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ELICITOR-INDUCED EXTENSIN INSOLUBILIZATION IN SUSPENSION-CULTURED TOMATO CELLS

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato; elicitor; extensin; peroxidase; cell wall.

Abstract—Insolubilization of cell wall hydroxyproline-rich glycoproteins is a common plant defence response when plants are challenged with either pathogens or elicitors derived from pathogens. A yeast elicitor that insolubilized cell wall extensin of suspension-cultured tomato cells was purified by 80% ethanol precipitation of yeast extract followed by anion-exchange chromatography, gel-filtration chromatography and reverse phase HPLC. MALDI/TOF-mass spectrum analysis of the purified elicitor preparation generated one major peak at 410±1 Da. Elicitor activity was inactivated at 100° for 10 min or after incubation with pronase. No loss of elicitor activity was observed after periodate treatment. Polyclonal antiserum raised against native tomato extensin was used to demonstrate extensin insolubilization *in vivo*. Salt-elutability of extensin from tomato cells was dependent upon concentration and time of incubation of the cells with elicitor. SDS-PAGE/western blotting of salt-eluted protein from elicited cells also demonstrated insolubilization of HRGPs in the cell wall. The elicitor was found to stimulate cell wall peroxidase activity and extensin insolubilization in isolated tomato cell walls and in intact cells. The elicitor also induced a transient oxidative burst which began after 5 min and was maximal after 20 min incubation with the cells. Other typical plant defence responses known to be triggered by elicitors, such as changes in H^+, K^+ or Ca^{2+} fluxes, enhanced activities of lipoxygenase, phenylalanine ammonia lyase and superoxide dismutase, were not observed when tomato cells were challenged with this elicitor. The Ca^{2+} channel blocker verapamil did not prevent elicitor-induced extensin insolubilization in whole cells. This study suggests that biochemical events, such as transient oxidative burst and enhanced peroxidase activity, which accompany extensin insolubilization involve the cell wall without any apparent plasma membrane participation. The above cell wall events may be sufficient to form the early plant defence responses to microbial challenge.

INTRODUCTION

Plants respond to pathogens and elicitors with a variety of defence responses such as cell wall hydroxyproline-rich glycoproteins (HRGP) insolubilization [1–4]. Several exogenous elicitors such as fusicoccin, derived from *Fussicoccum amygdali* [5], syringomycin, derived from *Pseudomonas syringae* [6] and elicitor preparations from suspension-cultured tomato cells infected with *Cladosporium fulvum* [7], stimulate plasma membrane H^+-ATPase activity. Elicitors from a variety of sources also influence H^+, K^+ and Ca^{2+} fluxes [8–13]. Several researchers have shown that fungal and bacterial elicitors stimulate H_2O_2 accumulation [8, 14–19] and superoxide radical formation in plants [8, 15, 17, 19]. Mader *et al.* [20] observed that some cell wall peroxidases catalyse the formation of H_2O_2. Fungal elicitors stimulate tomato lipoxygenase activity [21] and yeast-derived elicitors have been found to increase phenylalanine ammonia-lyase (PAL) activity in tomato [22]. Vera-Estrella *et al.* [9] found enhanced ferricyanide reduction, NADH oxidase, NADH-dependent cytochrome c reductase and ascorbate oxidase activities in tomato plasma membrane vesicles challenged with a fungal elicitor preparation. In this report, we have purified and par-

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tially characterised a yeast-derived elicitor of extensin insolubilization in the cell wall and investigated whether rapid cell wall or plasma membrane-mediated defence responses are associated with tomato cell wall extensin deposition.

RESULTS AND DISCUSSION

The ability of the elicitor to induce insolubilization of the soluble form of extensin in the cell wall of suspension-cultured tomato cells was measured as a decrease in elutability of this HRGP by $CaCl_2$. The $CaCl_2$-solubilized extensin was measured by ELISA (enzyme-linked immunosorbent assay) using polyclonal antibody against extensin. A crude elicitor preparation was obtained by ethanol-precipitation (80%, v/v) of water-dissolved yeast extract (Oxoid code L. 21). Basse and Boller [22] and Hahn and Albersheim [23] demonstrated that yeast extract was a convenient source of elicitors of plant stress responses. The elicitor was further purified by Amberlite anionic exchange chromatography. The bound fractions, eluted with NaCl, displayed elicitor activity. The concentrated pool of fractions were resolved into three A_{280}-absorbing peaks by Sephacryl S-200 gel-filtration chromatography (Fig. 1). Only peak II retained elicitor activity. This peak was further resolved by C_{18} reverse phase HPLC into three banded regions (A, B and C) as shown in Fig. 2. Fraction A and B exhibited elicitor activity. Band C displayed weak elicitor activity. As peak B was the most effective elicitor, it was chosen as a source of the purified elicitor preparation and used in all elicitation experiments to trigger extensin insolubilization in suspension-cultured tomato cells and to begin to unravel those biochemical processes that signal this cell wall event.

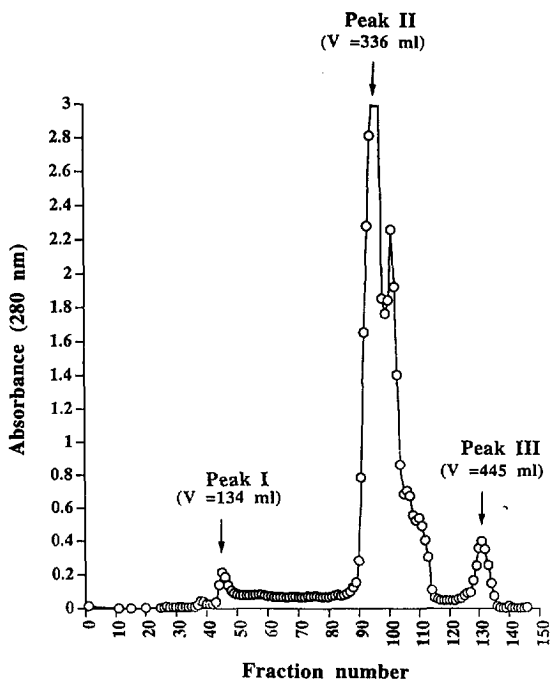

Fig. 1. Sephacryl S-200 gel-filtration showing further resolution of Amberlite-purified elicitor. V_0, void volume. Only peak II has elicitor activity.

Matrix-assisted laser desorption ionization/time of flight (MALDI/TOF)-mass spectrum analysis of this fraction generated one major peak at m/z 410 (Fig. 3). This represents the protonated molecular ion (see Experimental) which would indicate a M_r for the elicitor of 409 ± 1. Other peaks at m/z 177, 198 and 303 are derived from the gentisic acid matrix used in the analysis. Amino acid analysis showed that this elicitor fraction was very rich in Glu/Gln (49 mol%) Asp/Asn (20 mol%) and intermediate levels of Gly (10 mol%) and Phe (9 mol%) (Fig. 3). The predominant amino acids Glu/Gln, Asp/Asn, Gly and Phe are therefore predicted to exist in a ratio of 5:2:1:1. N-terminal amino acid sequencing of the purified elicitor (peak B; Fig. 2) found that its N-terminus was blocked.

Biological activity of elicitors from different sources are known to have different sensitivities to heat, pronase and periodate treatments: a fungal elicitor preparation of casbene production in castor bean was found to be heat labile, protease and periodate sensitive [24]. However, yeast-derived elicitors of ethylene biosynthesis in tomato were heat-stable but pronase and periodate-sensitive [22]. Our elicitor preparation was found to be heat-labile and protease sensitive but insensitive to periodate treatment, suggesting that the preparation is different to the partially-purified yeast elicitor reported by Basse and Boller [22]. These researchers estimated a mean M_r for the elicitor-active glycopeptides to be 2500 by only BioGel P-4 gel-filtration chromatography. No other corroborative analysis was undertaken to reduce the anomalous shape-dependent behaviour of glycopeptides relative to linear and acid-hydrolysed oligomeric α-1,4-glucan standards.

The activity of the elicitor was found to be dependent upon its concentration in the cell incubation (Fig. 4). It is also important that all experimental conditions in the ELISA assay, such as cell eluate dilution, are defined when elicitor activity is monitored. Up to 75 and 50% insolubilization of extensin was observed when tomato cells were incubated with 0.2 mg and 0.01 mg elicitor/g fresh weight (fr. wt) cells above 1/512 cell eluate dilution, respectively. Complete insolubilization of extensin at 1/64 dilution of cell eluate over a broad concentration profile of elicitor [up to 0.2 mg/g fr. wt cells; Fig. 4 (inset)] was not observed in 1 hr suggesting that either the cell eluate comprised an appreciable amount of antibody-cross-reactive non-extensin protein or that extensin insolubilization was not complete within the time span. For the ELISA assay, a serial dilution off salt-eluted protein applied on the ELISA plate was performed (a) because the salt-eluted protein was in 50 mM $CaCl_2$, pH 3.0, and not in the coating buffer (pH 9.2) which is intended to enhance binding of the protein to the ELISA plate and (b) to ensure that elicitation can be observed over a broad concentration range of cell-eluted protein.

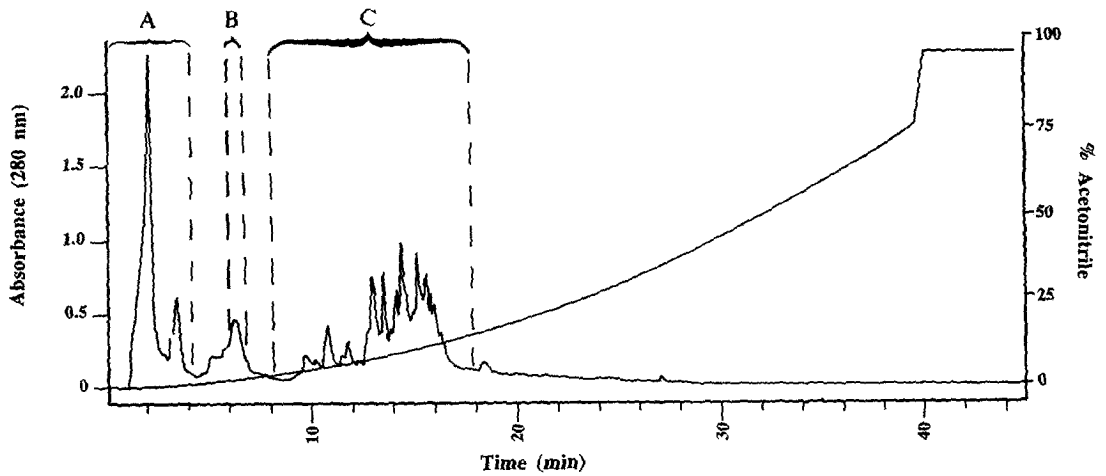

Fig. 2. C_{18}-reverse phase HPLC profile of Sephacryl S-200 resolved elicitor. The resolved bands A, B, and C were pooled separately and tested for elicitor activity.

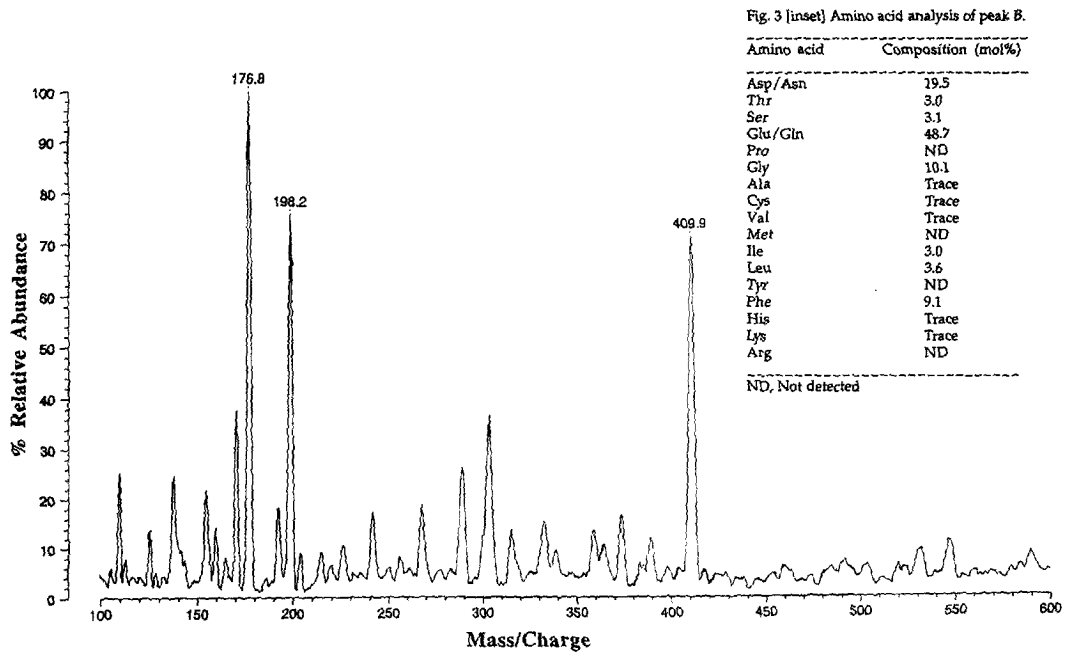

Fig. 3 [inset] Amino acid analysis of peak B.

Amino acid	Composition (mol%)
Asp/Asn	19.5
Thr	3.0
Ser	3.1
Glu/Gln	48.7
Pro	ND
Gly	10.1
Ala	Trace
Cys	Trace
Val	Trace
Met	ND
Ile	3.0
Leu	3.6
Tyr	ND
Phe	9.1
His	Trace
Lys	Trace
Arg	ND

ND, Not detected

Fig. 3. MALDI/TOF-mass spectrum and amino acid analysis [inset] of HPLC-resolved elicitor peak (B). For MALDI/TOF mass spectrometry, peak B was mixed with gentisic acid matrix.

Consistent with incomplete extensin insolubilization shown in the elicitor concentration dependence profile (Fig. 4), only 55% insolubilization of extensin was observed after 60 min of elicitation (Fig. 5). However, such a rapid response (within 1 hr) would suggest that extensin insolubilization at the cell wall constitutes a rapid plant defence response which is independent of *de novo* protein synthesis and subsequent secretion into the cell wall when plants are challenged with an elicitor. Bradley *et al.* [3] found a rapid increase in oxidative cross-linking of a 33 kDa HRGP, measured by the disappearance of a SDS-extractable, immunoreactive protein, when bean or soybean cells were extracted following treatment with fungal elicitors or glutathione. The cross-linking was complete within 10 min under optimal conditions with glutathione, and 20 min after treatment with fungal elicitor. We have demonstrated a decrease in three main immunoreactive proteins (20–45 kDa) from the $CaCl_2$-cell eluate following elicitor application for 1 hr (Fig. 6). The M_r of these proteins did not coincide with the known M_r of native tomato extensin and may therefore represent other HRGPs insolubilized in the cell wall. However, the intensity of one predominant protein band increased (Fig. 6). Elicitation experiments were also carried out with isolated cell walls (1 hr incubation) which showed a 15% decrease in the $CaCl_2$-elutable extensin relative to non-elicited cell

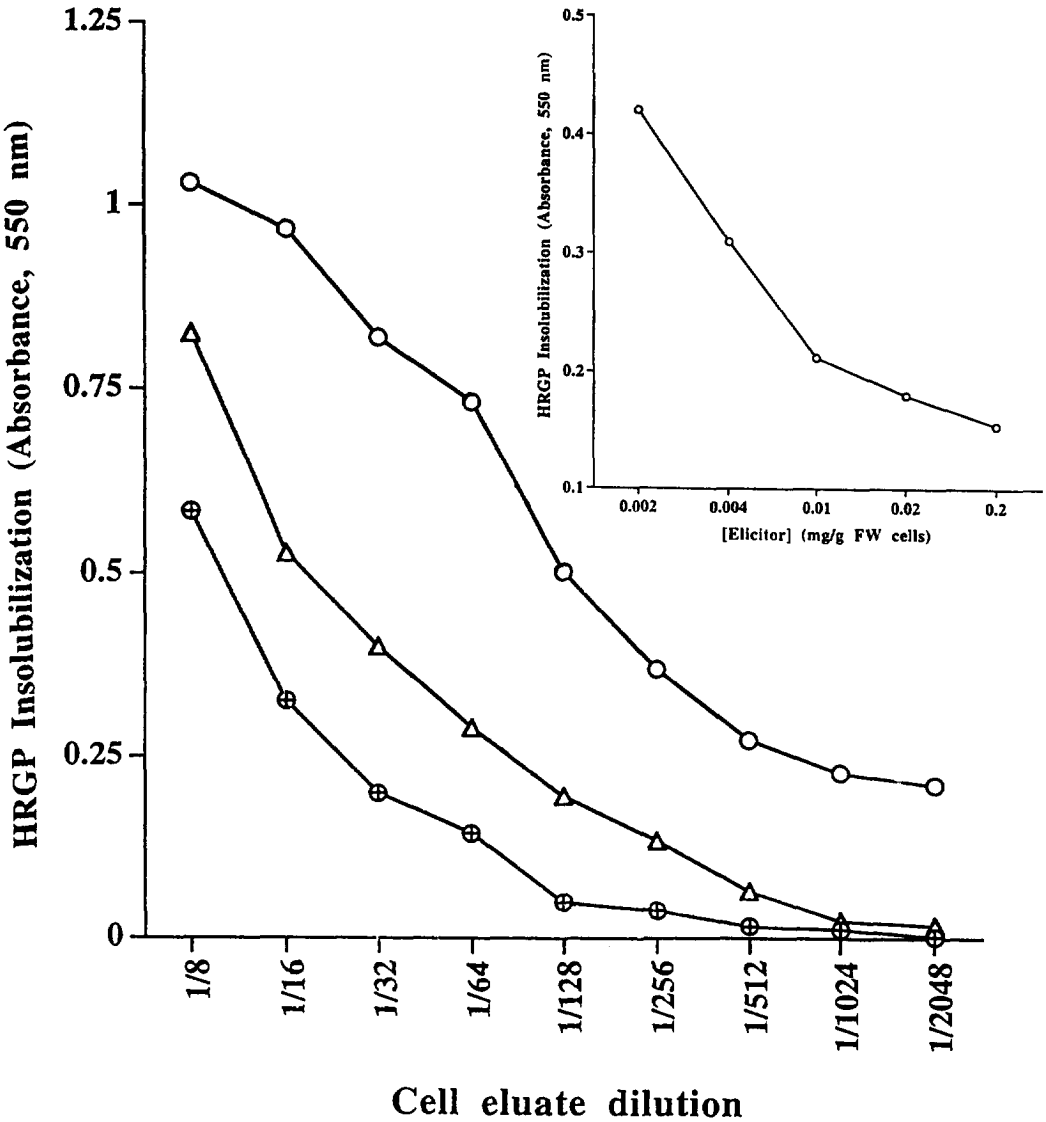

Fig. 4. Effect of elicitor concentration on insolubilization of extensin by tomato cells. Elicitor was incubated with 1 g cells for 1 hr at 25°. $CaCl_2$-cell eluate was assayed by ELISA using polyclonal antibody against extensin (see Experimental section). The assays were carried out at varying antigen dilutions. ○, control; ⊕, 0.2 mg elicitor/g fr. wt cell; △, 0.01 mg elicitor/g fr. wt cell. In Fig. 4 [inset], the elicitor concentration dependence of extensin insolubilization at 1/64 cell eluate dilution was performed with up to 0.2 mg/g fr. wt cells over 1 hr at 25°. The decrease in *A* at 550 nm indicates greater HRGP insolubilization.

walls. There was also an almost 20% increase in peroxidase activity of salt-eluates from isolated cell walls and intact cells relative to the control. These results suggest that the cell wall contains the necessary components for inducing rapid peroxidase-catalysed extensin insolubilization.

Treatment of tomato cells with the elicitor caused a transient oxidative burst (1.42 mM H_2O_2 equivalents), measured by luminol-mediated chemiluminescence, which began after 5 min of elicitation and peaked at 20 min (Fig. 7). This transient nature suggests a possible role for a number of reactive oxygen scavenging enzymes such as superoxide dismutase, catalase or peroxidase associated with the elicitation process. In order to determine whether or not an increase in H_2O_2 scavenging activity could abolish elicited extensin insolubilization, 0.1 μg/ml catalase was added to the elicitation assay. This enzyme did not show any inhibition of extensin insolubilization suggesting that extensin peroxidase may not be specific for H_2O_2 and that other organic peroxides may act as substrates for extensin cross-linking activity. Robertson [25] demonstrated that French bean cells exposed to an elicitor preparation from *Colletotrichum lindemuthianum* produced a transient burst of active oxygen species, equivalent to 120 μM H_2O_2 that began after 3 min and peaked at 10 min. They argued that the active oxygen species leads to a transient oxidative stress involved in oxidative metabolism, observed as a decrease in adenosine-5′-triphosphate (ATP) and $NADH/NAD^+$

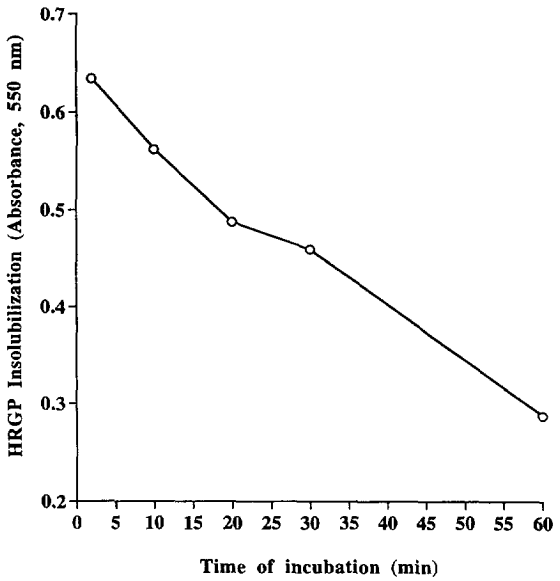

Fig. 5. Time-dependence of elicitor-induced extensin insolubilization with 1/16 cell eluate dilution following addition of 0.2 mg elicitor/g fr. wt cells over 1 hr. The decrease in *A* at 550 nm indicates greater HRGP insolubilization.

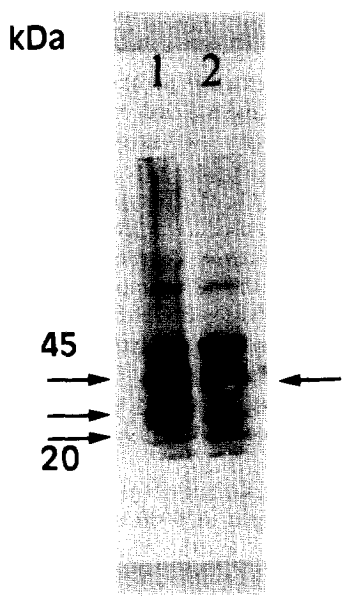

Fig. 6. SDS-PAGE western blot of salt-eluted protein after elicitation showing that three major bands decrease (shown by the arrows on the left of the gel) and one major band increases (shown by the arrow on the right of the gel) relative to the control. Lane 1, control cells; lane 2, elicited cells.

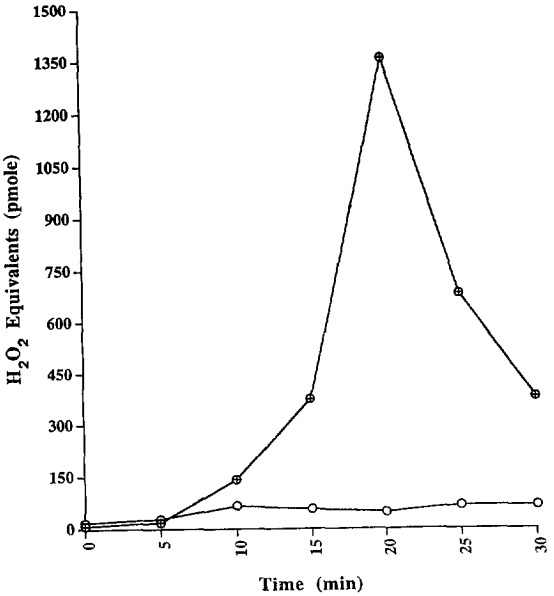

Fig. 7. Time-course for elicitor-induced oxidative burst after addition of elicitor. ○, control; ⊕, 0.2 mg elicitor/g fr. wt cell. Assay volume, 950 μl. See Experimental for details.

levels. Previous work has also demonstrated that H_2O_2 stimulated phytoalexin production by potato tuber slices [19], acts as a trigger for programmed cell death during the incompatible interaction and as a diffusible signal for inducing cellular protectant genes such as glutathione-*S*-transferase in adjacent cells [26]. Bradley *et al.* [3] demonstrated that H_2O_2 mediates a rapid insolubilization of cell wall structural proteins in suspension-cultured soybean and bean cells incubated with fungal elicitor within 10 min.

Extensin insolubilization begins almost immediately after challenging tomato cells with elicitor (Fig. 5). It is possible that small local increases in H_2O_2 production at discrete cross-linking sites in the cell wall are not detected by the chemiluminescence method for measuring an oxidative burst. Extensin insolubilization proceeds almost linearly throughout the first 60 min after elicitation, whereas production of activated oxygen species (peroxides, H_2O_2, superoxide and hydroxyl radicals) peaks after 20 min (1.42 mM H_2O_2 equivalents) and drops to 470 μM after 30 min. Everdeen *et al.* [27] found that 10–50 μM H_2O_2 was optimal for extensin cross-linking *in vitro*. Although, H_2O_2/other peroxide levels may only be a component of the observed oxidative burst, these levels would probably be sufficient for extensin insolubilization. The remaining components of the oxidative burst would be involved in the large number of activities described here [3, 19, 25, 26] and in other scientific papers.

Numerous reports have described elicitor-induced alterations in ion fluxes [7, 8, 10, 11, 12] which would suggest that altered transmembrane ion flux is associated with plant defence responses against elicitors. Our elicitor preparation did not alter H^+, K^+ or Ca^{2+} fluxes during the first 30 min incubation with the cells. In case the observed ineffectiveness of our elicitor on ion fluxes was due to changes being too small and localized to measure, plasma membrane-enriched vesicles were isolated by differential and sucrose density gradient centrifugation and the effect of the elicitor upon the plasma membrane H^+–ATPase and Ca^{2+}–ATPase activity was examined as described by Williams *et al.* [28]. Phosphohydrolase activity of the iso-

lated membranes were inhibited 50% by 100 μM vanadate suggesting that 50% of the membrane vesicles were derived from the plasma membrane. In agreement with whole cell studies, the elicitor had no observed effect upon the vanadate-sensitive plasma membrane H^+–ATPase and Ca^{2+}–ATPase activities. However, it is possible that these biological responses to the elicitor are coupled to a membrane receptor analogous to the fusicoccin receptor, which may have been lost during membrane isolation [5]. Similarly, cryptogein which causes an increase in extracellular pH in cultured tobacco cells does not inhibit the activity of the purified tomato plasma membrane H^+–ATPase suggesting that a cryptogein-binding site is lost during membrane isolation [12]. However, Vera-Estrella *et al.* [7] suggest that a tightly-bound membrane-associated elicitor binding site exists and they demonstrated induction of H^+ extrusion in whole cells and stimulation of plasma membrane H^+–ATPase activity in enriched tomato plasma membrane vesicles by a fungal elicitor. In order to address this possibility, plasma membranes were isolated from elicited and non-elicited tomato cells. However, no difference in specific ATPase activity was observed indicating that the plasma membrane H^+–ATPase was not involved in cell wall extensin insolubilization. In order to determine whether or not Ca^{2+} channels were involved in elicitor-induced extensin insolubilization, the Ca^{2+} channel blockers $LaCl_3$ and verapamil were included in the extensin insolubilization assay. However, we found that 100 μM $LaCl_3$ itself caused extensin insolubilization. Verapamil (100 μM) did not inhibit elicitor-mediated extensin insolubilization suggesting that Ca^{2+} channels are not involved in extensin insolubilization. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)/western blotting of verapamil and $LaCl_3$-incubated cells did not reveal any loss/gain of proteins immunoreactive to extensin polyclonal antisera (results not included).

The lack of any demonstrable effect on lipoxygenase, NADH oxidase, superoxide dismutase (SOD), malate dehydrogenase or ascorbate oxidase activities during the first 30 min following elicitation and PAL activity over a 24 hr incubation period suggests that these activities are not associated with rapid extensin insolubilization or the oxidative burst in our experiments.

In conclusion, rapid extensin insolubilization, a large oxidative burst and cell wall peroxidase activity were stimulated when isolated tomato cell walls were incubated with elicitor, suggesting that the cell wall itself can perceive and respond to pathogen challenge. In 1976, Elstner and Heupel [29] demonstrated a cell wall bound H_2O_2 generating system in the isolated cell walls of horseradish roots with the involvement of a cell wall peroxidase, malate dehydrogenase and apoplastic NAD^+ and NADH. Two electron transfer to O_2 or dismutation of $O_2^{\cdot-}$ [17] could also form H_2O_2 directly. However, no evidence for H_2O_2 or other peroxide production is presented in this paper. We have not attempted to characterize the peroxide species. We argue that a primary defence mechanism incorporating pre-existing HRGPs, peroxidase and an uncharacterized peroxide generating system in the cell wall of higher plants may be present.

EXPERIMENTAL

Material. Suspension culture of tomato (hybrid of *Lycopersicon esculentum* L. and *Lycopersicon peruvianum* L.) were a kind gift from Prof. Stephen Fry, Institute of Cell and Molecular Biology, University of Edinburgh. All chemicals and chromatography resins, unless specified, were purchased from Sigma.

Growth condition of tomato cells. Tomato suspension-cultured cells were grown [30] in a Murashige and Skoog-based tissue culture medium (basal salt mixture, Sigma, U.K.) [31] containing 20 g glucose/l and a supplement soln of 100 mg myo-inositol/l, 0.5 mg nicotinic acid/l, 0.5 mg pyridoxine HCl/l, 0.1 mg thiamine HCl/l, 2 mg glycine/l, 10 mg indole-3-acetic acid/l and 0.5 mg kinetin/l. Sterilised culture media (200 ml) in 500 ml conical flasks were subcultured with 7-day-old suspension-cultured cells and incubated on a rotary shaker at 110 rpm under continuous fluorescent lighting for 7 days at 22°.

Purification of extensin and extensin peroxidase. Soluble extensin and extensin peroxidase were salt-eluted from tomato cells and purified as previously described [30, 32]. The extensin peak was recognised by its ability to be cross-linked *in vitro* by extensin peroxidase in the presence of H_2O_2. Hydroxyproline and arabinose contents were also used as indicators of extensin [30].

Preparation of elicitor. Yeast extract (200 g) (Oxoid code L. 21, Unipath Ltd, Hampshire, U.K.) was dissolved in 400 ml H_2O, EtOH was added to a final concn of 80% (v/v) and the mixture was incubated for 4 days at 4°. The supernatant was decanted and the ppt, containing elicitor activity was recovered by centrifugation. It was dissolved in 120 ml H_2O and freeze dried [23].

The freeze-dried sample (89 g) was dissolved in H_2O (1 g/ml), centrifuged at 6000 rpm for 15 min and supernatant loaded on to an Amberlite IRA 416 (Cl^-) column (8 × 4 cm) equilibrated with degassed H_2O. The column was thoroughly washed with H_2O until the eluate was free of A_{280}. The column-bound material was eluted with 0.5 M NaCl. Frs were monitored at 280 nm, tested for elicitor activity and the active frs were pooled. This sample (10 ml) was then loaded on to a Sephacryl S-200 gel-filtration column (90 × 2.5 cm) pre-equilibrated with H_2O and elution continued with H_2O. Three distinct A_{280}-absorbing peaks (Fig. 1) were tested for elicitor activity. Peak II exhibited elicitor activity which was freeze-dried and then dissolved in H_2O to a final concn of 50 mg/ml. This sample was subjected to C_{18} reverse phase HPLC by applying 0.5 ml on to a Delta PAK C_{18} column (Waters, U.K., 15 × 0.2 cm) and eluted with a non-linear 0–100% MeCN (+0.1% TFA) gradient at a

flow rate of 0.5 ml/min. A_{280} absorbing fractions within three banded regions (Fig. 2), referred to as A, B and C, were pooled, freeze dried, each dissolved in H_2O (12.5 mg/ml, 12.7 mg/ml and 13.2 mg/ml A, B and C respectively) and tested for elicitor activity by taking 0.5 ml of each fraction. Peak B was acid-hydrolysed in 6 M HCl at 110° for 16 hr and its amino acid composition was analysed using an LKB Alpha-Plus Analyzer [Fig. 3 (inset)].

Cell elicitation and extensin insolubilization. Elicitor (0.5 ml) was added at a final concn of 0.04 mg/ml to 5 ml tomato cells (1 g fr. wt 4–8 day old tomato cells) in MS medium (0.2 mg dry wt (dry wt) elicitor/g fr. wt cell) or 5 ml cell wall suspension (0.2 mg dry wt elicitor/g fr. wt cell wall) and incubated for up to 1 hr on an orbital shaker (110 rpm) under light at 25°. H_2O replaced elicitor in the control experiments. Cells (1 g) were then H_2O-washed and eluted with 50 mM $CaCl_2$, pH 3.0 (1.5 ml) and the eluate recovered by centrifugation at 13000 rpm for 15 min. This step was repeated once more. The cell eluate (100 μl) was serially-diluted 1:1 in successive wells in the microtitre plates containing coating buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.2 g NaN_3 in 1 l H_2O, pH 9.6) and incubated overnight at 4°. Soluble extensin levels were measured by ELISA using polyclonal antibody raised in rabbit against extensin [32].

ELISA of $CaCl_2$*-eluted proteins.* The sample-coated microtitre plates were washed twice with 1 × PBS (10 × phosphate buffered saline (PBS); 80 g NaCl, 2 g KCl, 11.5 g Na_2HPO_4 and 2 g KH_2PO_4 dissolved in 1 l H_2O, pH 7.4). Washed plates were dried by tapping over filter paper and 100 μl 1% bovine serum albumin (BSA) blocking reagent in 1 × PBS was added and incubated for 1 hr at room temp. The plate was then washed with 1 × PBS and then primary extensin antisera (1/10000 dilution with 1% BSA in 1 × PBS) was added and incubated for 1 hr. After washing the plates with 1 × PBS to remove unbound primary antibody, 50 μl 1/1000-diluted secondary antibody in 1% BSA/PBS (goat anti-rabbit IgG conjugated with alkaline phosphatase; Sigma) was added to each well and incubated for 2 hr. The plate was then washed with 1 × PBS. Phenolphthalein phosphate substrate (50 μl; Serrono Diagnostics, Germany) was added and incubated for 45 min at room temp. Stop sln (185 μl) was then added to prevent further colour development and the plates were read at 550 nm.

SDS-PAGE and western blotting of $CaCl_2$*-eluted proteins.* SDS-polyacrylamide gels (7.5%) were developed in a Bio-Rad Mini-Protean II apparatus according to ref. [33]. Salt-eluted proteins from elicited and control tomato cells were boiled for 2 min in sample buffer × 2 [0.12 M Tris–HCl (pH 6.8), 2% (w/v) SDS, 20% (w/v) sucrose, 1% (v/v) 2-mercaptoethanol and 0.1% (w/v) bromophenol blue] and then microfuged at 13000 rpm for 5 min. The supernatants were loaded on to the gel and run for 1 hr at a constant 150 V. The gel was subsequently electro-blotted at a constant 100 V for 1 hr in transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine and 20% MeOH) on to prewetted PVDF membrane (Bio-Rad, U.K.) using a Bio-Rad Trans-Blot electrophoretic cell.

Following blotting, the PVDF membrane was washed in Tris tween buffered saline (TTBS) (20 mM Tris, pH 7.5, 500 mM NaCl, 0.05% Tween-20) for 30 min, immersed in blocking reagent (3% BSA in Tris-buffered saline (TBS) (20 mM Tris, pH 7.5, 500 mM NaCl) overnight and washed in TTBS for 15 min. The TTBS was decanted off and the membrane was incubated with a 1/30000 dilution of primary polyclonal antisera raised against native extensin for 1 hr at room temp. Unbound primary antibody was decanted off and the membrane was washed × 2 in TTBS for 5 min each time. The membrane was then incubated with goat anti-rabbit IgG alkaline phosphatase conjugate (1/3000 dilution in 1% gelatin-TTBS, Bio-Rad, U.K.) for 1.5 hr at room temp., washed twice in TTBS for 5 min each and once in TBS for 5 min at room temp. Colour development was achieved using the Bio-Rad alkaline phosphatase conjugate substrate kit. Development of the purple bands was stopped by immersing the membrane in H_2O for 10 min.

Plasma membrane isolation. Plasma membrane vesicles were isolated by differential centrifugation, as in ref. [34]. Post-mitochondrial microsomal pellet was fractionated by discontinuous sucrose density centrifugation. Plasma membrane vesicles were collected at the 32–38% (w/v) sucrose interface, diluted to 2 mg membrane protein/ml, flash frozen and stored in liquid N_2.

Tomato cell wall preparation. Tomato cells (100 g) were ground in liquid N_2 in a pestle and mortar. H_2O (150 ml) was added to the ground cells and the suspension centrifuged at 2000 *g* for 10 min. The resultant supernatant was decanted and the pellet was washed by Buchner filtration with 3 l H_2O. The cell wall prepn (fr. wt 3.5 g) was resuspended in 17.5 ml H_2O (200 mg/ml). In control experiments, this corresponds to 48 mg dry wt cell wall/ml. The ionically-bound status of the soluble extensin and extensin peroxidase was preserved by avoiding treatment of samples with salts at any stage of cell wall prepn.

Protein concns. Determined by the method of ref. [35] using BSA (fraction V, essentially fatty acid free) as the protein standard.

MALDI-TOF MS. Mass spectra of reverse-phase HPLC purified elicitor fraction B were obtained with a Kratos Analytical Kompact MALDI TOF 1 mass spectrometer. Elicitor (0.5 μl, 13 mg/ml) was mixed with 0.5 μl gentisic acid and air-dried on to a stainless steel target and ionized by UV photons from a N_2 laser source at 337 nm. The spectra obtained were an average of 50 laser shots.

Heat, pronase and periodate treatment of the elicitor. Purified elicitor (1 ml, 55 mg/ml) was heated for 10 min at 100° and assayed for elicitor activity. The elicitor (100 μg) was also incubated with 200 μg pronase (0.1 M Tris–HCl pH 8.0, 5 mM $CaCl_2$) in a 1 ml

incubation vol. at 37° for 48 hr under toluene [22]. Elicitor (100 μg/ml) was treated with 10 mM $NaIO_4$ in 67 mM NaOAc buffer, pH 5.5 (1 ml incubation vol.) at room temp. in the dark for 30 min [22]. The reaction was stopped with 20 mM $Na_2S_2O_5$ and $NaIO_4$-treated elicitor was assayed for its ability to trigger extensin insolubilization.

Assays pH, K^+ and Ca^{2+} flux. Extracellular cell suspension medium pH and K^+ were determined continuously using elicited and control cells resuspended in 0.3 mM Tris–Mes, pH 6.5 containing 3% (w/v) sucrose [9] over a 30 min incubation period with a H^+-selective and K^+-selective ion electrode. Extracellular K^+ and Ca^{2+} were also measured with an inductively coupled plasma emission spectroscopy (Tracesan Plasma Spectrometer Thermo Electron Ltd, Cheshire, U.K.). Cells were resuspended in 250 mM mannitol-buffered medium (0.3 mM Tris–Mes, pH 6.5 containing 3% (w/v) sucrose) with and without exogenous Ca^{2+} and K^+ (100 ppm) and then incubated with elicitor in order to investigate its effect upon Ca^{2+} and K^+ flux. Aliquots were withdrawn at 5 min intervals over a 30 min incubation period for ion analysis. Non-elicited and elicited cells (100 mg) were pre-incubated with Ca^{2+} channel blockers (either 100 μM $LaCl_3$ or verapamil) for 5 min before adding 0.13 mg/ml elicitor for 1 hr at 25°. Following this treatment, the levels of soluble HRGPs in the salt-eluted protein fraction were monitored by ELISA.

Plasma membrane ATPase activity. Plasma membrane vesicles (10 μg membrane protein) from elicited and non-elicited cells were incubated in 300 μl nucleoside-5′-triphosphate (NTP) mix (5 μM Gramicidin D, 10 mM NaN_3, 100 μM Na molybdate, 3 mM Tris–ATP (or guanosine-5′-triphosphate [GTP]), 3 mM $MgSO_4$, 50 mM KCl and Tris/Mes, pH 6.5) for 1 hr at 21°. The reaction was terminated with 0.9 ml Ames soln [36] (1 vol. 10% (w/v) ascorbic acid and 6 vol. 0.42% (w/v) acid NH_4 molybdate) containing 1% (w/v) SDS. A_{820} was determined after a further 30 min incubation [9]. ATP was replaced with GTP and the assay pH was raised to pH 7 for measurements of GTPase activity [28].

Ascorbate oxidase activity. Plasma membrane vesicles (50 μg protein) were incubated with reaction medium containing 250 μM ascorbate, 50 mM Na–Pi buffer pH 7.0, 1.0 mM H_2O_2 and 3.8 μmol EDTA for 30 min at 25° [7]. A decrease of A_{290} in the assay mixt. was measured.

Malate dehydrogenase activity. Elicited and non-elicited washed cells (20 g fr. wt each) were flash frozen in liquid N_2, homogenised with a pestle and mortar and suspended in 0.1 M K–Pi buffer. It was then centrifuged at 13 000 *g* for 15 min at 4°. Supernatant (50 μl) was then incubated in 1 ml 0.1 M K–Pi buffer (pH 7.4) containing 100 μM NADH and 6 mM oxaloacetate for 30 min at 25°. Malate dehydrogenase activity was determined by the oxidation of NADPH at 340 nm at 5 min intervals for 30 min.

Oxidative burst. Luminol (8.8 mg, 5-amino-2,3-dihydro-1,4-phthalazinedione) was dissolved in 1 ml NaOH (1 M) and diluted 1/100 in 30 mM Tris–Mes buffer pH 7 [37]. Elicited and non-elicited cell suspensions (0.1 g fr. wt cells/ml MS medium; 800 μl aliquots) were withdrawn at 5 min intervals for up to 30 min and transferred to the luminometer (LKB Wallac 1251 luminometer). Chemiluminescence was measured after addition of 150 μl luminol and expressed as H_2O_2 equivalents.

Superoxide dismutase activity. Superoxide anion generation was determined spectrophotometrically by the reduction of cytochrome c [20]. Elicited and non-elicited H_2O-washed cells (0.1 g fr. wt) were incubated with 1 ml 50 mM K–Pi buffer (pH 7.8), 20 μM cytochrome c (Type VI, horse heart) and 0.1 mM EDTA. After 5 min intervals, the cells were removed from the suspension by centrifugation at 13 000 *g* for 10 sec and the supernatant was assayed for cytochrome c reduction at 550 nm.

NADH oxidase activity. Elicited and non-elicited plasma membrane vesicles (25 μg protein) or water-washed intact tomato cells (0.1 g fr. wt) was suspended in 1 ml assay medium containing 20 mM Tris–Mes buffer pH 6.5, 150 μM NADH and 1 μM KCN [7]. After centrifugation at 13 000 *g* for 10 sec, NADH oxidation was assayed at 340 nm over a 30 min incubation period at room temp. Control experiments contained all ingredients except vesicles and cells.

Peroxidase activity. Non-elicited and elicited cells (0.1 g fr. wt) and isolated cell walls (0.1 g fr. wt) were H_2O-washed. Protein was eluted with 1 ml 50 mM $CaCl_2$ pH 3.0. Cell wall peroxidase activity was measured in 20 mM K–Pi buffer (pH 7.0) containing 33 μM H_2O_2 and 13.3 mM guaiacol over a 2 min time course at 470 nm.

Phenylalanine ammonia lyase activity. Non-elicited and elicited cells (20 g fr. wt each) were homogenised with a mortar and pestle in 100 mM Tris/HCl (pH 8.8) containing 5 mM 2-mercaptoethanol. Homogenate was centrifuged at 13 000 *g* and the supernatant (100 μl) was incubated with 10 mM L-phenylalanine, 100 mM Tris–HCl (pH 8.8) at 25° for 30 min. PAL activity was monitored at 290 nm at defined time intervals. D-phenylalanine replaced the L-isomer in control assays.

Lipoxygenase activity. Elicited and non-elicited cells (20 g fr. wt each) were incubated with 0.1 ml Triton X-100 for 15 min at 25° and any particulate material was then pelleted for 15 min at 13 000 *g*. Supernatant (aliquots of 100 μl, 200 μl and 500 μl) was incubated with 1 ml reaction medium containing 5 mM linolenic acid and 0.05% (v/v) Tween 20 in 0.1 m K–Pi buffer pH 6.2 for 30 min at 37°. Lipoxygenase activity was monitored at 234 nm [38].

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