Plant Physiol. (1995) 109: 1115–1123

Purification and Partial Characterization of Tomato Extensin Peroxidase[1]

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Early plant defense response is characterized by elevation of activity of peroxidases and enhanced insolubilization of hydroxyproline-rich glycoproteins, such as extensin, in the cell wall. The insolubilization process (cross-linking between soluble extensin precursor molecules) is catalyzed by extensin peroxidases. We have ionically eluted extensin peroxidases from intact water-washed suspension-cultured tomato (hybrid of *Lycopersicon esculentum* Mill. and *Lycopersicon peruvianum* L. [Mill.]) cells and purified them to homogeneity by molecular sieve and cation-exchange chromatography. Four ionic forms of peroxidase (PI, PII, EPIII, and EPIV) were resolved; only the latter two cross-linked tomato soluble extensin. The molecular weight (34,000–37,000), amino acid composition, and isoelectric point (9.0) of the extensin peroxidases were determined. Substrate specificities of the enzymes were investigated: soluble extensin and potato lectin (a hydroxyproline-rich glycoprotein with a domain that strongly resembles extensin) were cross-linked by only two forms of the enzyme, whereas bovine serum albumin, aldolase, insulin, a number of other marker proteins, and proteins eluted from tomato cells (except extensin) could not be cross-linked. We have also isolated a yeast elicitor that enhances total peroxidase activity and extensin insolubilization within 1 h of challenge in cultured cells of tomato. A highly sensitive enzyme-linked immunosorbent assay technique using polyclonal antiserum raised against soluble tomato extensin was used to demonstrate extensin insolubilization in vivo. A tomato cell-wall peroxidase that cross-links extensin has been purified and may have a role in plant defense.

Plants generate a large array of inducible defense responses following mechanical damage or pathogen attack. These include synthesis of antimicrobial compounds (phytoalexins), deposition of callose, lignin, and cell-wall-bound phenolics, accumulation of HRGPs in the cell wall, and synthesis of protease inhibitors and hydrolytic enzymes (Lamb and Dixon, 1994). Extensin (an HRGP) exists in two developmental forms: soluble extensin, which is ionically bound to the cell wall, and an insoluble form that is deposited in the cell wall by a peroxidase/H_2O_2-mediated cross-linking process (Cooper and Varner, 1984).

In an earlier study, extensin and an associated peroxidase were eluted from cell walls of suspension-cultured tomato (hybrid of *Lycopersicon esculentum* Mill. and *Lycopersicon peruvianum* L. [Mill.]) cells (Brownleader et al., 1993). Cross-linking of extensin was then demonstrated by an FPLC gel-filtration assay in the presence of H_2O_2 (Brownleader et al., 1993). Everdeen et al. (1988) had earlier identified an extensin-cross-linking enzyme that they referred to as extensin peroxidase; their nomenclature is used in this paper.

Peroxidases (EC 1.11.1.7) have been found ionically and covalently bound to the plant cell wall (Ridge and Osborne, 1971), but their function in the plant cell wall is not completely understood. The precise role of individual isoforms of peroxidases remains unclear owing to the lack of information on the localization of the enzymes and the availability of their specific substrates in vivo. They are required for lignin biosynthesis, but they also occur in cell walls that do not form lignin (Whitmore, 1971). Subsequent work by Whitmore (1976) demonstrated that peroxidases catalyze the linking of ferulic acid to cell walls of pine (*Pinus elliottii*). Cationic peroxidases have been found to be most effective in IAA catabolism, and anionic isoforms are believed to be associated with lignification (Gaspar et al., 1991; Medina et al., 1993). Ros-Barceló et al. (1987, 1989) observed that lupin acidic peroxidases are located primarily in the cell wall, whereas basic peroxidases are primarily vacuolar and bound to the tonoplast (Ros-Barceló et al., 1991). However, Ferrer et al. (1992) demonstrated that two strongly basic peroxidases are located in the cell wall of etiolated lupin hypocotyls. Peroxidases are also believed to play a significant role in plant disease resistance. Increased peroxidase activity and elevated HRGP deposition in the cell wall have been demonstrated after challenging the plant with pathogens (Esquerré-Tugayé and Lamport, 1979; Reimers et al., 1992; Lamb et al., 1993).

This paper is concerned with the purification and characterization of ionically bound cell-wall peroxidase activities from cultured tomato cells that cross-link the HRGP

[1] M.D.B. is the recipient of a special research fellowship from The Leverhulme Trust. This work was supported by Biotechnology and Biological Sciences Research Council.

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Abbreviations: ABTS, 2,2′-azino-bis(3-ethyl benzthiazoline-6-sulfonic acid); CM-cellulose, carboxymethyl-cellulose; EPIII, EPIV, "extensin peroxidase" III and IV; EtOH, ethanol; FPLC, fast protein liquid chromatography; HRGP, hydroxyproline-rich glycoprotein; HRP, horseradish peroxidase; L-DOPA, L-dihydroxyphenylalanine; PI, PII, peroxidase I and II; MeOH, methanol; RZ, A_{405}/A_{280}.

extensin and that may play a role in plant disease resistance.

MATERIALS AND METHODS

Materials

Callus of tomato cells (hybrid of *Lycopersicon esculentum* Mill. and *Lycopersicon peruvianum* L. [Mill.]) was a gift from Dr. Stephen Fry (Department of Botany, University of Edinburgh, UK). CM-cellulose cation-exchange resin was obtained from Whatman and Superose-12 and Sephacryl S-200 gel-filtration resin were purchased from Pharmacia. Protogel for SDS-PAGE was obtained from National Diagnostics (Manville, NJ). Calibration marker kits for IEF and SDS-PAGE were obtained from Pharmacia. Polyclonal antiserum against extensin was prepared at Royal Holloway (University of London). Alkaline phosphatase-labeled goat anti-rabbit secondary antibody was purchased from Sigma. Phenolphthalein phosphate substrate and stop solution for ELISA were purchased from Serono Diagnostics (Geneva, Switzerland).

Growth Conditions of Cell Suspension Culture

Tomato suspension cells were grown in Murashige and Skoog tissue culture medium (basal salt mixture) (Murashige and Skoog, 1962) comprising 20 g/L Glc and a supplement solution of 100 mg/L *myo*-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 0.1 mg/L thiamine HCl, 2 mg/L Gly, 10 mg/L IAA, and 0.5 mg/L kinetin. Aliquots of sterilized medium (200 mL) in 500-mL conical flasks were inoculated with 7-d-old suspension cells and incubated on a gyratory shaker at 110 rpm under continuous fluorescent lighting for 7 d at 25°C.

Elution of Soluble Extensin Monomer and Extensin Peroxidase

Ionic desorption of soluble extensin and extensin peroxidase from cell walls of intact water-washed tomato cells in suspension cultures was accomplished with 50 mM $CaCl_2$, adjusted to pH 3.0 with 6 M HCl, by Büchner filtration. The filtrate was subsequently dialyzed in 5 L of 30 mM hydrochloric acid solution (pH 3.0) overnight. The low pH was chosen to prevent cross-linking of extensin by peroxidase if H_2O_2 or organic peroxides were present in the filtrate, and this was followed by dialysis against distilled water for an additional 3 h (Brownleader and Dey, 1993).

Purification of Extensin and Extensin Peroxidase

After dialysis, the salt-eluted protein was concentrated by CM-cellulose (CM-52; strong cation-exchange resin) chromatography. The column (5 × 3.3 cm) was equilibrated with degassed 20 mM McIlvaine's buffer (Na_2HPO_4-citric acid buffer), pH 5.5 (McIlvaine, 1921). Protein was eluted by addition of 20 mM McIlvaine's buffer, pH 5.5, containing 0.5 M NaCl. Elution was monitored spectrophotometrically at a wavelength of 280 nm to detect protein. The CM-cellulose resin had a large protein-binding capacity and could also be used for batch processing.

The eluted protein was concentrated by Filtron ultrafiltration (10-kD Omega membrane, Filtron Technology Corp., Northborough, MA) to a final volume of 5 mL and the sample was applied to a S-200 Sephacryl gel-filtration column (105 × 2.5 cm) equilibrated with degassed 0.1 M sodium acetate buffer, pH 5.0, containing 0.1 M NaCl and 0.02% (w/v) sodium azide. Fractions containing either extensin or extensin peroxidase were monitored spectrophotometrically at a wavelength of 280 nm to detect protein and pooled separately. Four recognized criteria were used to identify extensin, and this has been discussed in our previous paper (Brownleader and Dey, 1993). Extensin eluted earlier than extensin peroxidase and was recognized by the fact that it was cross-linked by extensin peroxidase in the presence of H_2O_2. No other proteins tested, e.g. BSA or aldolase, could be cross-linked. Hyp and Ara contents, amino acid analysis, and the presence of the putative sequence motif -Ser(Hyp)$_4$- in the N-terminal end of the peak fractions reported to be extensin were also used as indicators of the identity of this HRGP. The pooled fractions containing extensin were dialyzed against distilled water and concentrated to a final volume of 3 mL by Filtron ultrafiltration (10-kD Omega membrane). The extensin sample was then freeze-dried. All peaks were assayed for their ability to oxidize guaiacol. Only one peak oxidized guaiacol, and the corresponding fractions containing peroxidase that eluted after extensin were pooled and then dialyzed against water. In addition, this peak absorbed strongly at 405 nm, indicating that the protein component contained a heme group.

The dialyzed fractions corresponding to high A_{405} and containing high guaiacol-oxidizing (2-methoxyphenol) activity were loaded onto a preparative CM-52 ion-exchange column (5 × 3.3 cm) pre-equilibrated with degassed 20 mM McIlvaine's buffer, pH 6.0. The proteins were eluted with a linear 0 to 0.4 M NaCl gradient at a flow rate of 3 mL/min and monitored spectrophometrically at 280 and 405 nm to detect protein and heme, respectively. At this stage, the peak that cross-linked extensin was pooled and dialyzed against water. Two milliliters of this sample were loaded onto an analytical Mono S HR 5/5 FPLC column (0.5 × 5 cm; 1-mL bed volume) that was equilibrated with 20 mM McIlvaine's buffer, pH 5.5. The protein was eluted with a linear 0 to 0.4 M NaCl gradient at a flow rate of 0.4 mL/min. This analytical column produced a better resolution of proteins than the preparative CM-52 cellulose resin.

Routine Assay of Peroxidase Activity

Activity of peroxidase fractions during purification was routinely monitored by oxidation of guaiacol. The assay contained 20 mM potassium phosphate buffer, pH 7.0, 13.3 mM guaiacol, and 33 μM H_2O_2. The reaction was initiated by addition of peroxidase (2 μL) and the color development was monitored over a 2-min time period at 470 nm.

A wide range of low-molecular-weight, nonprotein substrates were tested for the tomato peroxidase preparations, and the rates of reaction were compared with those of HRP

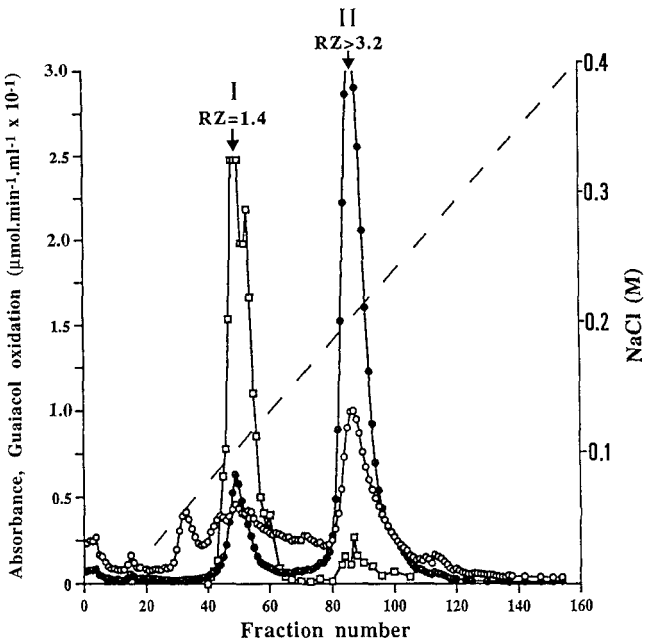

Figure 1. CM-cellulose cation-exchange chromatography of Sephacryl S-200-purified peroxidase showing resolution of peroxidase into forms I and II. ○, A_{280}, indicating protein content. ●, A_{405}, indicating heme content. □, Guaiacol oxidation.

(see "Results and Discussion"). These substrates include a number of naturally occurring low-molecular-weight compounds of plants. There were two objectives: first, to shed light on the possible in vivo functions of the enzymes, and second, to find a more convenient substrate for spectrophotometric assay in order to distinguish between peroxidases that do and do not cross-link extensin. Substrate specificity of peroxidase was monitored with the following low-molecular-weight substrates: 2 mM ABTS (A_{420}), 0.7 mM ascorbic acid (A_{290}), 20 mM catechol (A_{450}), 3.6 mM 4-methyl catechol (100% EtOH, A_{420}), 0.12 mM coniferyl alcohol (1 M NaOH, A_{264}), 0.09 mM ferulic acid (1 M NH_3, A_{287}), 0.5 mM NADH (A_{340}), 13.3 mM guaiacol (A_{470}), 3 mM *p*-phenylenediamine (100% EtOH, A_{487}), 0.1 mM syringaldazine (A_{550}), 0.09 mM syringaldehyde (DMSO, A_{550}), 0.02 mM L-DOPA (0.5 M HCl, A_{310}), 0.2 mM L-Tyr (0.1 M NaOH, A_{290}), 0.2 mM D-Tyr (A_{290}), 0.12 mM 4-aminoantipyrene (A_{380}), 0.06 mM vanillin azine (100% MeOH, A_{470}), 0.16 mM 4-hydroxy-3-methoxybenzyl alcohol (95% EtOH, A_{300}), 0.27 mM 3-hydroxy-4-methoxy benzyl alcohol (100% MeOH, A_{290}), 0.22 mM 4-hydroxy-3-methoxy benzylamine HCl (1 M NaOH, A_{295}), and 0.1 mM L-Tyr-ethyl ester HCl (100% EtOH, A_{240}). Distilled water was the solvent unless stated otherwise in parentheses. The solvent concentration never exceeded 0.2% (v/v) in the assay. The incubation medium contained 0.2 M potassium phosphate buffer, pH 7.0, and 0.6 mM H_2O_2. Reactions were monitored over a 2-min time course at the appropriate wavelength.

Assay of Extensin Cross-Linking by Extensin Peroxidase

Approximately 35 µg of tomato extensin or 53 µg of potato lectin was incubated with 0.2 M sodium phosphate buffer, pH 7.0, in the presence of 75 µM H_2O_2. The reaction was initiated with extensin peroxidase (5 µL, 13 µg) in a total volume of 100 µL. After incubation for 30 min in the dark at room temperature, the incubation mixture was injected onto a Superose-12 gel-filtration FPLC column (Pharmacia, 30 × 1 cm) using a Rheodyne (Cotati, CA) volume injector. The reaction was performed in the dark to prevent decomposition of H_2O_2 by light. The column was equilibrated with 0.2 M sodium phosphate buffer, pH 7.0, containing 0.005% (w/v) sodium azide at a flow rate of 0.2 mL/min, and the eluate was monitored at 280 nm. The lower-molecular-weight extensin monomer eluted slower than extensin cross-linked after incubation with peroxidase and H_2O_2.

SDS-PAGE

Purity and molecular weight of extensin peroxidase were determined by 12% acrylamide SDS-PAGE as described by Laemmli (1970). The calibration markers used for molecular weight determination were α-lactalbumin (M_r 14,000), trypsin inhibitor (M_r 20,000), carbonic anhydrase (M_r 30,000), ovalbumin (M_r 43,000), BSA (M_r 67,000), and phosphorylase *b* (M_r 94,000).

IEF

The pI of extensin peroxidase was established using a Pharmacia Phast gel system (pH 3–9). EPIII and EPIV

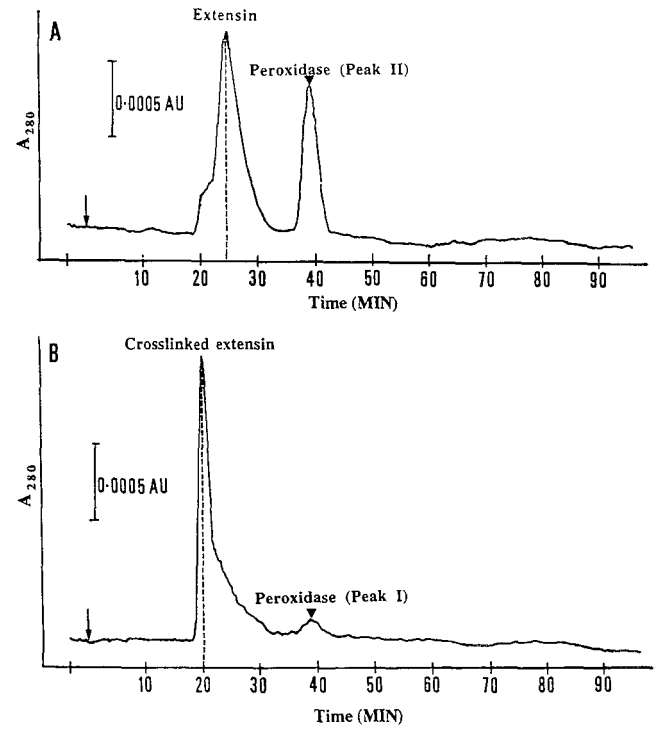

Figure 2. Extensin cross-linking assay in vitro by Superose-12 gel-filtration FPLC using 20 µg of peak II enzyme (A) (see Fig. 1) or 1 µg of peak I enzyme (B), demonstrating that peak I and not peak II constitutes extensin peroxidases. The lower-molecular-weight extensin monomer eluted at a larger retention time than the cross-linked extensin.

(0.15–0.3 μg) were loaded onto a prefocused gel that was developed for 12 min at 2000 V (2.5 mA). The gel was subsequently fixed in 20% TCA for 5 min at 20°C, washed in MeOH:acetic acid (30:10%, v/v) for an additional 2 min at 20°C, stained in 0.02% (w/v) Coomassie brilliant blue R350 (Phast gel Blue R) dissolved in MeOH:acetic acid (30:10%, v/v), and allowed to incorporate 0.1% copper sulfate for 10 min at 50°C. The gel was then destained with MeOH:acetic acid (30:10%, v/v) for an additional 10 min at 50°C. Calibration markers were purchased from Pharmacia and designed for use with pH 3.0 to 9.0 IEF gels.

Protein Determination

Protein concentration was estimated by the dye-binding method of Bradford (1976). BSA (fraction V, essentially fatty acid free) was used as the protein standard.

N-Terminal Amino Acid Sequencing

N-terminal sequencing of the extensin peroxidases was determined by a pulsed-liquid protein sequencer (477A; Applied Biosystems).

Amino Acid Analysis

Samples of the tomato peroxidases were acid hydrolyzed in 6 M HCl at 110°C for 16 h. The acid hydrolysates were analyzed on an LBK Alpha-Plus Analyzer (Pharmacia LKB Biotechnology, St. Albans, UK) using sodium buffers and ninhydrin determination.

Partial Purification of Elicitor

Brewer's yeast extract (100 g), an autolysate of *Saccharomyces cerevisiae*, was dissolved in 200 to 300 mL of distilled water. EtOH was added to a final concentration of 80% (v/v), and the precipitate was allowed to settle for 4 d at 4°C. The supernatant was decanted, and the resultant precipitate was dissolved in 100 mL of distilled water and freeze-dried. This preparation is regarded as the partially purified elicitor.

Cell Elicitation and Extensin Insolubilization

Freeze-dried elicitor was dissolved in distilled water to 20 mg/mL, autoclaved at 121°C for 15 min, and allowed to cool. Hahn and Albersheim (1976) have demonstrated that the crude yeast elicitor is stable at 121°C for 30 min. Elicitor was added at a final concentration of 0.5 mg/mL to tomato cells (estimated 2.5 mg elicitor/g fresh weight cells) and incubated for 1 h on an orbital shaker under light at 100 rpm. Water replaced the elicitor in the control experiment. The tomato cells were washed by Büchner filtration with an excess of distilled water. Water-washed cells (4 g) were eluted with 50 mM $CaCl_2$, pH 3.0 (1 mL), and the eluate recovered by centrifugation. This gentle elution was repeated two more times. The eluate (100 μL) was then coated onto a microtiter plate, diluted 1:1 in coating buffer (1.59 g of Na_2CO_3, 2.93 g of $NaHCO_3$, 0.2 g of NaN_3 in 1 L of distilled water, pH 9.6), and incubated overnight at 4°C. Soluble extensin was measured by ELISA. Cell eluate (100 μL) was assayed for peroxidase activity as before. Surplus filtrate was stored at −20°C.

ELISA

The eluate was coated onto ELISA plates, and after overnight incubation, the microtiter plates were washed twice with PBS, pH 7.4. PBS was prepared as a concentrated stock solution (80 g of NaCl, 2 g of KCl, 11.5 g of Na_2HPO_4, 2 g of KH_2PO_4) and diluted 10 times prior to use. The plates were dried by shaking them on filter paper, and then 100 μL of 1% BSA blocking reagent in PBS was added and incubated at room temperature for 1 h. The plate was then washed with PBS and incubated with primary extensin antiserum (1:20,000 dilution with 1% BSA in PBS) for 1 h. The plate was then washed in PBS to remove the primary antibody. Fifty microliters of secondary antibody (alkaline-phosphatase-labeled goat anti-rabbit IgG) diluted 1:1000 in 1% BSA in PBS, pH 7.4, was pipetted into the wells and incubated for 2 h. The plate was then washed in PBS. Phenolphthalein phosphate substrate (50 μL) was added and incubated for 45 min. Stop solution (185 μL) was then added to stop any further color development, and the plate was read at 570 nm.

Production of Antibodies against Tomato Extensin

Polyclonal antiserum was raised in rabbit to the purified native extensin (Brownleader and Dey, 1993). Extensin (100 μg) was dissolved in 250 μL of PBS (0.15 M NaCl, 20 mM sodium phosphate, pH 7.0), mixed with 250 μL of Freund's complete adjuvant, and injected subcutaneously into a rabbit. Two other injections of the protein were given as above after 4-week intervals. Blood serum was then collected and stored at −70°C. The antiserum had a titer of 1:5600 with 50 ng of extensin.

RESULTS AND DISCUSSION

Purification of Extensin Peroxidase

The activity of extensin peroxidase was assessed by determining the ability of the enzyme to produce the cross-linked high-molecular-weight form of the soluble extensin as the end product when both were incubated in vitro in the presence of H_2O_2. The mild conditions of intact cell elution with $CaCl_2$ (0.05 M) accounted for only 2% of the total protein of the whole-cell homogenate (Everdeen et al., 1988). Therefore, a significant purification of both extensin and extensin peroxidase was achieved by this procedure. The S-200 enzyme preparation was resolved into two active peaks (I and II) by CM-cellulose chromatography (Fig. 1). Although both peaks oxidized guaiacol, only peak I was able to cross-link soluble extensin in vitro and was therefore used as a source of extensin peroxidase for further purification. It is important to note that the RZ value of peak II (3.2) is 2.3-fold higher than the RZ value of peak I. Figure 2 demonstrates that 1 μg of peak I protein was able to cross-link extensin in vitro, whereas 20 μg of peak II had no effect on extensin oligomerization. Peak I was further

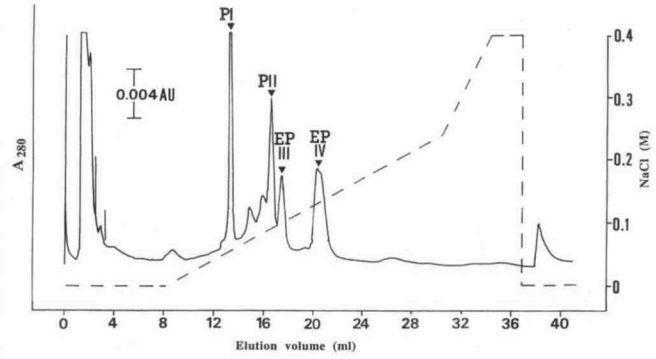

Figure 3. Mono S FPLC of peak I from CM-cellulose column (Fig. 1).

purified by Mono S FPLC. Four main protein peaks were eluted with a NaCl gradient (Fig. 3). All peaks possessed peroxidase activity when assayed with guaiacol. The extensin cross-linking assay demonstrated that PI and PII (up to 20 μg) did not cross-link, whereas EPIII (RZ 1.5) and EPIV (RZ 1.8) (1–9 μg) cross-linked extensin. It is difficult to relate the RZ values of the enzymes to their purity because this estimation depends on the amount and the ionic form of the heme as well as the proportion of the aromatic amino acids in the protein.

All four peaks displayed single protein bands by SDS-PAGE (Fig. 4A). The molecular masses estimated for EPIII and EPIV were 37 and 34 kD, respectively (Fig. 4B). These values coincide well with the molecular masses determined by gel filtration (results not shown). Therefore, EPIII and EPIV are not multisubunit proteins.

Partial Characterization of Extensin Peroxidase Isoforms

The pI values of both EPIII and EPIV were approximately 9.0 (results not shown), which confirms that these are basic peroxidases and is consistent with the ionic binding of the enzymes to cationic column media (Figs. 1 and 3). The N termini of the isozymes were found to be blocked when subjected to amino acid sequencing. This is typical of many peroxidases. The amino acid compositions of EPIII and EPIV were similar (Table I); they lacked Cys (no detectable cysteic acid after acid hydrolysis in 6 N HCl at 110°C for 24 h), which is unusual when compared with the published results of a number of cationic peroxidases (Table II). Another notable feature of the two enzyme forms is the lower levels of Asp/Asn and higher levels of Glu/Gln. These amino acid residues together with Arg and His are considered to be important in catalysis within the plant peroxidase superfamily (Welinder and Gajhede, 1993). The conserved residues in this family are Gly, Pro, Asp, and Arg, and these contribute to the required protein backbone conformation. Extensin peroxidases also appear to be rich in Gly (12.7–14.6 mol%) in contrast to other cationic peroxidases, except barley peroxidase P8.5 (Kerby and Sommerville, 1992), which had an average Gly content of 3 to 4 mol%. The overall amino acid composition of the two enzymes (EPIII and EPIV), except for Cys, resembled more closely that of the barley peroxidase P8.5 than those of other cationic peroxidases from horseradish or peanut (Table II).

Substrate specificity of EPIII and EPIV demonstrated that only soluble tomato extensin can be cross-linked. Other proteins eluted from tomato cells and fractionated by Sephacryl S-200 gel filtration could not be cross-linked (the negative results are not presented in this paper). Some marker proteins such as BSA (a glycoprotein), aldolase, and insulin were also not cross-linked. However, potato lectin, an HRGP (Allen and Neuberger, 1973; Allen et al., 1978; Matsumoto et al., 1983) that is structurally related to extensin, was cross-linked by both extensin peroxidases (Fig. 5). The lectin protein has two domains: a Ser/Hyp glycoprotein domain, which has a strong resemblance to extensin, and a Cys-rich, nonglycosylated domain. Extensins are devoid of Cys (Lamport, 1980) and because the Ser/Hyp glycoprotein domain of the lectin resembles extensin, it is likely to contain the cross-linking site. Exhaustive pronase treatment of potato lectin following reductive alkylation was shown to release a M_r 33,000 glycopeptide that is rich in Ser and Hyp but not in Gly or Cys (Allen et al., 1978). It is interesting that 33 kD is the same size as the HRGP reputedly insolubilized in soybean cell wall after fungal challenge (Bradley et al., 1992). The nature of the linkage involved in the cross-linking process has not yet been identified. Although the existence of intrapeptide isodity-

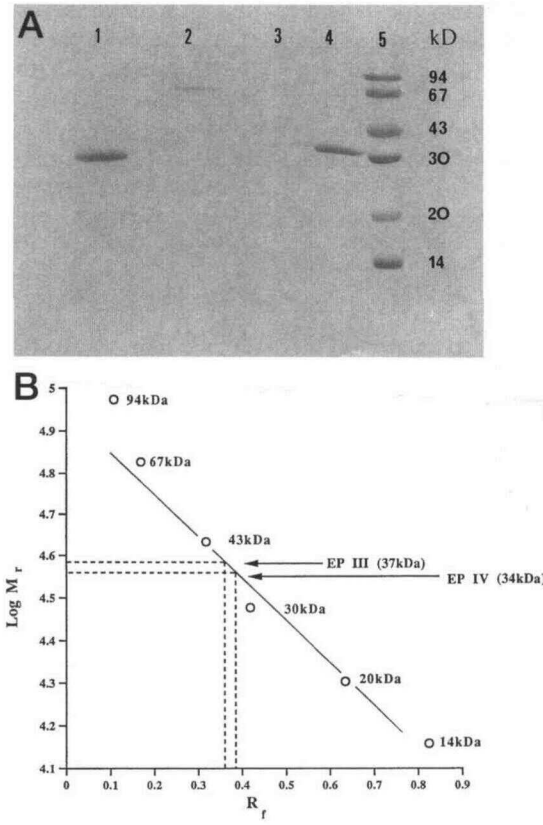

Figure 4. SDS-PAGE of Mono S FPLC-resolved extensin peroxidases. A, Lane 1, 3.7 μg of PI; lane 2, 1.5 μg of PII; lane 3, 0.6 μg of EPIII; lane 4, 3.7 μg of EPIV; lane 5, molecular mass markers. B, The standard curve derived from the marker proteins.

Table I. *Amino acid analysis of Mono S-purified peroxidases from suspension-cultured tomato cells*

Amino Acid	Mono S PI	Mono S PII	Mono S EPIII	Mono S EPIV
		mol %		
Asp/Asn	15.1	11.9	10.6	10.1
Thr	7.6	7.0	5.8	5.2
Ser	7.8	7.4	5.9	8.7
Glu/Gln	7.7	11.1	12.8	10.0
Pro	4.1	5.3	4.7	8.7
Gly	8.4	11.4	12.7	14.6
Ala	12.9	6.5	12.0	8.5
Cys	1.7	1.1	ND	ND
Val	5.2	7.0	7.8	6.5
Met	ND[a]	ND	1.0	N
Ile	5.3	5.7	5.7	3.8
Leu	8.7	8.9	8.8	6.4
Tyr	ND	0.25	2.7	1.8
Phe	4.3	3.7	4.2	3.8
His	1.3	1.7	1.9	1.7
Lys	2.2	4.8	7.3	6.2
Arg	7.9	4.9	3.4	3.9
Trp	NA[b]	NA	NA	NA

[a] ND, Not detected. [b] NA, Not assayed.

rosine linkages have been known to exist in cell-wall insoluble extensin (Fry, 1983), such linkages have not been reported between extensin molecules. Comparison of the nature of in vitro cross-linking of extensin with the in vivo process that occurs in the cell wall must await further structural analyses, which are in progress in our laboratories. Proteolytic digestion of extensin and further analysis of the proteolytic fragments will allow us to determine the in vitro cross-linking site of extensin before establishing whether the same type of cross-link exists in insoluble cell-wall extensin. However, with respect to the in vivo role of the extensin peroxidase, it is noteworthy that both soluble extensin and the enzyme are bound to the cell wall, which is also the site of extensin insolubilization.

Table III clearly demonstrates that the four forms of the tomato peroxidase can be distinguished from HRPs by their total inability to act upon NADH. EPIII and EPIV can be distinguished from the non-cross-linking forms, PI and PII, by the high rates obtained with ascorbic acid, ferulic acid, and guaiacol. EPIV shows higher rates than EPIII with 4-methyl catechol, guaiacol, and syringaladazine. It is not clear, for example, why EPIII and EPIV display a preference for 4-methyl catechol rather than catechol. The methyl group does not appear to alter the chemical properties of the catechol significantly except to increase slightly the hydrophobicity. The addition of the methyl group may enhance a possible hydrophobic interaction with the active site. Other Mono S-resolved isoforms also show a considerable degree of preference for 4-methyl catechol compared with HRPs. Sterjiades et al. (1993) reported the specific activity of HRP (Sigma P-8250, containing at least five isoforms) using catechol and 4-methyl catechol as 122 and 1248 units/mg, respectively; the values for *Acer* acidic peroxidase with the two substrates were 0.18 and 89.6, respectively. Subtle alterations in the structure of phenolic substrates appear to have a marked effect on peroxidase catalytic activity. Guaiacol appears to be a better substrate than *p*-phenylenediamine. Experiments identifying the exact structural requirements of substrates for EPIII and EPIV would be interesting. EPIII can also be distinguished from EPIV because the former reacts with considerably higher rates with ABTS, ascorbic acid, coniferyl alcohol, and ferulic acid. Zimmerlin et al. (1994) have purified a cationic peroxidase from French bean cell wall that can yield dehydrogenation oligomers of ferulic acid. Coumaric acid and

Table II. *Published amino acid analysis of cationic peroxidases*

Amino Acid	P8.5[a]	Cationic[b] HRP	HRP B[c]	HRP C[c]	Peanut Peroxidase[d]
Asp/Asn	ND	15.4	15.5	14.5	15.9
Thr	9.2	7.7	6.6	5.8	7.5
Ser	11.3	8.4	5.0	4.7	6.5
Glu/Gln	ND	6.4	6.6	6.2	6.9
Pro	5.1	5.3	4.2	4.0	4.7
Gly	10.0	5.3	2.5	2.2	4.7
Ala	9.2	7.2	4.4	4.0	7.4
Cys	2.7	2.5	0.9	0.9	2.8
Val	7.3	6.5	4.4	4.1	5.4
Met	1.1	1.9	1.0	0.9	0.8
Ile	3.4	4.0	3.7	3.6	3.9
Leu	7.7	11.5	10.9	10.1	9.5
Tyr	1.0	1.6	1.8	2.0	2.1
Phe	3.3	6.5	8.8	8.3	8.9
His	1.4	1.2	1.1	1.0	2.5
Lys	4.2	1.9	1.8	1.8	3.3
Arg	4.0	6.5	8.5	8.2	6.2
Trp	ND	0.3	ND	ND	ND

[a] Cationic extracellular barley peroxidase (Kerby and Sommerville, 1991). [b] Cationic horseradish peroxidase C1 (Mazza and Welinder, 1980). [c] Cationic horseradish peroxidase B and C (Shannon et al., 1966). [d] Cationic peanut peroxidase (van Huystee and Maldonado, 1982).

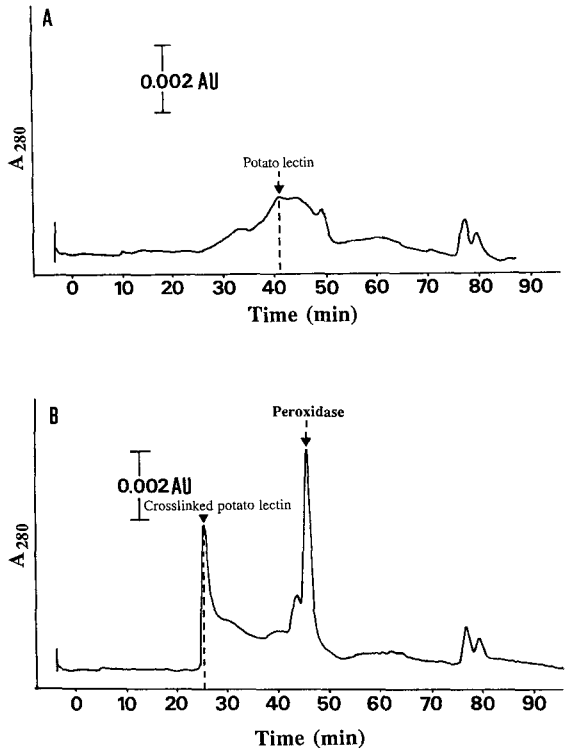

Figure 5. Assay of cross-linking of potato lectin by S-200-purified extensin peroxidase preparation using a Superose-12 FPLC column. The absence of a sharp, defined peak of potato lectin in A is due to aggregation into higher-order oligomeric forms.

ferulic acid have been reported to be linked to Gal and Ara moieties of plant cell-wall pectic polysaccharides, and they are suggested to be the cross-linking points between the pectic neutral side chains (Bolwell, 1993). It is possible that such feruloyl and coumaroyl residues exist in extensin linked to the monosaccharides of this glycoprotein and that these are involved in interpeptide linkages that are formed during the cross-linking process catalyzed by extensin peroxidase.

A number of phenolic compounds (Table IV) were also tested as substrates in an attempt to distinguish the tomato peroxidases from those of acidic and basic HRPs. The tomato enzyme preparation at the Sephacryl S-200 step was used to obtain preliminary results. The rates obtained with the tomato preparation are in general different from those of horseradish acidic enzymes (Table IV). Although some rates obtained with our preparation are comparable to those of the basic HRPs, those with 2- and 4-hydroxy-3-methoxy-benzylalcohol, vanillin azine, and 4-aminoantipyrine are much lower than with HRP X, a basic peroxidase. Therefore, this investigation suggests that there are four phenolic substrates that can be used to distinguish spectrophotometrically between peroxidases from the two sources.

In Vivo Cross-Linking of Extensin

The results presented in the previous section refer to in vitro cross-linking of cell-wall-derived soluble extensin. To demonstrate that extensin is insolubilized in the intact cell wall, this phenomenon was stimulated in suspension-cultured tomato cells with a biotic elicitor. Insolubilization was measured by a decrease in salt-elutibility of extensin. The eluted extensin was quantitated by ELISA assay using polyclonal antiserum raised against tomato extensin.

Cultured tomato cells challenged for 1 h with a partially purified elicitor (see "Materials and Methods") from Brewer's yeast (*S. cerevisiae*) showed an overall increase in peroxidase activity by 20% (data not shown) and in extensin insolubilization by up to 30% relative to the control (no elicitor) (Fig. 6). At the present time, we can only speculate that an increase in extensin peroxidase activity contributes to this 20% increase in total peroxidase activity. A serial dilution of salt-eluted protein on the ELISA plate was performed (a) because the salt-eluted protein was in 50 mM $CaCl_2$, pH 3.0, and not in coating buffer (pH 9.2), which is intended to enhance binding of the protein to the ELISA plate, and (b) to ensure that the elicitation can be observed over a broad concentration range of cell-eluted protein. A standard curve generated for extensin concentration was not linear, and it would prove difficult to express the

Table III. *Substrate specificity of peroxidases isolated from tomato suspension-cultured cells and HRPs.*

A comparison of the ionic forms of tomato peroxidase with those of HRP. Values are expressed as μmol min^{-1} mg^{-1} protein under the assay conditions described in "Materials and Methods." HRP VII and HRP VIII are acidic peroxidases, and HRP IX and HRP X are basic peroxidases.

Substrate	HRP VII	HRP VIII	HRP IX	HRP X	CM-Cellulose Peak II	CM-Cellulose Peak I			
						Mono S PI	Mono S PII	Mono S EPIII	Mono S EPIV
ABTS	241	132	74	251	8	216	33	1043	295
Ascorbic acid	502	330	297	1214	0.0	27	21	281	57
Catechol	140	285	445	1424	7	718	6	267	342
4-Methyl catechol	297	108	723	1265	11	1396	48	1209	3108
Coniferyl alcohol	84	182	26	56	2	41	2	76	2
Ferulic acid	50	17	28	59	5	4	6	978	513
NADH	363	30	204	628	1	0	0	0	0
Guaiacol	49	143	222	649	1	132	66	486	665
p-Phenylenediamine	263	305	384	796	7	472	32	184	143
Syringaldazine	2	18	15	113	1	67	33	62	472
Syringaldehyde	0	0	0	0.0	0	0	0	0	0

Table IV. *Substrate specificity of peroxidase isolated from tomato suspension-cultured cells and HRPs.*

A comparison of the unresolved forms of tomato peroxidase with those of HRP. Values are expressed as $\Delta A\ min^{-1}\ mg^{-1}$ protein under the assay conditions described in "Materials and Methods."

HRP VII and HRP VIII are acidic peroxidases, and HRP IX and HRP X are basic peroxidases.

Substrate	HRP VII	HRP VIII	HRP IX	HRP X	S-200 Peroxidase
4-Hydroxy-3-methoxy-benzylamine	11	38	47	198	117
3-Hydroxy-4-methoxy-benzyl alcohol	8	3	4	269	27
4-Hydroxy-3-methoxy-benzyl alcohol	47	44	72	229	85
L-Tyr ethyl ester	20	3	8	8	7
L-Tyr	5	2	1	4	2
D-Tyr	1	1	1	1	1
L-DOPA[a]	15	93	9	9	13
Vanillin azine	381	696	453	563	165
4-Aminoantipyrene	9	76	11	318	3

[a] Under identical conditions, phenolase and tyrosinase oxidized L-DOPA with rates of 1 and 87 $\Delta A\ min^{-1}\ mg^{-1}$, respectively.

change in *A* of salt-eluted protein in microgram amounts of extensin. This is particularly true if one considers that the antiserum is polyclonal and may cross-react with other HRGPs present in the salt-eluate. The response to the elicitor over the short period of 1 h of incubation suggests that the process is not likely to involve de novo protein synthesis.

Extensin insolubilization is a recognized plant defense response (Esquerré-Tugayé et al., 1979; Lamb et al., 1993). Several researchers have stimulated plant defense events such as phytoalexin accumulation, ethylene biosynthesis, and Phe-ammonia lyase activity with elicitor derived from yeast (Hahn and Albersheim, 1978; Basse and Boller, 1992). In this context, it is important to note the distinction between the peroxidases of the pathogen and those of the host plant and that the pathogen peroxidases may contribute to the increased activites of total peroxidases in disease resistance. This prompted us to use a yeast elicitor, devoid of peroxidases, to investigate peroxidase-mediated cell-wall extensin deposition. Our results showing a rapid in vivo insolubilization of extensin may indicate a rapid generation of signals at the infection site that may act as a primary defense barrier to the invading pathogen. Modulation of extensin peroxidase activity could occur by altering the local levels of H_2O_2 or extensin, modulating the amount of a specific inhibitor of extensin cross-linking (Brownleader et al., 1993), or creating a more favorable pH for extensin cross-linking.

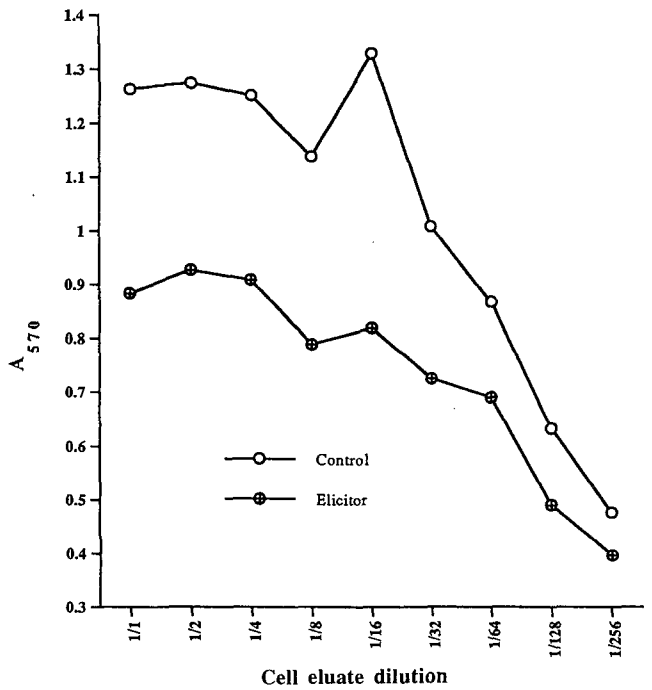

Figure 6. Effect of yeast elicitor on extensin insolubilization. Control represents nonelicited cells. Soluble extensin in the cell eluates was assayed by ELISA.

ACKNOWLEDGMENTS

We thank Mr. Ian Moss (Charing Cross and Westminster Medical School) for performing the amino acid analysis and Mrs. Margaret Pickering (Royal Holloway, University of London) for performing the peptide sequencing.

Received June 7, 1995; accepted August 23, 1995.

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