peroxidase activity of Spring cabbage consists of only cationic isoenzymes whereas the soluble peroxidase activity consists of both cationic and anionic isoenzymes (McLellan & Robinson, 1983). In other *Brassica* species the soluble peroxidase fraction has also been shown to be more heat stable than the ionically bound fraction (McLellan & Robinson, 1981), while in oranges, the bound peroxidases were more stable than those in the soluble fraction (McLellan & Robinson, 1984).

The purpose of the present investigation was to purify, from Spring cabbage, the soluble peroxidase isoenzymes, in order to investigate further the relative heat stabilities of both a cationic and an anionic isoenzyme.

## MATERIALS AND METHODS

Spring cabbage (*Brassica oleracea capitata* var. *Offenham* BG283) was grown locally from F1 hybrid seed. Sephadex G100 and DEAE Sephacel were obtained from Pharmacia Ltd, Milton Keynes, and SP Trisacryl from LKB, Selsdon, South Croydon. Bovine serum albumin and polyvinylpolypyrrolidone were purchased from Sigma Chemical Co., Poole, Dorset; *o*-dianisidine was from Koch Light Laboratories, Colnbrook, Bucks and all other chemicals were obtained in the AnalaR grade where available from BDH Chemicals Ltd, Poole, Dorset.

Peroxide activity was assayed using *o*-dianisidine as the hydrogen donor substrate, as described previously (McLellan & Robinson, 1981). Total protein was estimated using the Biuret method, with bovine serum albumin as protein standard.

## Purification

100 g Spring cabbage was homogenised in 200 ml 0·01M sodium phosphate buffer at pH 6·0, containing 1% (w/v) polyvinylpolypyrrolidone, using a Waring blender. The homogenate was filtered through muslin and centrifuged at 15 000 *g* for 25 min at 4°C. The supernatant was collected and soluble peroxidases precipitated between 50% and 90% saturation with ammonium sulphate. Peroxidase was obtained in the residue following centrifugation of the 90% saturated fraction at 15 000 *g* for 25 min at 4°C. The residue was redissolved in 0·1M sodium phosphate buffer at pH 6·0 and applied to a Sephadex G100 column (Pharmacia C16/70), previously equilibrated with the same buffer. A single peak of peroxidase was eluted from the column with 0·1M sodium phosphate buffer at pH 6·0. The fractions containing peroxidase activity were pooled, dialysed against 0·05M Tris-HCl buffer at pH 7·2, concentrated using an Amicon concentrator with a PM10 membrane and applied to a DEAE Sephacel column (Pharmacia C16/40) previously equilibrated with 0·05M Tris-HCl buffer at pH 7·2. The column was eluted at 8 ml $h^{-1}$ using a gradient of 0–0·25M sodium chloride in a total volume of 240 ml of the Tris buffer. Peroxidase activity was eluted in two major peaks (Fig. 1) detected using the *o*-dianisidine method of assay. The first peak, which was not retained by the anion exchanger, contained mainly cationic peroxidase isoenzymes. These cationic isoenzymes were concentrated using an Amicon concentrator with a PM10 membrane, dialysed against 0·05M sodium acetate buffer at pH 5·2 and fractionated by cation exchange chromatography on a SP Trisacryl column (Pharmacia C16/40), previously equilibrated with the acetate buffer. Elution from the cation exchanger was with a linear ionic strength gradient, using 0–0·25M sodium chloride in a total volume of 200 ml of the 0·05M acetate buffer. The peroxidases were eluted in two peaks; the fractions comprising each peak were pooled separately and retained for assessment of homogeneity by isoelectric focusing.

The second peak of peroxidase activity, which had been obtained by the initial anion exchange fractionation on DEAE Sephacel, and contained anionic peroxidases, was concentrated using an Amicon concentrator with a PM10 membrane. The concentrate was dialysed against 0·05M Tris-HCl buffer at pH 7·2, and reapplied to the DEAE Sephacel column, previously equilibrated with the same Tris buffer. The column was eluted using a gradient from 0–0·25M sodium chloride in a total volume of 240 ml of the Tris buffer. A small peak of peroxidase activity was eluted with the void volume and was discarded. The fractions comprising the major peak of peroxidase activity determined using the *o*-dianisidine method of assay were pooled and designated DEAE2, which was retained for assessment of

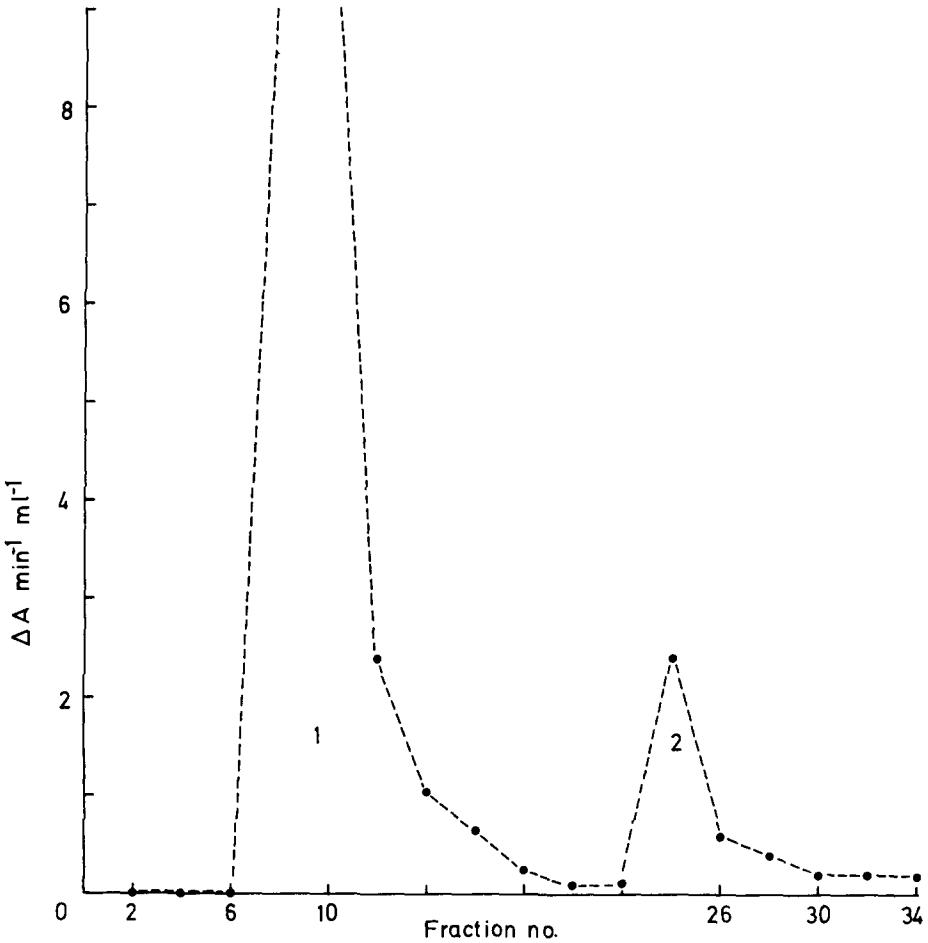


**Fig. 1.** Separation of Spring cabbage peroxidases on DEAE Sephacel. ●---●, peroxidase activity.

homogeneity by isoelectric focusing. The purification scheme is shown in Fig. 2, together with typical specific activities for the fractions at each stage of the purification. These were calculated after assays with the *o*-dianisidine method for peroxidase (McLellan and Robinson, 1981), and the Biuret method for protein.

## Isoelectric focusing (IF)

IF was carried out using an LKB Ultrophor Electrofocusing unit with an LKB Multitemp Thermostatic Circulator, as recommended by the manufacturers (LKB, South Croydon). All samples were dialysed against 0·01M sodium phosphate buffer at pH 6·0 prior to analysis by IF.

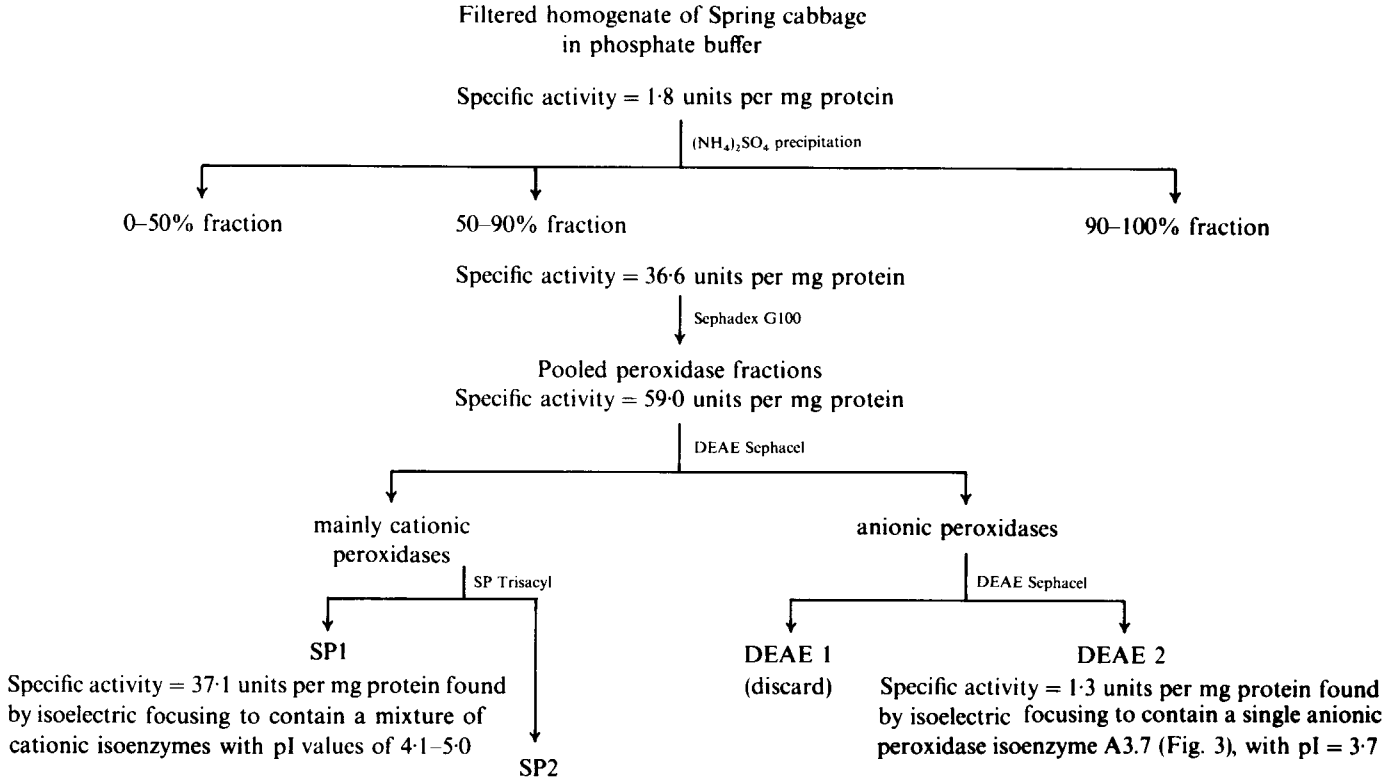


**Fig. 2.** Flow diagram for the purification of peroxidase isoenzymes from Brussels sprouts. Details of each step are given in the text.

The gels were stained for peroxidase activity using *o*-dianisidine, as described previously (McLellan & Robinson, 1987).

### Heat inactivation and regeneration of peroxidase activity

Prior to heat treatments all samples were dialysed against 0·01M sodium phosphate buffer at pH 6·0. Experiments to investigate the inactivation and regeneration of peroxidase activity were then carried out as described previously (McLellan & Robinson, 1981).

## RESULTS AND DISCUSSION

Isoelectric focusing in thin-layer polyacrylamide gels was used to identify the peroxidase isoenzymes in each of the three preparations obtained (Fig. 3). The fraction DEAE2 was found to contain a single peroxidase isoenzyme with an isoelectric point (pI) of approximately 3·7. This isoenzyme represented the most anionic peroxidase in a crude preparation of Spring cabbage (McLellan & Robinson, 1983) and is designated A3.7 (Fig. 2). The peroxidase samples obtained following cation exchange chromatography, SP1 and SP2, were found to contain anionic isoenzymes and a single cationic isoenzyme, respectively. The isolated cationic isoenzyme (pI = 9·9) represents the most cationic peroxidase isoenzyme detected in a crude

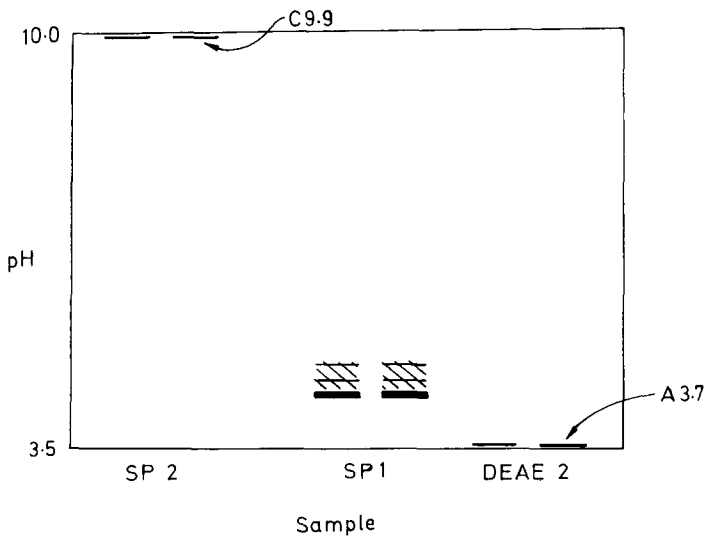


**Fig. 3.** Analysis of purified isoperoxidase preparations using isoelectric focusing in thin layer polyacrylamide gels.

preparation of Spring cabbage and is designated C9.9 (Fig. 2). The nomenclature chosen therefore includes the pI values of the isoenzymes.

The specific activity of isoenzyme A3.7 for the oxidation of *o*-dianisidine was 1·3 units per mg protein compared to 27·7 units per mg protein for C9.9. This is partly explained by the higher concentration of protein in A3.7; however, the peroxidase activity in C9.9 was some 6-fold greater than that in A3.7. Similarly, low specific activities were found for anionic isoperoxidases

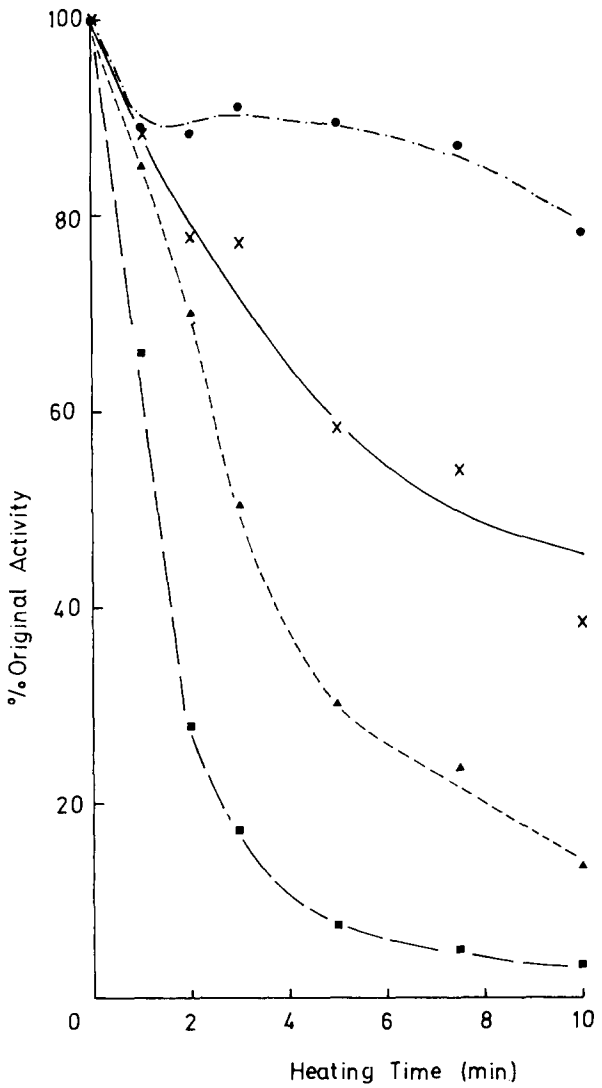


**Fig. 4.** Heat inactivation of Spring cabbage peroxidase isoenzyme A3.7. ●—·—·—●, inactivation at 60°C. ×———×, inactivation at 65°C. ▲– – –▲, inactivation at 67°C. ■— — —■, inactivation at 70°C.

purified from Brussels sprouts (McLellan & Robinson, 1987). Isoenzyme C9.9 appears to represent a much greater percentage (3·6%) of the total peroxidase activity in extracts of Spring cabbage than A3.7 (0·6%), when estimated by the *o*-dianisidine method of assay.

Isoenzyme A3.7 was found to be quite heat stable; only a 50% inactivation resulted from heating at 67°C for 3 min (Fig. 4). This degree of

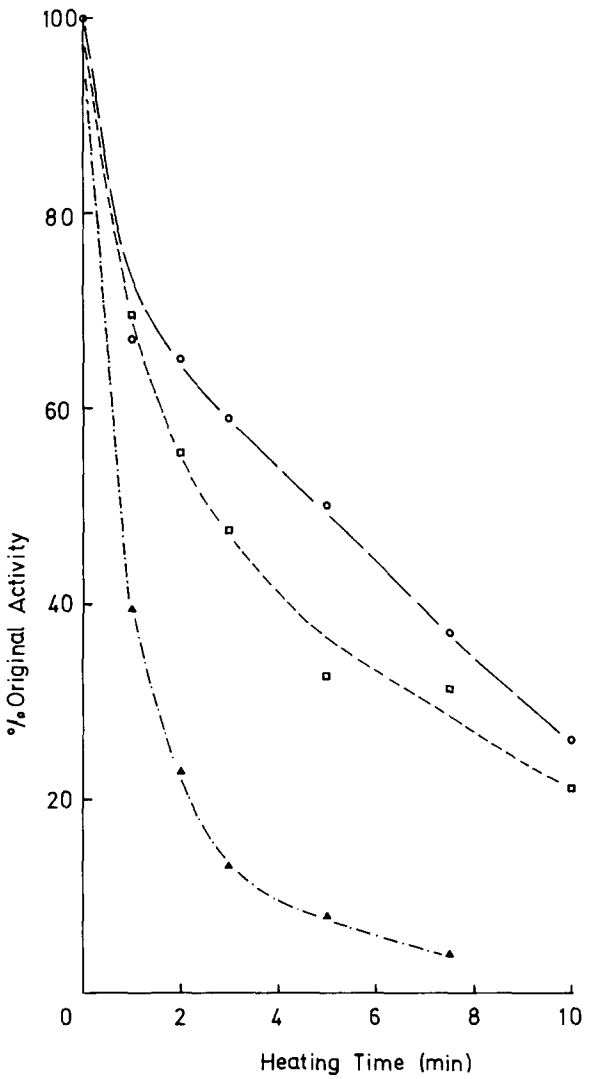


**Fig. 5.** Heat inactivation of Spring cabbage peroxidase isoenzyme C9.9. ○———○, inactivation at 53°C. □----□, inactivation at 56°C. △—·—·—·—△, inactivation at 60°C.

heat stability is comparable to that obtained previously when only 50% of the peroxidase activity present in crude extracts of soluble Spring cabbage was destroyed after heating at 70°C for 3 min (McLellan & Robinson, 1981). Isoenzyme C9.9, which represents a large proportion of the total peroxidase activity, was, however, considerably more heat labile, and was 50% inactivated after heating for 5 min at 53°C (Fig. 5). Regeneration of

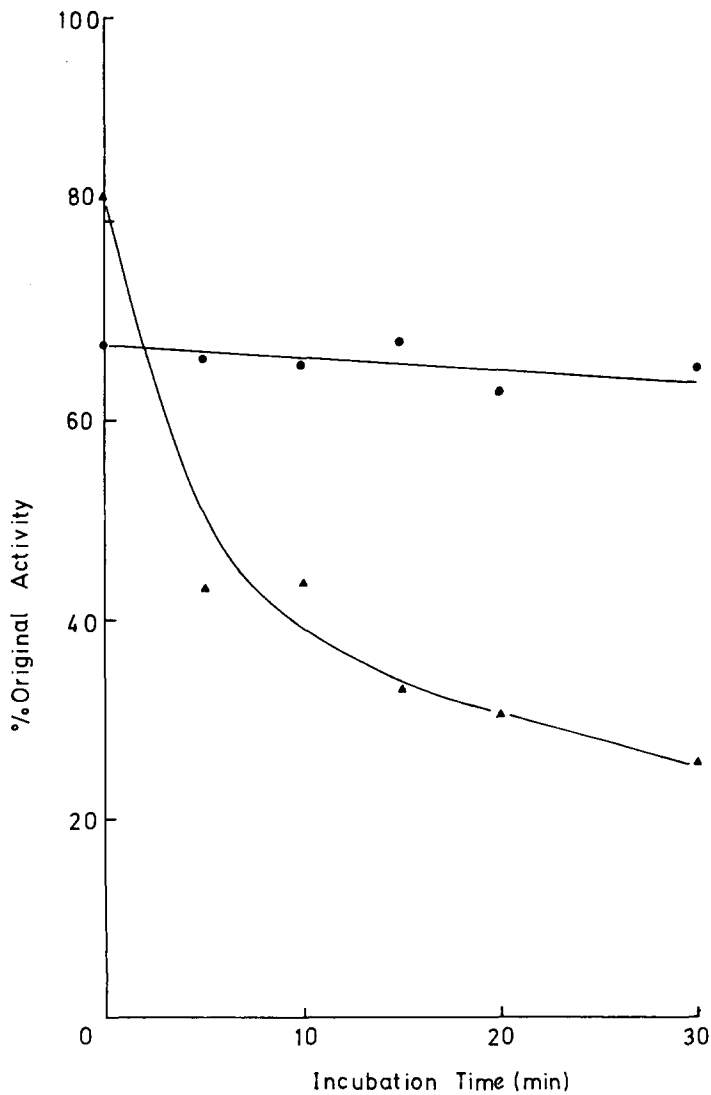


**Fig. 6.** The relationship between residual peroxidase activity and time of incubation at 30°C after partial inactivation by heating at higher temperature. ●——●, isoenzyme A3.7. ▲——▲, isoenzyme C9.9.

peroxidase activity following heat inactivation was not observed for purified isoenzyme A3.7 or C9.9 (Fig. 6). Indeed, while little change was apparent in the activity of A3.7 during the 30 min incubation period studied, the activity of C9.9 showed a marked decrease. This further emphasises the heat lability of C9.9. Under similar experimental conditions, regeneration was observed for peroxidase activity in crude Spring cabbage extracts. This may indicate that regeneration might be dependent on the presence of some unknown factor which was removed during our purification procedure.

We have previously reported that Spring cabbage ionically bound peroxidases are less stable to heat than Spring cabbage soluble peroxidases (McLellan & Robinson, 1981) and that the ionically bound enzyme preparations contain only cationic isoenzymes, while the soluble preparations contain cationic and anionic isoperoxidases (McLellan & Robinson, 1983). The present investigation shows that the purified cationic isoenzyme is also less stable than the anionic isoenzyme. Similarly, Lee & Pennesi (1984) have reported the presence of peroxidase isoenzymes with different heat stabilities in cauliflower. Their isoenzyme C represented the majority of peroxidase activity detected using the guaiacol method of assay and was relatively heat labile, while the less abundant cauliflower peroxidase A was heat stable. Powers *et al.* (1984) have extracted a 'basic' and a 'salt' fraction of peroxidase activity from asparagus and found these to have similar heat stabilities, but only the salt fraction was able to regenerate following heat inactivation.

A model system consisting of at least two enzyme fractions with different heat stabilities has been used to explain the biphasic kinetics of peroxidase inactivation in corn on the cob (Naveh *et al.*, 1982). The present investigation has shown the presence of isoperoxidases with different heat stabilities in Spring cabbage extracts. The extent of inactivation achieved by heating isoenzyme C9.9 at 60°C closely resembled that achieved by heating isoenzyme A3.7 at 70°C. However, our isolated peroxidase isoenzymes still show a biphasic inactivation process in response to heat (Figs 4 and 5). Hence, although vegetable extracts do contain peroxidase isoenzymes with different heat stabilities, this may not fully explain the biphasic nature of peroxidase inactivation by heat.

## ACKNOWLEDGEMENTS

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