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Purification of Brussels sprout isoperoxidases

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Extracts of Brussels sprouts contain a complex mixture of isoperoxidases. Purification of the anionic and cationic groups of isoenzymes by ion-exchange chromatography has resulted in identification of four distinct isoperoxidases (A_1, A_2, C_1 and C_2) of different molecular size. Each preparation showed single staining bands for isoperoxidase activity and by the more sensitive silver staining technique for protein. However, only the C_2 isoperoxidase preparation showed a single band by Western blotting against polyclonal horseradish antibody, whereas the other isoenzymes still showed cross contamination with other isoperoxidases although minor in the instances for the A_1 and A_2 isoperoxidases. Also these results show that, even after extensive chromatographic purification of peroxidases, it is still necessary for assessment of homogeneity, prior to structural studies, to use the more sensitive Western antibody technique. Amino acid sequencing yielded a short identical sequence for A_2 and C_1which was also 95% identical to some previously detected drought/salt stress proteins.

INTRODUCTION

Plant peroxidases are known to be comprised of a range of isoenzymes differing in number from one plant to another and have been observed to differ with respect to thermal stability, pH optima, substrate specificity, amino acid composition and their physiological roles in plant tissues (Shannon *et al.*, 1996; Haard, 1977; Vamos-Vigyazo, 1981).

The catalytic characteristics and thermal properties of the individual isoenzymes have received considerable attention due to their thermostability and ability to recover enzymic activity after partial heat-inactivation and hence their potential effect on food quality (Burnette, 1977). Most of the information on the molecular structure has been based on the assumption that plant peroxidases are homologous to yeast cytochrome c peroxidase (Finzel *et al.*, 1984) and more recently the use of DNA sequencing of cloned genes which has allowed the primary structure to be elucidated for cultured peanut cell peroxidase (Esnault *et al.*, 1991) tobacco peroxidase (Lagramini *et al.*, 1987) and induced-wheat peroxidase (Hertig *et al.*, 1991). Immunological methods have also become a powerful tool for detecting the protein moiety of enzymes, as well as for determining cellular localisation.

For vegetables and fruits the various kinds of isoperoxidases including the anionic and cationic types with low and high pI values respectively can be extracted as both soluble and ionically bound forms. The present investigation reports the purification and isolation of 2 cationic and 2 anionic isoperoxidases from extracts of Brussels sprouts and their immunological cross reactivity with a commercial horseradish peroxidase antibody.

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MATERIALS AND METHODS

Brussels sprouts (*Brassica oleracea* var. *gemmifera*) were purchased from Leeds market. Sephadex G100, Sephacryl S100, Q Sepharose, SP Trisacryl, *o*-dianisidine were obtained from Sigma UK. Carrier ampholytes and all other materials and equipment for analytical isoelectric focusing were supplied by Pharmacia. All other chemicals were obtained from BDH Chemicals. Polyclonal rabbit anti-horseradish peroxidase, polyclonal sheep anti-rabbit: IgG alkaline phosphatase conjugate and monoclonal sheep anti-Mouse: IgG3 Alkaline Phosphatase Conjugate was purchased from SEROTEC.

Enzyme assay

Peroxidase activity was estimated using *o*-dianisidine at pH 6.0 at 25°C (McLellan and Robinson, 1981). Absorbance changes were recorded at 460 nm using a Pye Unicam SP 200 UV spectrophotometer.

Protein determination

Total protein content of the crude and purified extracts was estimated using a dye-binding method (Bradford, 1976).

Analytical isoelectric focusing in thin-layer polyacrylamide gels

All samples were dialysed against 100 mM sodium phosphate buffer at pH 6.0 prior to analysis. Isoelectric focusing (IEF) was carried out using an Ultraphor Electrophocusing unit connected to a multi-temperature thermostatic circulator set at 4°C. Following IEF the pH gradient in the gel was measured at 0.5 cm intervals using a surface pH electrode. The focused gels were stained for peroxidase activity with *o*-dianisidine (McLellan and Robinson, 1987).

Ion exchange chromatography—Cationic ion exchange -SPTrisacryl

Preswollen SPTrisacryl was degassed and packed into a C16/40 column. The collected fractions obtained by gel-filtration were dialysed against 25 mM Mes buffer (pH 6.0) at 4°C before application to the column. Elution was with buffer alone for at least 2 h for unbound anionic isoperoxidases and then further with a linear gradient (0–0.2 M NaCl) in Mes (2[N-morpholine]ethane) sulphonic acid (200 ml) for the bound cationic isoenzymes; 4 ml fractions were collected.

Anion-exchange—Q-Sepharose

Pre-swollen Q-Sepharose was packed into a Pharmacia C16/40 column and extensively equilibrated with 25 mM Tris/HCl at pH 7.5 at a flow rate of 30 ml h^{-1} until the pH value of the eluant was 7.5. Elution was first with the Tris/HCl buffer at a flow rate of 30 ml h^{-1} for at least 2 h followed by a 0–0.5 M NaCl gradient in a total volume of 300 ml of buffer to facilitate elution of the anionically bound peroxidases. Fractions (4 ml) were collected.

Staining for protein

The purity of the separated isoenzymes showing a single band for peroxidase activity after staining with *o*-dianisidine, following IEF focusing, was assessed by staining for protein using a silver stain method (Morrissey, 1981) incorporating the recycling section of Heukeshoveen and Dernick (1985).

Isoelectric focusing

The isoelectric points of isoperoxidases were determined by both measuring, at 5 mm intervals, the pH gradient across the focused gel using a glass surface electrode and by the use of 11 proteins of known pI value.

Molecular weight

These were determined by gel filtration using Sephacryl 100 (S100) following the Pharmacia gel filtration calibration kit instruction manual. Bovine serum albumin (BSA), ovalbumin, chymotrypsinogen and ribonuclease were used as standards.

Extraction of soluble peroxidases

The outer leaves and any damaged tissues were removed from Brussels sprouts and the remainder chopped into pieces of approximately 1 cm^3 in size. Samples (50 g) were homogenised in ice cold 10 mM sodium phosphate buffer (100 ml) at pH 6.0 for 3 min using a Waring Blender on a high speed setting. One per cent (w/v) polyvinylpyrrolidine was added to the extraction buffer to improve the stability of the enzyme. The suspension was filtered through a double layer of muslin and centrifuged at 15 000 *g* for 20 min at 4°C. Supernatants were collected and are described as the soluble fraction. Also the effect of a range of concentrations of calcium chloride (0 to 1 M added to the extraction buffer) on the release of peroxidase activity was monitored in 1 g:2 ml and 1 g:3 ml Brussels sprout to buffer ratios.

Purification of peroxidase isoenzymes

For concentrating the enzymes, precipitation was achieved with ammonium sulphate (50 to 90% saturation). For gel filtration, Sephadex G100 (8 g) was swollen in 100 mM sodium phosphate buffer (200 ml) at pH 6.0 and packed into a Pharmacia C 16/70 chromatography column at a flow rate of 30 ml h^{-1}. The soluble fraction was dialysed against the 100 mM phosphate buffer at 4°C prior to application to the column. Elution was carried out at 4°C using the phosphate buffer at a flow rate of 30 ml h^{-1} and 4 ml fractions collected. The fractions containing peroxidase activity were pooled and concentrated using an Amicon concentrator (membrane PM10) to 10 ml at 4°C and stored at −18°C.

Cross reactivity with horseradish peroxidase antibody

Immunodiffusion was carried out between horseradish peroxidase antibody and Brussels sprout peroxidase (BSP) at room temperature in a 1% agarose gel based on the method described by Ouchterlony and Nilsson (1978). 5 μl BSP (0.1 mg ml^{-1}) was added to each antigen well in an undiluted, 1:2, 1:4, 1:8 and 1:16 dilution in HPLC grade water. The sixth well acted as a control and contained HPLC grade water. The centre well contained 7 μl of the horseradish peroxidase antibody in either an undiluted state, a 1:2 or a 1:10 dilution with HPLC grade water. After 15 h, cross-reactivity was visualised by the formation of precipitin lines.

Western blotting

Samples (100 μl) were applied to a SDS-polyacrylamide gel for electrophoresis at 20 mA constant current for 7 h. The focused gel was equilibrated for 15 min in 25 mM Tris 192 mm glycine containing 20% methanol (Towbin *et al.*, 1979). Blotting was carried out overnight at 15V, 0.1 A and then at 50V, 0.5 A for 1.5 h. The nitrocellulose (NC) membrane was removed from the assembly, then placed (protein side down) and sealed into a polythene bag holding 3% (w/v) bovine serum albumin in phosphate buffered saline (20 ml) containing the primary antibody at a dilution of 1:1000. This was agitated for 2 h and then held in a dish containing 3% bovine serum albumin (100 ml) in phosphate-buffered saline (100 ml) and agitated for 1 h (Blake *et al.*, 1984). The NC membrane was held at room temperature overnight. The NC membrane was removed from the bag and washed 3 times, for 10 min with phosphate-buffered saline containing Tween 20 (0.1% v/v). The NC membrane was re-sealed in a polythene bag with the secondary antibody at a dilution of 1:2000 in 20 ml of 3% w/v bovine serum albumin in phosphate buffered saline and agitated for 2 h, removed and washed five times for 5 min per wash with phosphate-buffered saline containing Tween 20 (0.1% v/v). The binding of the secondary antibody to the primary antibody was visualised by first gently blotting the NC membrane with filter paper, which was then transferred to the colour development substrate solution (Fast bromo-chloro-indoylphosphate).

RESULTS AND DISCUSSION

Maximum extraction (68.8% of the total activity) was achieved using 10 mM phosphate buffer at pH 7.5 containing 0.8 M calcium chloride for a 1 g:2 ml ratio of Brussels sprout to buffer.

Chromatography of soluble peroxidases

The elution profile for gel filtration showed the separation of three distinct regions containing peroxidase activity (Fig. 1). For further purification aimed at maintaining yield, all the fractions collected from gel filtration that contained peroxidase activity were combined and separated into cationic and anionic groups by cationic ion exchange chromatography (Fig. 2). Peak A consisted of unbound anionic isoperoxidases, as revealed by isoelectric focusing, Peak B contained a small quantity of cationic isoenzymes while the main cationic isoperoxidases C_2 and C_1 were eluted in peaks C and D, respectively (Fig. 3). Further purification of the cationic isoperoxidases was achieved by re-chromatography using a salt gradient to 0.125 M NaCl.

The anionic components collected in peak A (Fig. 2) from the SP Trisacryl column were separated by anion exchange chromatography using Q-Sepharose anion-exchanger (Fig. 4) into two major groups (peaks E and G) and a minor component, peak F. Both Peaks, F and G, were eluted by a salt gradient increased to 0.3 M NaCl. Isoelectric focusing showed that peak E, eluted prior to the application of the NaCl gradient, contained, not surprisingly, some cationic isoperoxidases with occasionally small quantities of anionic isoenzymes. Isoelectric focusing of the separately-collected peaks F and G showed that these fractions contained single anionic isoperoxidases (Fig. 5)—which are defined as isoperoxidases A_2 and A_1, respectively. The isoenzymes with pI values between 3.46 and 9.5 were readily detected with the highly sensitive *o*-dianisidine stain for peroxidase in the following IEF. The fractions were further

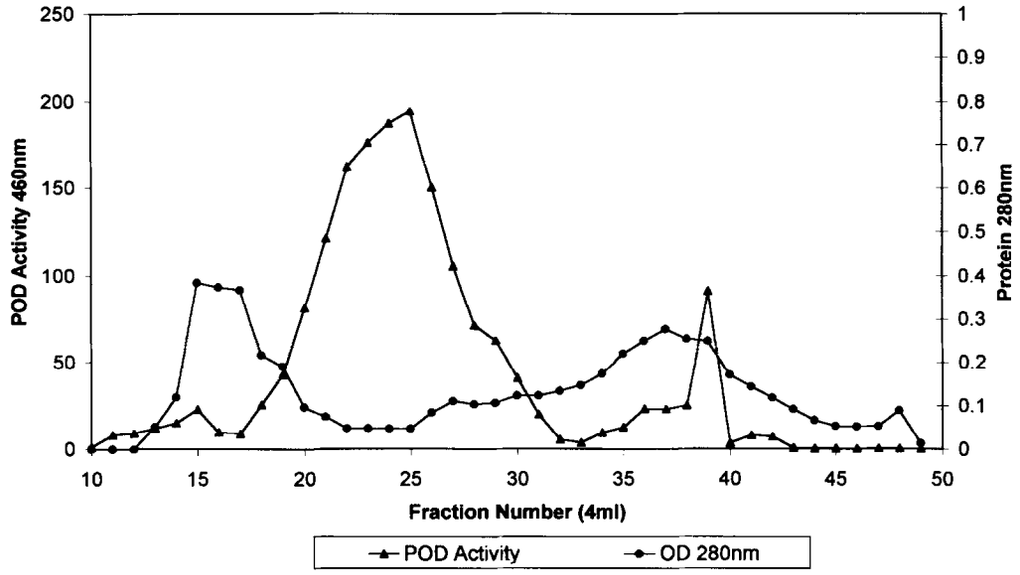

Fig. 1. Gel filtration of peroxidases on G100 Sephadex. Protein was measured at 280 nm. and peroxidase activity was detected by oxidation of *o*-dianisidine with H_2O_2 at 460 nm.

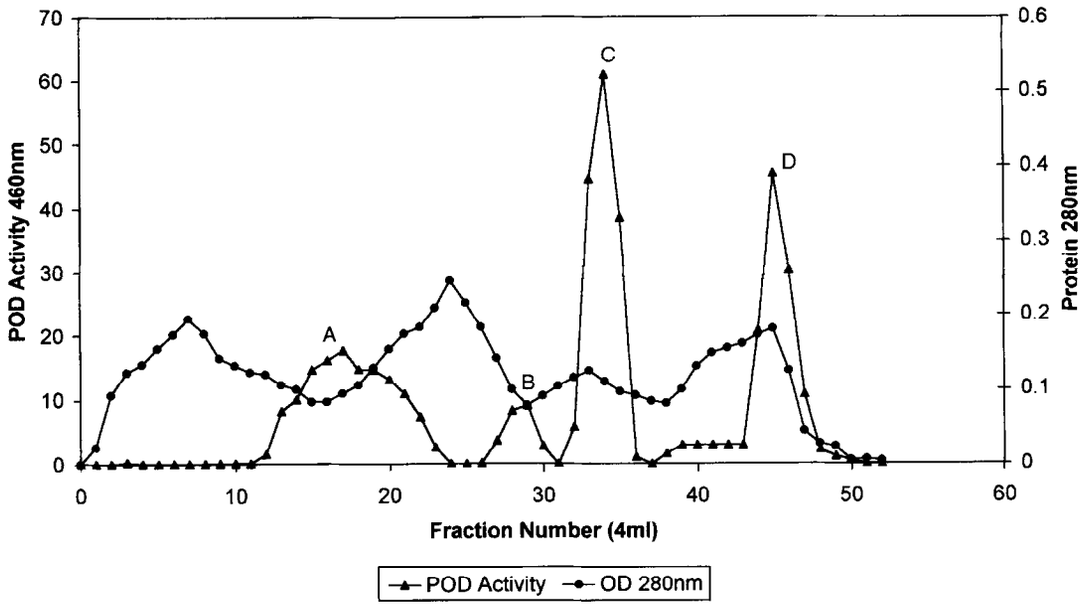

Fig. 2. Cation-exchange chromatography of peroxidases on SP Trisacryl. Protein was measured at 280 nm. and peroxidase activity was detected by oxidation of *o*-dianisidine with H_2O_2 at 460 nm.

assessed for contaminating proteins by a silver staining method. The isoperoxidases, $A_1$and C_2 preparations, showed mainly single silver stained bands (Fig. 6). A purification table is shown in Table 1, where it is seen that the isoperoxidases A_1 and C_2 possessed the highest specific activity.

The molecular weights determined by gel filtration and the pI values by isoelectric focusing for the four isoperoxidases are shown in Table 2. The wide range of values for the molecular weights is surprising as peroxidases are generally considered to have a molecular weight of approximately 40 000. As peroxidases are glycoproteins it seems possible that the higher molecular weight for the A_1 isoenzyme may be due to extra carbohydrate residues. Other research (in preparation) has shown that the A_1 isoperoxidase is also the most thermostable. The molecular weight of the C_2 isoenzyme was found to be nearly 50% less than the A_1isoperoxidase and this finding is similar to that reported for mango cationic isoperoxidases (Khan and Robinson, 1994).

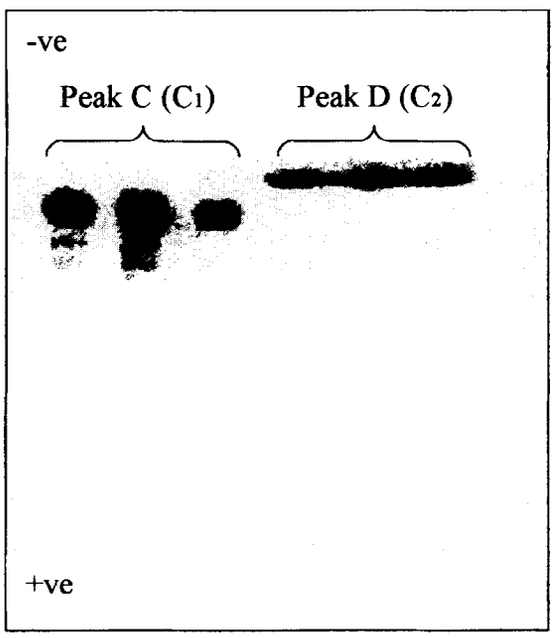

Fig. 3. Isoelectric focusing of cationic isoperoxidases. Peroxidase activity on the gel was detected by oxidation of *o*-dianisidine with H_2O_2.

For each purified isoperoxidase, precipitation lines were observed with Rabbit Anti-HRP (Polyclonal Antibody) but not with the monoclonal antibody at any of the concentrations investigated. Whereas the horseradish peroxidase reacted equally with both the polyclonal and the monoclonal antibodies (Mouse Anti-HRP). The results are summarised in Table 3. The commercial HRP, acting as the antigen, reacted with both the monoclonal and polyclonal antibodies to the same extent, which was at a 1:2 and 1:10 dilution of the antibody with the antigen in an undiluted state, at a 1:4, 1:16 and at a 1:64 dilution. The A_1, A_2 and C_1 antigen samples cross reacted with the undiluted polyclonal antibody to the same extent in an undiluted state or a 1:2 or a 1:4 dilution for precipitin lines to be formed. The C_2 sample only cross-reacted with the antibody when it was undiluted or in a 1:2 dilution. In each case water acted as the control.

Western blotting is a more sensitive method for detection of antigen-antibody interactions and is less affected by dilution due to diffusion. Following electrophoresis, cross-reactivity between the purified isoperoxidases and the polyclonal horseradish peroxidase antibody was observed for each isoenzyme (Fig. 7),

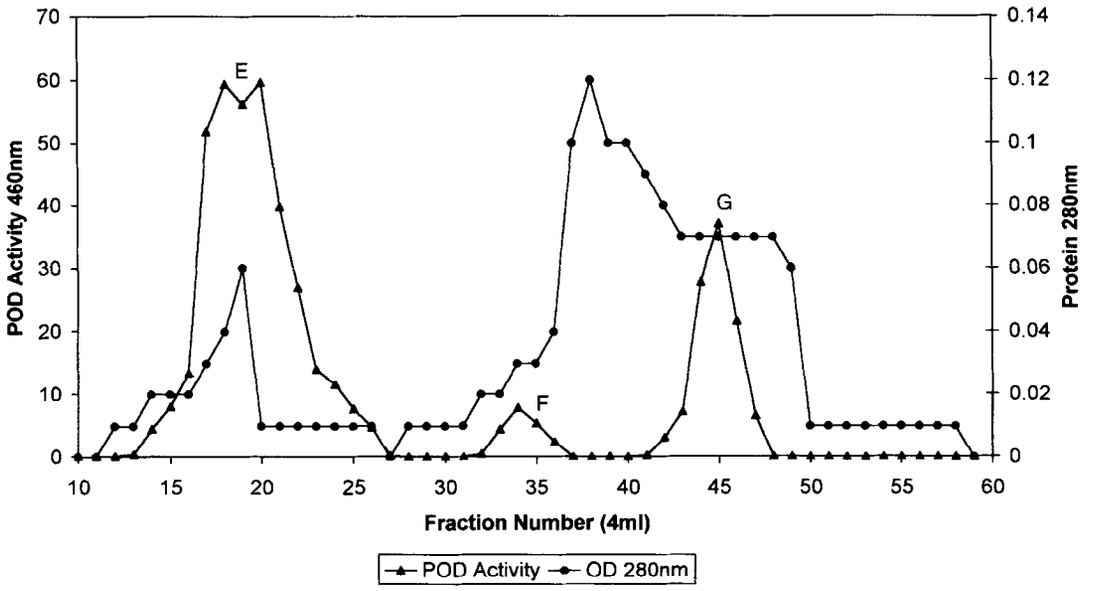

Fig. 4. Anion exchange chromatography of peroxidases on Q Sepharose. Protein was measured at 280 nm. and peroxidase activity was detected by oxidation of *o*-dianisidine with H_2O_2 at 460 nm.

Fig. 5. Isoelectric focusing of anionic isoperoxidases, A_1 and A_2. Peroxidase activity on the gel was detected by oxidation of *o*-dianisidine with H_2O_2.

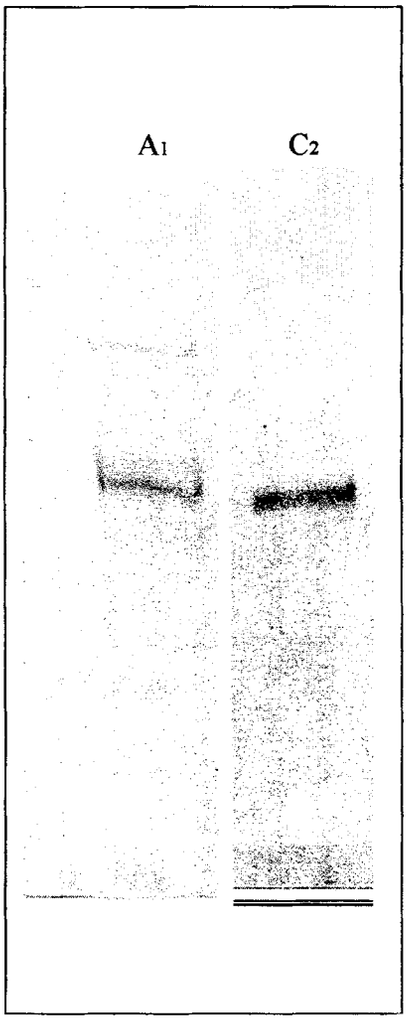

Fig. 6. Isoelectric focusing electrophoretogram for detection of proteins in isolates of A_1 and C_2 isoperoxidases. Protein bands were detected by silver staining.

Table 1. Purification of the cationic and anionic isoperoxidases

Fraction	Vol. ml	Peroxidase activity 460 nm	Recovery %	Protein mg/ml	Specific activity	Purification factor
Crude extract	200	236	100	4.2	56.1	1.0
$(NH_4)_2SO_4$ fraction	15	1256	39.7	10.9	115.2	2
Gel filtration fractions	45	316	30.13	1.02	309.8	5
A_1	10	8.88	0.19	0.006	1480	26
A_2	5	3.5	0.037	0.008	437.5	8
C_1	10	6.9	0.146	0.007	985.7	18
C_2	10	7.3	0.15	0.004	1825	33

although no cross reactivity was detected with the monoclonal horseradish peroxidase antibody. Only the C_2 isoperoxidase preparation yielded just one band whereas, for the other three isoperoxidases (A_1, A_2 and C_1), mainly one band plus traces of the other isoenzymes, one plus three minor bands and four main bands plus traces of three others were observed, respectively.

The cross-reactivity observed between the preparations of the purified isoperoxidases and the polyclonal antibody to horseradish peroxidase indicates that all the isoenzymes must share some of the same antigenic determinants and some of those present on horseradish peroxidase enzyme. The results obtained by Western blotting, using the polyclonal antibody, demonstrated the greater sensitivity of this method for detecting small amounts of proteins, as the purified samples (previously showing only one protein band following silver staining) displayed more than one antigen for the purified isoenzymes with importantly, the exception of isoperoxidase C_2. It was also shown that denaturing of the isoenzyme did not remove the antigenic determinants required for the polyclonal antibody to bind to the isoenzymes. The results also indicate that, in some cases, minor contaminating proteins in preparations A_2 and $C_1$are mainly the other isoperoxidases (A_1 and C_2) binding to the antibody. It is also clear that extensive tests for homogeneity of peroxidase preparations need to be carried out prior to structural studies of the isoperoxidases. As each of the isoperoxidases reacts with the horseradish antibody, yield may be increased through an antibody binding step but complete resolution is only likely to be achieved using highly refined ion-exchange chromatography.

Table 2. Molecular weights and isoelectric points of four isoperoxidases

Isoperoxidase	Molecular weight	Isoelectric point
A_1	48 300	3.46
A_2	33 400	4.88
C_1	32 000	9.5
C_2	26 800	8.75

Table 3. Antibody to antigen concentrations yielding precipitin lines by diffusion against rabbit-anti HRP antibody

Antigen	Antibody dilution	Antigen (concentration) yielding precipitin lines
HRP	Undiluted,	—
	1:2	Undiluted, 1:4, 1:16, 1:64
	1:10	Undiluted, 1:4, 1:16, 1:64
	1:100	—
BSP A_1	Undiluted, 1:2	Undiluted, 1:2, 1:4
	1:10	—
		—
BSP A_2	Undiluted, 1:2	Undiluted, 1:2, 1:4
	1:10	—
		—
BSP C_1	Undiluted, 1:2	Undiluted, 1:2, 1:4
	1:10	—
		—
BSP C_2	Undiluted, 1:2	Undiluted, 1:2
	1:10	—
		—

The symbol '-' indicates that no precipitation was observed, hence cross-reactivity was not detected.

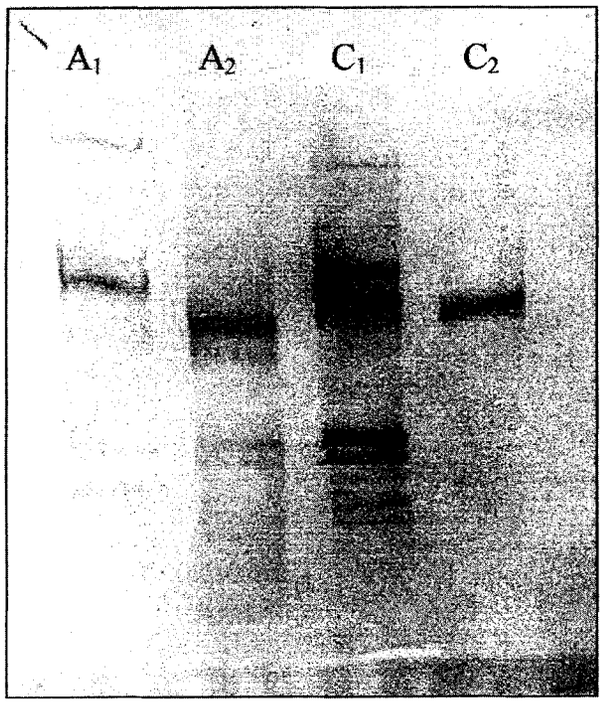

Fig. 7. Western Blot of isolated isoperoxidases. Polyclonal antibody raised against horseradish peroxidase was used to detect isoperoxidases A_1, A_2 $C_1$and C_2 isoperoxidases.

Table 4. Homologous sequences for Brussels sprout isoperoxidases and drought-induced proteins

A_1: D V N G G g A i L	This sequence has not been identified
Isoperoxidases A_2 and C_1	E N R V x D T D G N P L R T T A Q Y L I L P
	: :
Drought-induced turnip: isoperoxidases A_2 and C_1	E N R V D D T D G N P L R T T A Q Y L I L P
	E N R V x D T D G N P L R T T A Q Y L I L P
	: : : : : : : : : : : : : : :
Drought-induced radish: isoperoxidases A_2 and C_1	L E E V K D S N G N P V R V G A Q Y F I Q P
	E N R V x D T D G N P L R T T A Q Y L I L P
	: : : : : : : : : : : : : : :
Drought-induced rape:	R E Q V K D S N G N P V K R G A K Y F I Q P

x = unidentified residue.
Lower case letter = tentative assignment.

As cross-reactivity was not observed with the monoclonal antibody, the antigenic determinant for the horseradish monoclonal antibody may have been either unique to horseradish peroxidase or, alternatively, the Brussels sprout isoperoxidase antigenic determinant for the monoclonal antibody might have been inaccessible within the molecules. The results are not unexpected as the monoclonal antibody has been raised against a single specific antigenic determinant on horseradish peroxidase which therefore seems not to be present or detectable in Brussels sprout isoperoxidases.

Amino acid sequencing

Due to small amounts of protein present, the A_2 and C_1 preparations could be sequenced only to a limited extent. An identical sequence for 21 amino acids was found (Table 4) and surprisingly was 95% identical to a drought/salt stress-induced protein from turnip and 60 and 43% identical to drought-induced proteins from radish and rape, respectively, rather than horseradish peroxidase. However, this is not the first time that a correlation has been reported between plant stress proteins and peroxidases, as Lopez *et al.* (1994) found that the sequence of a stress protein (P28.5) from radish exhibited a strong homology to an ascorbate peroxidase. Also Mittler and Zilinkas (1994) observed that pea cytosolic ascorbate peroxidase increased during drought stress.

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