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Purification and Heat Stability of Brussels Sprout Peroxidase Isoenzymes

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ABSTRACT

Using gel filtration and ion-exchange chromatography, a total of four peroxidase isoenzymes were isolated from extracts of Brussels sprouts. The isoenzymes were found to vary in their substrate specificities and heat stability properties. Three of the isoenzymes showed biphasic inactivation in response to heating, while the fourth isoenzyme was relatively heat labile and inactivated in a more linear manner with time. Regeneration following heat inactivation was not observed for the isolated isoenzymes.

INTRODUCTION

Peroxidase (EC 1.11.1.7) is recognised to be one of the most heat stable enzymes in fruit and vegetables and may cause quality deterioration during storage (Burnette, 1977). Peroxidase has a low specificity for hydrogen donor substrate and will oxidise aromatic amines, e.g. *o*-dianisidine, and phenols, e.g. guaiacol, as well as other organic compounds, at the expense of hydrogen peroxide. The precise nature of the natural peroxidase substrates is unclear and peroxidase has been implicated in several physiological roles. The catalytic action of peroxidase has been claimed to affect vegetable texture (Haard, 1977) and correlations have been reported between peroxidase activity and quality loss in some vegetables, e.g. corn-on-the-cob (Lee & Hammes, 1979).

In 1962 the inactivation of peroxidase in extracts of corn-on-the-cob was shown to be a biphasic process (Yamamoto *et al.*, 1962) and, since then,

heat inactivation for a variety of vegetables including Brussels sprouts and cabbage (McLellan & Robinson, 1981), potatoes (Kahn *et al.*, 1981) and asparagus (Wang & Lu, 1983) has been shown to be biphasic. Partial regeneration of peroxidase activity following heat treatment has also been reported by a number of workers (Lu & Whitaker, 1974; Tamura & Morita, 1975; Vamos-Vigyazo *et al.*, 1979; McLellan & Robinson, 1981), which indicates that a portion of the peroxidase activity undergoes reversible inactivation. Considerably more severe heat treatment is required for irreversible inactivation (Adams, 1978).

In extracts of plant material, peroxidase activity is associated with a number of isoenzymes (Delincee & Radola, 1970; Heidrich *et al.*, 1983; McLellan & Robinson, 1983*a*) and consequently it has been suggested that non-linear heat inactivation of peroxidase activity with time is due to the existence in vegetable extracts of separate isoenzymes with differing heat stabilities (Yamamoto *et al.*, 1962). Peroxidase isoenzymes may also differ with respect to the rate of catalysis of the oxidation of different hydrogen donor substrates. Horseradish peroxidase isoenzymes have been isolated and studied extensively (Shannon *et al.*, 1966; Kay *et al.*, 1967; Morita *et al.*, 1971) and, in particular, horseradish peroxidase isoenzyme C has been well characterised (Clarke & Shannon, 1976; Welinder, 1976). Four peroxidase isoenzymes isolated from horseradish have been claimed to possess different stabilities towards heat (Yoon & Park, 1982). Recently, Lee and co-workers have isolated a heat labile and a heat stable peroxidase isoenzyme from cauliflower (Lee *et al.*, 1984; Lee & Penesi, 1984).

In extracts of Brussels sprout, McLellan & Robinson (1983*a*) have reported a total of nine soluble isoenzymes which all possess peroxidase activity although, more recently, thin-layer isoelectric focusing has shown up to 17 isoperoxidases in extracts of Brussels sprouts (unpublished data). The present investigation reports a method for the isolation of four peroxidase isoenzymes from Brussels sprouts, and a comparison of their heat stability.

MATERIALS AND METHODS

Materials

Brussels sprouts (*Brassica oleracea gemmifera* var. *Lancelot*) were grown outdoors from F1 hybrid seed. Sephadex G100 and DEAE Sephacel were purchased from Pharmacia (GB) Ltd, Hounslow, Middlesex; SP Trisacryl and Ampholine Carrier Ampholytes (pH 3·5–10·0 and pH 2·5–4·5) were purchased from LKB Instruments Ltd, Selsdon, South Croydon, Surrey.

o-Dianisidine was obtained from Koch Light Laboratories Ltd, Colnbrook, Bucks; Bovine Serum Albumin from Sigma Chemical Co., Poole, Dorset and 2,2'-Azino-di-3-ethyl-benzothiazoline sulphonate(6) (ABTS) from BCL, Lewes, East Sussex.

All other chemicals were obtained, in the Analar grade where available, from BDH Chemicals Ltd, Poole.

Methods

Damaged outer leaves were removed from the Brussels sprouts and discarded. 100 g Brussels sprouts were homogenised in 200 ml ice-cold 0·01M sodium phosphate buffer, pH 6·0, containing 1% (w/v) polyvinyl polypyrrolidone (PVPP) for 1 min in a Waring blender at high speed. The homogenate was filtered through a double layer of muslin and 120 ml of the resulting filtrate was centrifuged at 15 000 *g* for 25 min at 6°C. The supernatant fluids following centrifugation were collected and ammonium sulphate added to 50% saturation. Centrifugation at 15 000 *g* for 25 min at 6°C yielded a residue which was discarded, and a supernatant to which ammonium sulphate was added to 90% saturation. This fraction was retained overnight at 4°C before centrifuging as before, discarding the supernatant and redissolving the residue in a minimal volume of 0·1M sodium phosphate buffer, pH 6·0.

Sephadex G100 (8 g) was swollen in 250 ml 0·1 M sodium phosphate buffer, pH 6·0, degassed and packed into a Pharmacia C16/70 chromatography column. The redissolved residue obtained from ammonium sulphate precipitation was applied to the Sephadex G100 column. Elution was carried out at room temperature using 0·1M sodium phosphate buffer at a flow rate of 30 ml h^{-1} and fractions were collected at 10-min intervals. The peroxidase was eluted in a single peak and those fractions showing peroxidase activity were pooled, concentrated to approximately 10 ml using an Amicon concentrator with a PM10 membrane and dialysed overnight at 4°C against 0·05M Tris HCl buffer at pH 7·8.

DEAE Sephacel was degassed, packed into a Pharmacia C16/40 column and extensively equilibrated with 0·05M Tris HCl buffer at pH 7·8. The concentrated and dialysed peroxidase sample obtained from the Sephadex G100 column was applied to the DEAE Sephacel. Elution was then carried out using 0·05M Tris HCl buffer, pH 7·8, for 2 h at a flow rate of 10 ml h^{-1} and then with a gradient of 0–0·3M NaCl in a total volume of 300 ml of the same buffer. Fractions were collected at 30-min intervals throughout. Peroxidase activity was eluted in four peaks (Fig. 1) and the fractions comprising each peak were pooled for analysis by isoelectric focusing (Fig. 2).

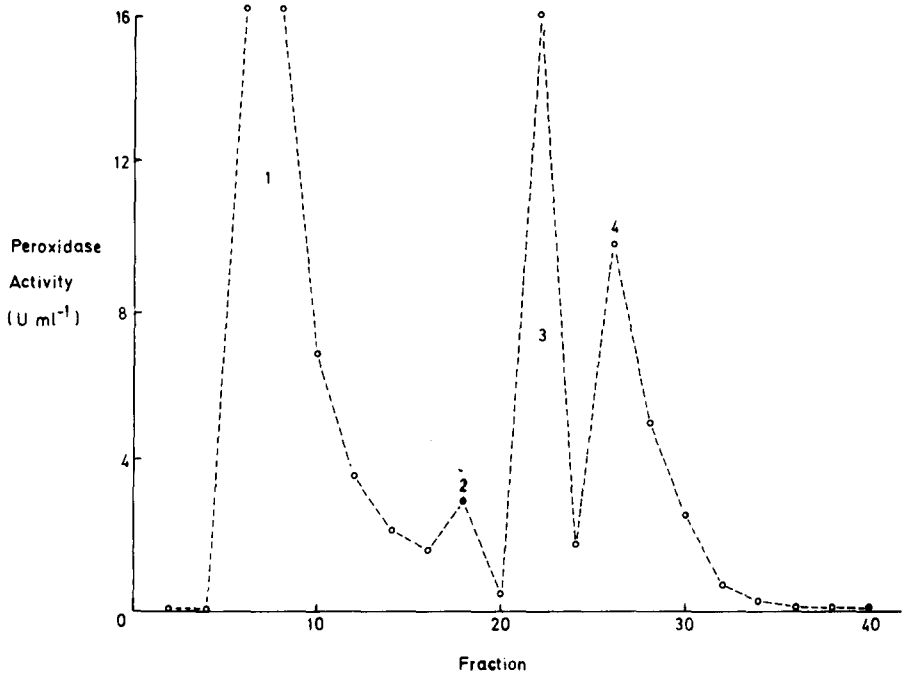

Fig. 1. Separation of Brussels sprout peroxidase isoenzymes on DEAE Sephacel. O ----- O, peroxidase activity.

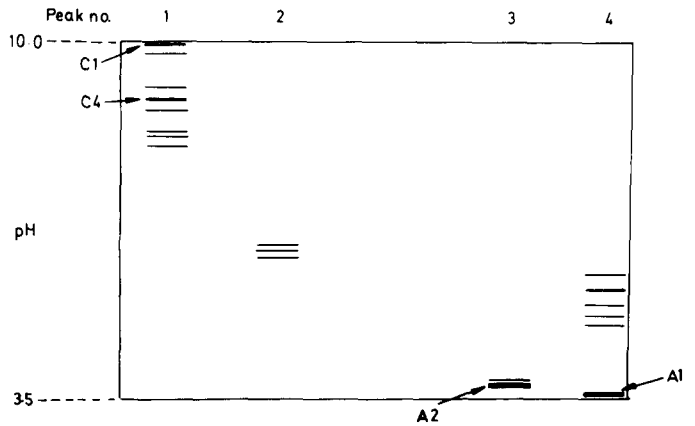

Fig. 2. Isoelectric focusing of peroxidase fractions obtained following ion-exchange chromatography on DEAE Sephacel. Peak numbers refer to DEAE Sephacel elution profile (Fig. 1).

The components of peak 1, containing cationic peroxidase isoenzymes, were concentrated and dialysed against 0·05M sodium phosphate buffer at pH 6·0. The fractions comprising peak 3 were pooled, concentrated and dialysed against 0·05M Tris HCl buffer at pH 7·8 and those comprising peak 4 were pooled, concentrated and dialysed against the same 0·05M Tris buffer. SP Trisacryl was degassed, packed into a Pharmacia C16/40 column and extensively equilibrated with 0·05M sodium phosphate buffer at pH 6·0. The dialysed peak 1 from DEAE Sephacel was applied and the column eluted initially with the phosphate buffer at pH 6·0 (1·5 h) and then with a linear gradient of 0–0·25M NaCl in a total volume of 240 ml of the same buffer. A flow rate of 40 ml h^{-1} was used throughout and fractions were collected every 10 min. The fractions comprising the two peaks of peroxidase activity were separately pooled, designated C1 and C4 and analysed using isoelectric focusing (Fig. 3). For further purification of peaks

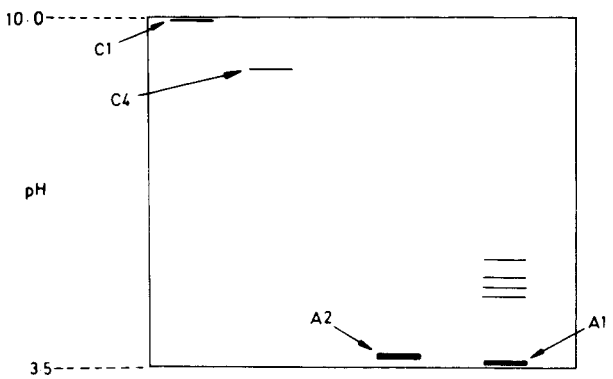

Fig. 3. Isoelectric focusing of isoenzyme preparations following the second ion-exchange chromatography fractionations.

3 and 4 using anion exchange chromatography the same DEAE Sephacel column was re-equilibrated as already described, and the dialysed peak 3 applied. Elution was carried out as before, resulting in a single major peak of activity designated A2. Similarly, rechromatography of peak 4, using DEAE Sephacel, resulted in a single major peak of peroxidase activity. The fractions comprising the peak were pooled and designated A1. Fraction A1 was shown, using isoelectric focusing, to contain a number of peroxidase isoenzymes (Fig. 3). The DEAE Sephacel column was therefore reequilibrated once more using 0·02M Tris HCl, pH 7·8. Fraction A1 was reapplied and finally eluted with a linear gradient of 0–0·25M NaCl in a total volume of 240 ml 0·02M Tris HCl buffer at pH 7·8. The major peak of peroxidase activity obtained was pooled and designated 'rechromatographed A1'.

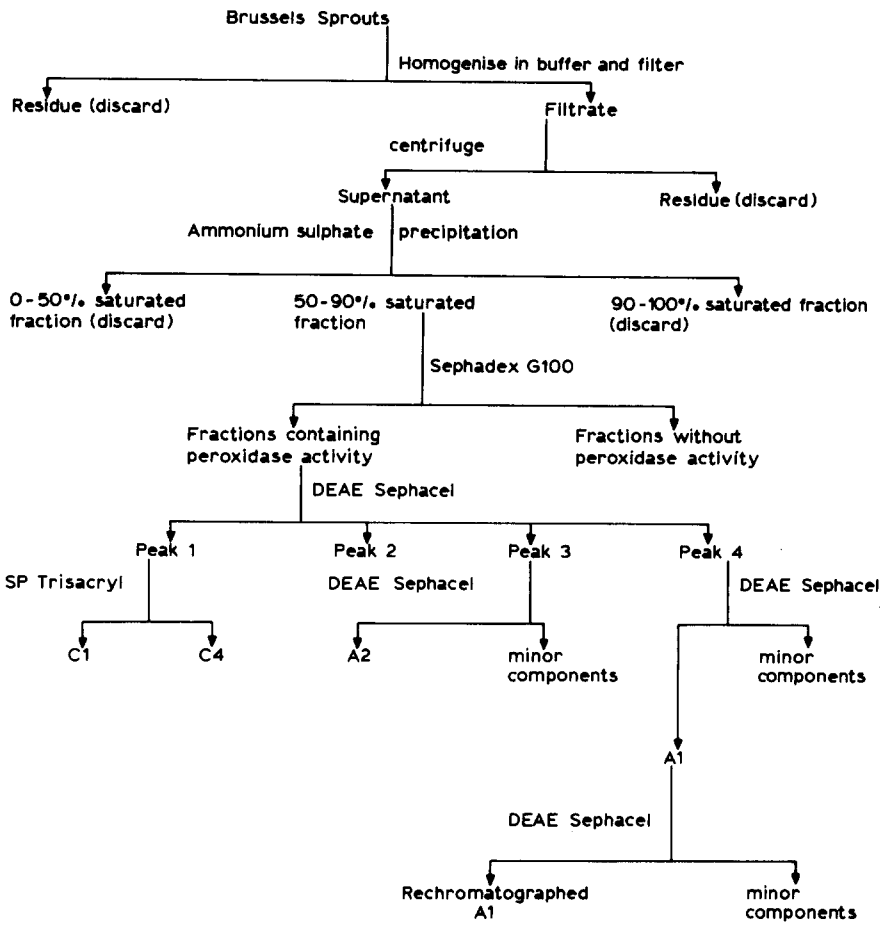

Fig. 4. Flow diagram for the purification of peroxidase isoenzymes from Brussels sprouts. Details of each step are given in the text.

All purified peroxidase samples were dialysed against 0·01M sodium phosphate at pH 6·0 in preparation for heat inactivation and substrate specificity studies. An outline of the purification procedure is given in Fig. 4.

Peroxidase activity estimation

o-*Dianisidine method*

Peroxidase activity was estimated at 25°C as described previously (McLellan & Robinson, 1981) and absorbances were measured using a Pye Unicam SP8 200 UV Spectrophotometer. 1 unit of peroxidase activity is defined as an absorbance change of 1 per minute.

Guaiacol method
0·2 ml peroxidase solution was added to 1·4 ml 0·1% (w/v) H_2O_2 in 0·1M sodium acetate buffer at pH 5·6. The reaction was started by the addition of 1·4 ml 0·5% (v/v) aqueous guaiacol solution, and the initial change in absorbance at 420 nm was measured at 30°C. 1 unit of peroxidase activity is defined as an absorbance change of 1 per minute.

ABTS method
2·2 ml 0·1M sodium phosphate buffer at pH 7·9, 0·7 ml aqueous ABTS solution (100 mM) and 0·1 ml peroxidase solution were mixed in a test tube. The reaction was initiated by the addition of 0·1 ml H_2O_2 solution prepared by dissolving 0·1 ml 30% H_2O_2 in 100 ml distilled H_2O. The reagents were mixed and the initial absorbance change at 436 nm was measured at 25°C using a Pye Unicam SP8 200 UV Spectrophotometer. 1 unit of activity is defined as a change in absorbance of 1 per minute.

Protein determination

Total protein was estimated according to the Biuret method (Scopes, 1982). A standard curve was prepared using known concentrations of Bovine Serum Albumin.

Isoelectric focusing in thin-layer polyacrylamide gels

Isoelectric focusing was carried out as recommended in LKB Instruction Note 1818-P using an LKB Ultrophor Electrofocussing unit equipped with an LKB MultiTemp thermostatic circulator. All samples were dialysed against 0·01M sodium phosphate buffer at pH 6·0 prior to analysis by isoelectric focusing. Following isoelectric focusing, the pH gradient in the gel was measured by removing a section 1 cm wide across the whole width of the gel. This section was divided into 2 mm pieces and the ampholytes eluted from each piece into 1 ml distilled H_2O. The pH of the resulting fractions was then measured and a calibration plot of pH against distance into gel was prepared.

Staining of polyacrylamide gels for peroxidase activity

The whole gel was immersed in 150 ml 0·1M sodium phosphate buffer at pH 6·0; 40 ml 0·2% (w/v) *o*-dianisidine in 80% (v/v) methanol was added and the gel allowed to stand for 5 min. The staining reaction was then initiated by the addition of 20 ml 0·3% (w/v) H_2O_2 and allowed to proceed for 30 min at room temperature. The gel was then removed from the

staining solution and washed with distilled water. The presence of peroxidase activity was indicated by the appearance of brown permanent bands, and the isoelectric points of the peroxidases were estimated by comparing the position of the stained bands with the pH calibration plot.

Heat inactivation and regeneration

Pyrex tubes (15 × 1·6 cm, wall thickness 1·8 mm) were preheated in a water bath to the temperature required for inactivation (see 'Results') before adding 0·2 ml peroxidase isoenzyme sample to each tube. Heat treatment was then allowed to proceed for the desired time period before removing the tubes and placing in ice water. The estimation of peroxidase activity surviving heat treatment was carried out immediately using the *o*-dianisidine method of assay.

For regeneration, heat treated samples were held at 30°C for the required period of time before cooling and assaying for peroxidase activity as described above. The time periods used in both inactivation and regeneration methods are given in the 'Results'.

RESULTS

Purification

A five-fold increase of peroxidase activity, as measured using the *o*-dianisidine method of assay, was achieved by salt fractionation and gel filtration. The isolation of individual purified peroxidase isoenzymes was then carried out using ion exchange chromatography. A typical elution profile obtained for the initial separation of anionic and cationic isoenzymes using DEAE Sephacel is shown in Fig. 1. Further analysis of each of the four chromatographically separated peroxidase components showed the presence of a number of peroxidase isoenzymes (Fig. 2). Peak 1 contained the cationic isoenzymes which did not adsorb to the DEAE Sephacel under the conditions used. These cationic isoenzymes were further separated by ion-exchange chromatography on SP Trisacryl yielding two purified isoenzymes. These isoenzymes, designated C1 and C4, were found, using isoelectric focusing at pH 3·5–10·0, to have isoelectric points of 9·8 and 9·0, respectively (Fig. 3) and to be free of contamination by other peroxidase isoenzymes. The components of peak 3, obtained from the initial ion-exchange fractionation, contained mainly isoenzyme A2 (Fig. 2). After rechromatography on DEAE Sephacel, isoenzyme A2 was found, using isoelectric focusing, to have a pI of approximately 3·9 and to be free

TABLE 1
Purification of Peroxidase Isoenzymes from Brussels Sprouts

Fraction	*Volume (ml)*	*Activity ($U\ ml^{-1}$)*[a]	*Total activity (U)*[a]	*Per cent recovery*	*Total protein ($mg\ ml^{-1}$)*	*Specific activity ($U\ mg^{-1}$ of protein)*[a]	*Purification factor*
Homogenate	120	228	27 360	100	7·6	30	1
50–90% $(NH_4)_2SO_4$ precipitate	9·2	1780	16 376	60	16·8	106	3·5
Pooled peroxidase fractions from Sephadex G100	52	248·4	12 917	47	1·7	146	4·9
Ion exchange:							
Rechromatographed A1	22	7·0	154	0·56	0·08	87·5	2·9
A2	12	17·6	211	0·77	1·26	14·0	0·5
C1	33·3	45	1 498	5·5	0·04	1125	37·5
C4	26·7	56	1 495	5·5	0·08	700	23·3

[a] 1 unit of peroxidase activity is defined as an absorbance change of 1 per minute measured using the *o*-dianisidine method of assay.

of contamination with other peroxidases (Fig. 3) as determined by the *o*-dianisidine method of assay. After rechromatography of peak 4 on DEAE Sephacel a number of peroxidase isoenzymes were present in the major peak of peroxidase activity (Fig. 3) and a further fractionation on DEAE Sephacel using a lower ionic strength buffer as eluant was necessary to free isoenzyme A1 from the other contaminating peroxidase isoenzymes. The pI of isoenzyme A1 was found to be 3·6 and the purity of the isoenzyme A1 sample was confirmed after isoelectric focusing on polyacrylamide gels with a pH gradient of 2·5–4·5. The results of a typical purification are shown in Table 1. While the yield and purification factors for the cationic peroxidase isoenzymes were considered adequate, low recoveries of anionic peroxidase isoenzymes were obtained.

The stability to heat of the four isolated isoenzymes was investigated. For isoenzymes A1, A2 and C1, heat-treatment for 10 min at 70°C (Fig. 5) resulted in a 70–90% loss of measureable peroxidase activity. However, as

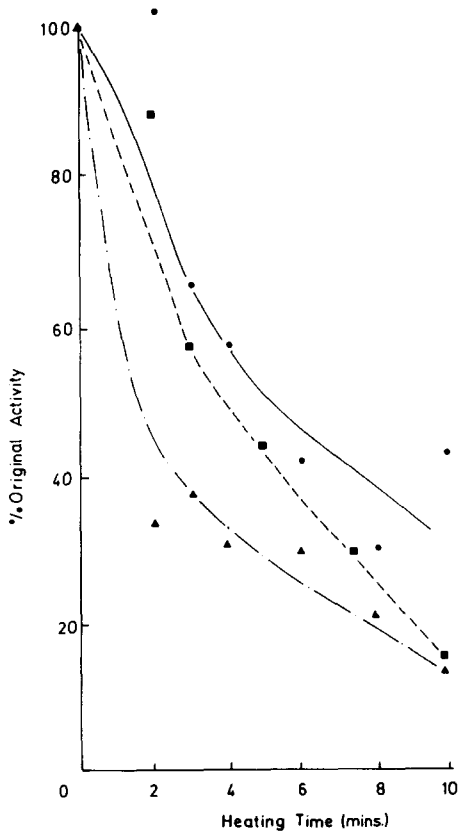

Fig. 5. Heat inactivation of Brussels sprout peroxidase isoenzymes A1, A2 and C1 at 70°C ●———●, Isoenzyme A1. ■– – –■, Isoenzyme A2. ▲—·—·—▲, Isoenzyme C1.

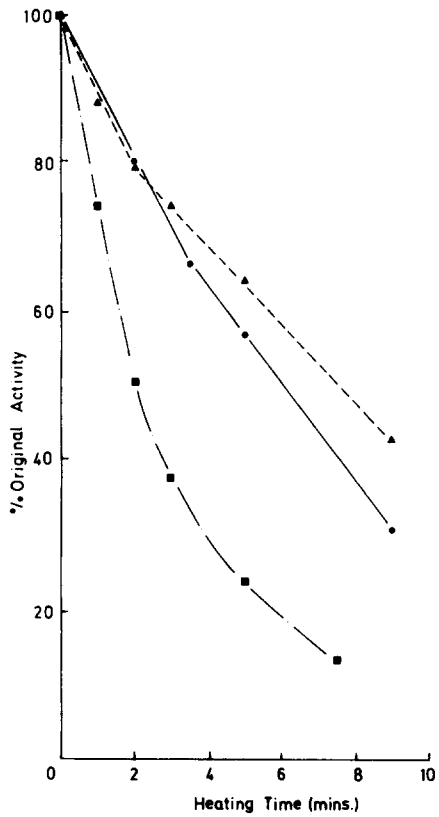

Fig. 6. Heat inactivation of Brussels sprout peroxidase isoenzyme C4. ▲——▲, 50°C. ●——●, 55°C. ■—·—·—■, 60°C.

the activity of the anionic isoenzymes was difficult to measure due to a non-linear reaction rate after the first 1–2 min of the assay, the measured results are inconsistent. Thus the results shown for A1 and A2 in Fig. 5 are an average of triplicate determinations. Isoenzyme C4 was considerably more heat labile (Fig. 6), some 90% inactivation being achieved following heating at 60°C for 7·5 min.

Surprisingly, regeneration of peroxidase activity was not observed for any of the purified isoenzymes when held at 30°C for 1 h, following 80% inactivation by heating, although partial regeneration of peroxidase activity in crude extracts of Brussels sprout has been observed following identical conditions of heat treatment (McLellan & Robinson, 1981).

Oxidation of different substrates

The peroxidase activities for the four isoenzymes measured with three different H donor substrates are shown in Table 2. Isoenzyme A1 appeared

TABLE 2
Specific Activity of Peroxidase Isoenzyme Preparations
(Measured using three methods of assay)

Sample	*Total protein* ($mg\ ml^{-1}$)	*Specific activity* ($U\ mg^{-1}$)[a]		
		o-Dianisidine assay method	*Guaiacol assay method*	*ABTS assay method*
A1	0·08	87	0	0
A2	1·26	14	7	1·4
C1	0·04	1125	250	78
C4	0·08	700	75	5

[a] 1 unit of peroxidase activity is defined as an absorbance change of 1 per minute measured using the assay method shown.

unable to oxidise either guaiacol or ABTS at concentrations of the isoenzyme giving a readily detectable level for the oxidation of *o*-dianisidine. Isoenzyme A2 was found to have a very low specific activity in all three assays due to the presence of substantial amounts of protein ($1{\cdot}26\ mg\ ml^{-1}$). The cationic isoenzymes were able to catalyse the oxidation of all three hydrogen donors, although isoenzyme C4 only slowly oxidised the hydrogen donor, ABTS. The specific activity values shown in Table 2 represent results obtained using different buffer conditions and substrate concentrations for the various assay methods. Thus, these results are not suitable for direct comparison, but the results do demonstrate relative differences in the specificities of the isoenzymes towards different hydrogen donor substrates.

DISCUSSION

Previous studies have shown heat inactivation of peroxidase in extracts of Brussels sprouts to be a biphasic process (McLellan & Robinson, 1981). Two pH optima were detected when the oxidation of *o*-dianisidine by Brussels sprout peroxidase was measured at different pH values (McLellan & Robinson, 1983*b*). It has been proposed that this is due to the presence of multiple forms of peroxidase in vegetable extracts. Furthermore, inconsistent results have sometimes been obtained when the method of assay for peroxidase activity has been varied by the use of different hydrogen donor substrates, which may indicate the presence of isoenzymes with different preferred substrates. Therefore, some of the individual peroxidase isoenzymes present in extracts of Brussels sprouts have been

isolated for further study. In the present investigation, this was achieved using ion-exchange chromatography where the main aim has been to obtain isoenzymes which were free of other peroxidases rather than the removal of all extraneous protein. The recovery and purification factor of the less abundant anionic isoenzymes was low, implying either the presence of a high level of protein impurities or a low specific activity for these particular isoperoxidases. For isoenzymes A1, C1 and C4, the total protein contents were similar and were relatively low. This indicates that isoenzyme A1 does not readily oxidise the hydrogen donor, *o*-dianisidine, while the cationic isoenzymes have a greater ability to catalyse *o*-dianisidine oxidation. However, for isoenzyme A2 the total protein in the sample was indeed high and so the low apparent specific activity probably reflects substantial protein contamination in the sample.

The nomenclature used for the isolated isoenzymes reflects their positions when separated by isoelectric focusing on polyacrylamide gels. C1 and C4 are cationic isoenzymes and are the 1st and 4th bands, respectively, from the cathode following separation of a crude Brussels sprout extract by isoelectric focusing (pH 3·5–10·0) and staining using *o*-dianisidine. Similarly, the anionic isoenzymes A1 and A2 are the 1st and 2nd isoenzyme bands from the anode under the same conditions of isoelectric focusing (Fig. 2).

The heat stability studies show isoenzyme C4 to be heat labile (Fig. 6) and to be inactivated in a more linear manner than the other isolated isoenzymes. The non-linear heat inactivation plots for A1, A2 and C1 may have arisen from an unknown characteristic property of only the more thermostable isoenzymes.

The peroxidase activity of the purified isoenzymes did not regenerate following heat inactivation and two possible explanations are proposed for this behaviour. First, as regeneration of peroxidase activity occurs in crude extracts of Brussels sprouts then some protective factor which is removed during the purification may be responsible for regeneration of peroxidase activity. Alternatively, the ability to regenerate may be restricted to some isoenzymes that have been lost during the purification procedures and thus have not yet been isolated and identified.

The specific activity of the purified isoenzymes was calculated using three different hydrogen donor substrates. This showed that some isoenzymes with a high specific activity for one substrate have a much lower specific activity for another substrate, relative to that of other isoenzymes. For example, for *o*-dianisidine the specific activity of isoenzyme C1 was less than twice that of isoenzyme C4 while, for ABTS, the specific activity of C1 was some 16-fold greater than that of isoenzyme C4. Furthermore, isoenzyme A1 was only detected by the *o*-dianisidine method of assay. This

research therefore offers an explanation for the variation in results which have been obtained for peroxidase activity measurements by different researchers using the different methods of assay. In addition, the use of any particular method may influence the results obtained by favouring the detection of only a particular isoenzyme or group of isoenzymes. Therefore it is now clear that experimental results for assay of peroxidases are only directly comparable if they have been obtained using the same hydrogen donor substrate.

It is apparent from this investigation that the multiple forms of peroxidase found in plants differ in their heat stability and in their ability to oxidise different substrates at the expense of hydrogen peroxide.

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