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Purification, characterization and accumulation of three virus-induced cucumber peroxidases

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Abstract

Three anionic peroxidases (EC 1.11.1.7), named Prx1, 2, and 3, which are rapidly accumulated in cucumber (*Cucumis sativus* L., cv. Laura) reacting hypersensitively to tobacco necrosis virus, were purified to homogeneity. The three enzymes had an isoelectric point about 4.3, and the relative molecular masses of Prx1, 2, and 3 estimated by SDS-PAGE were 40 700, 38 000, and 37 100, respectively. These peroxidases had a similar pH stability, but differed in their specific activity, pH optimum, and thermal stability. By Ouchterlony double diffusion tests with antisera raised against the three purified enzymes, close serological relationships have been demonstrated between the three peroxidases.

Introduction

In a number of plant species, pathogen attack or stress is accompanied by the synthesis of a broad spectrum of host-encoded products called "defence-related" proteins (Bowles 1990). Among them, the peroxidases have been studied extensively in higher plants. Under pathogenic or stress conditions peroxidases play a crucial role in at least four defence-related events that occur in the extracellular matrix: clearance of H_2O_2, suberin synthesis (Kolattukudy 1987), lignin synthesis (Higuchi 1981), and construction of intermolecular linkages (Fry 1986).

Formation of disease symptoms in plants infected with a pathogen (*e.g.* fungi, bacteria, viruses or viroids) are frequently accompanied by increases in peroxidase

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Abbreviations: ABA - abscisic acid; DEAE-cellulose - diethylaminoethylcellulose; SDS-PAGE - sodium dodecyl sulphate-polyacryl-amide gel electrophoresis; HR - hypersensitive response; EDTA - ethylenediamintetraacetic acid
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activity concomitant with alterations in several other oxidative and hydrolytic enzymes (Van Loon and Callow 1983). The accumulation of a group of acidic peroxidases, inherited as a single locus (Dane 1983), has been correlated with induced resistance in muskmelon, watermelon or cucumber plants inoculated with *Colletotrichum lagenarium* (Smith and Hammerschmidt 1988).

By native and two-dimensional SDS-polyacrylamide gel electrophoresis of intercellular fluid extracts of virus-infected cucumber cotyledons, five major acidic virus-elicited proteins have been identified (Repka *et al.* 1990). As shown by analysis of enzymatic activities of intercellular fluids from virus-infected cucumber fractionated on DEAE-cellulose, three of the five acidic virus-elicited proteins appear to be peroxidases (Repka *et al.* 1991).

The purpose of the present study was to purify and characterize by relative molecular mass, isoelectric point and serology the three cucumber anionic peroxidases whose activity is strongly enhanced during a hypersensitive response. With the aid of prepared antibodies, peroxidase accumulation in the apoplastic space of virus-infected cucumber plants also was studied.

Material and methods

Plant material: Cucumber plants (*Cucumis sativus* L. cv. Laura) were grown from seed in a temperature-controlled glasshouse (20 - 32 °C), in 10 cm pots containing a sterilized standard potting compost.

Inoculation of plants: Cotyledons of *ca.* 7-d-old plants were dusted using carborundum as an abrasive and then rubbed with a foam pad containing a leaf homogenate from cucumber plants infected with tobacco necrosis virus (TNV) or purified TNV (500 μg cm^{-3}). Control plants were treated similarly with a homogenate of non-infected leaves or with virus-isolation buffer.

Isolation of intercellular fluid (ICF): The ICF from virus-infected and control leaves was collected by vacuum infiltration following the procedure of Repka *et al.* (1993). The ICF thus obtained was used immediately or freeze-dried and stored at -20 °C.

Protein and peroxidase activity determination: Protein concentration in the extracts was determined colorimetrically according to Bradford (1976) with bovine serum albumin (BSA) as the standard. Peroxidase activity of ICF extracts was determined using guaiacol as a substrate and enzyme activity was expressed as change in absorbance according to Frič and Fuchs (1970).

Polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF): Polyacrylamide slab gels, 0.75 - 1 mm thick, were prepared for both native and denaturing gel systems. Discontinuous PAGE of acidic proteins was performed at 4 °C under native conditions according to Laemmli (1970), with the exception that SDS was omitted in all buffers and the sample buffer did not contain 2-mercaptoethanol. Electrophoresis

was performed using a 4 % stacking gel and a 10 % separation gel. Each lane was loaded with equal amounts of protein and brom phenol blue as a marker.

For denaturing gels, the SDS system of Laemmli (1970) was used with a 12 % acrylamide gel. A protein standard kit (*BIO-RAD, SDS-PAGE Standards-LMW*) was used as M_r marker. Gels were stained for peroxidase activity *in situ* (in the native system) or for protein upon completion of electrophoresis.

The ICF extracts and purified proteins were subjected to vertical isoelectric focusing on 5 % polyacrylamide gels containing ampholytes in the pH range 3 - 10 (*BioLyte, BIO-RAD*). After the prefocusing of the gel, the samples to which equal amounts of native sample buffer was added were subjected to electrofocusing for 4 h at a constant power of 7 W at 15 °C. After the focusing was completed, a 1 cm wide strip from the margin of the gel was cut off and sliced with a razor blade into 0.5 cm wide slices. The slices were incubated overnight in 1 cm^3 distilled water at room temperature. The pH of the eluates was measured and the pI of the protein of interest was extrapolated from the resulting pH gradient curve. Separated proteins were stained for peroxidase activity as described for PAGE or with Coomassie Brilliant Blue.

Polyacrylamide gel stains: The peroxidase isozymes were visualized in gels by soaking the gel in a solution of guaiacol (20 min.), then H_2O_2 was added to the solution and bands appeared immediately. Due to the instability of peroxidases detected in PAGE gels, the stabilization of the peroxidase pattern was performed with the double-staining method described by Shimoni and Reuveni (1988).

For determination of proteins, the gels after electrophoresis were fixed, stained with Coomassie Blue and destained according to Repka *et al.*(1993). Alternatively, the gels containing proteins were stained with the silver nitrate staining procedure of Damerval *et al.*(1987).

Purification of cucumber peroxidases: Immediately after isolation, ICF (50 - 100 cm^3) was centrifuged for 30 min at 20 000 *g* and solid ammonium sulphate was added to the supernatant to reach 80 % saturation. The precipitated proteins were collected by centrifugation for 30 min at 15 000 *g*, dissolved in buffer A (50 mM TRIS-HCl, pH 7.5, 1mM EDTA) and the suspension dialysed against several changes of the same buffer. The protein solution was subjected to ion-exchange chromatography using a column of DEAE-cellulose (*Whatman*) equilibrated with buffer A. The adsorbed protein fraction was eluted with 200 cm^3 of a linear gradient from 0 to 0.5 M NaCl in buffer A. Active fractions (4 cm^3) were pooled and lyophilized. The peroxidase fraction was resuspended in buffer B (50 mM TRIS-HCl, pH 7.5, 1 mM EDTA, 0.05 2-mercaptoethanol) and further purified on a column of Sephadex G-75 (*Pharmacia*). Proteins were eluted with the same buffer and active fractions were pooled and lyophilized. Proteins were resuspended in an electrophoretic buffer C (0.5 M TRIS-HCl, pH 6.8) and subjected to preparative slab or tube gel electrophoresis. After the electrophoresis was completed, the peroxidases were directly detected by their reddish-brown colour, excised from the gel with a razor blade and

electroeluted. Highly purified proteins thus obtained were stored at -20 °C for future use.

Optimum pH, pH-stability and temperature-stability: The dependency of the enzyme activity on pH and temperature was determined in Britton-Robinson's buffer according to Ito *et al.* (1991) with the exception that 10 % guaiacol was used as a donor.

Preparation of antisera: Polyclonal antibodies against purified peroxidases were obtained as follows. The purified proteins, 0.5 mg in 1 cm^3 of phosphate buffered saline (PBS, pH 7.4), were mixed with an equal volume of complete Freund's adjuvant (*Difco*) and injected intramuscularly into rabbits. Five booster injections, each containing 0.1 mg of peroxidase in 0.3 cm^3 of PBS and 0.3 cm^3 of incomplete Freund's adjuvant (*Serva*) were administered at 10 d intervals. Blood collections were made 10 d after the last booster injection. The titre of antibodies was estimated by drop precipitation test and by Ouchterlony double diffusion analysis.

Immunodiffusion, immunoblotting and dot-blotting techniques: Ouchterlony (1958) double diffusion was carried out on glass slides (8 × 8 cm) or in a Petri dish coated with a 3 mm layer of 1 % agarose in PBS. Antigen and antibody were allowed to diffuse towards each other at room temperature for 24 h in a moist Petri dish, then transferred to 4 °C until precipitate formation was maximal. Unreacted proteins were washed-out from agarose in 0.1M NaCl, then with water, slides were allowed to dry at room temperature and precipitation lines visualized by staining with Coomassie Brilliant Blue.

Proteins separated in the native gel system were blotted on to nitrocellulose membranes (*Schleicher & Schuell*, 0.45 μm) using 0.7 % acetic acid as a transfer buffer. Following transfer, the membrane was heated at 80 °C for at least 8 h to inactivate endogenous peroxidases. After blocking with a solution containing either 2 % BSA or 5 % non-fat dry milk (Blotto), the Western blots were washed and incubated with the appropriate rabbit antiserum. The blots were then washed again with a several changes of TEN buffer (50 mM TRIS-HCl, pH 7.4, 5mM EDTA and 150 mM NaCl) containing 0.05 % Tween-20 and incubated with a peroxidase-conjugated swine anti-rabbit IgG (*SWaR-Px, Sevak* Praha) diluted 1:1000. Once again the blots were washed with buffer containing 0.05% Tween-20, and finally rinsed with buffer without Tween-20. Staining was in PBS, containing 0.5 % 3,3'-diamino-benzidine (*Fluka*), 0.02 % H_2O_2 and 0.03 % $CoCl_2$ as an intensifier. Colour development was stopped by washing with distilled water. The stained blots were dried and protected from light.

For quantitative and rapid screening of large numbers of specimens, the dot-blot technique was employed. In all cases, antigen was applied with membranes installed in the Dot-blot apparatus. Following a short incubation and removal of antigen solution by suction, the membrane was washed, blocked and processed as described for immunoblots.

Results

Induction of peroxidase activity in the apoplast during the hypersensitive response of cucumber cotyledons: As compared to both controls, inoculation of cucumber cotyledon tissue with TNV resulted in the expression of enhanced peroxidase activity within 72 h (Fig. 1). Maximum enzyme activity, which increased by 5.5-fold, was observed on the sixth day of infection.

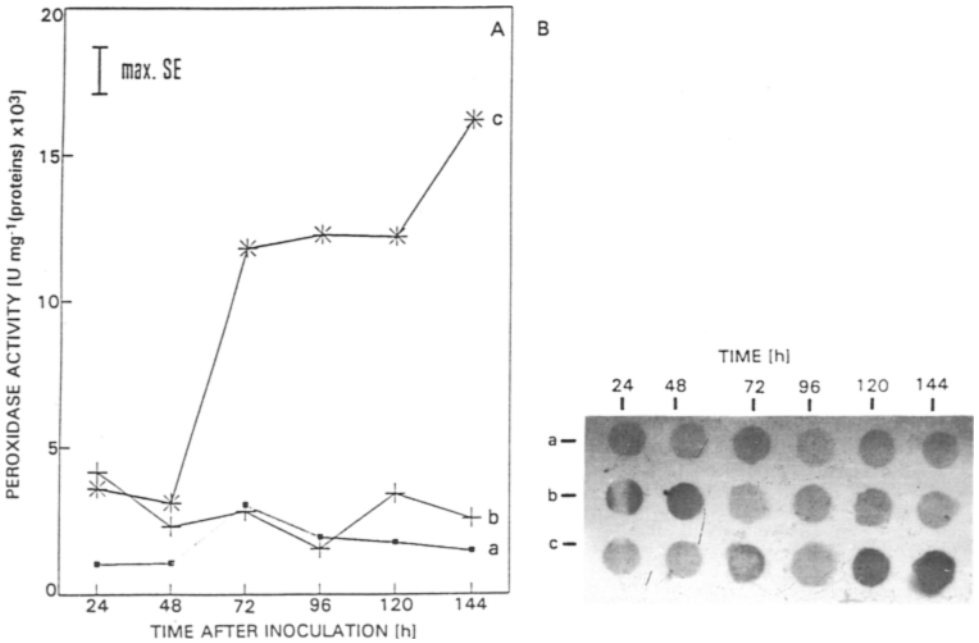

Fig. 1. Time course curve (A) of TNV-induced apoplastic peroxidase activity after inoculation of cucumber cotyledons with virus isolation buffer (*crosses*) or with purified TNV (*asterisks*). Non-treated control is denoted by dotted line. Data are the means of three experiments; vertical bar represents maximal S.E. ** ($P > 0.01$) according to *t*-test. Insert (B). Dot-blot analysis of the total ICF peroxidase accumulation (activity stained); these samples were dotted in the same order as marked in the graph.

For qualitative analysis of extracts, proteins from healthy and virus-infected cotyledons were subjected to electrophoresis on native polyacrylamide gels and stained for total proteins with Coomassie blue (Fig.2). By comparison of both peroxidase- and total protein-stained patterns from ICF extracts prepared from virus-infected cotyledon tissues, three major anionic peroxidase isozymes were revealed and termed Prx1, Prx2 and Prx3.

Purification of virus-elicited peroxidases: Peroxidases were eluted from the DEAE-cellulose column with a linear salt gradient as a single peak at 0.15 M NaCl (Fig.3). Active fractions were pooled, lyophilized and dissolved in equilibration buffer for a Sephadex G-75 column. Peroxidase activity was eluted again as a single peak containing only three peroxidase isozymes as demonstrated by gel electrophoresis

(Fig.4). Further purification was easily achieved by an additional preparative gel electrophoresis and electroelution. Highly purified and electrophoretically homogeneous preparations of particular peroxidases were thus obtained. The specific activities [U mg^{-1}(protein)] were 275 000, 325 000 and 315 000 for Prx1, Prx2 and Prx3, respectively.

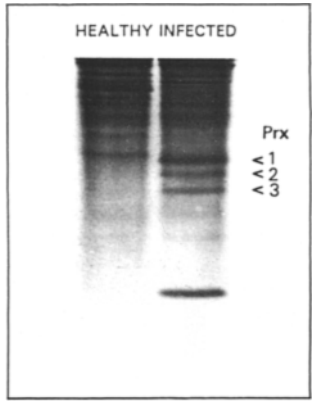

Fig. 2. Comparative 12 % polyacrylamide slab gel electrophoresis under native conditions. Proteins were extracted from ICF of healthy and TNV-infected cucumber cotyledons. Each lane was loaded with 100 μg of proteins and gel was stained with Coomassie Blue.

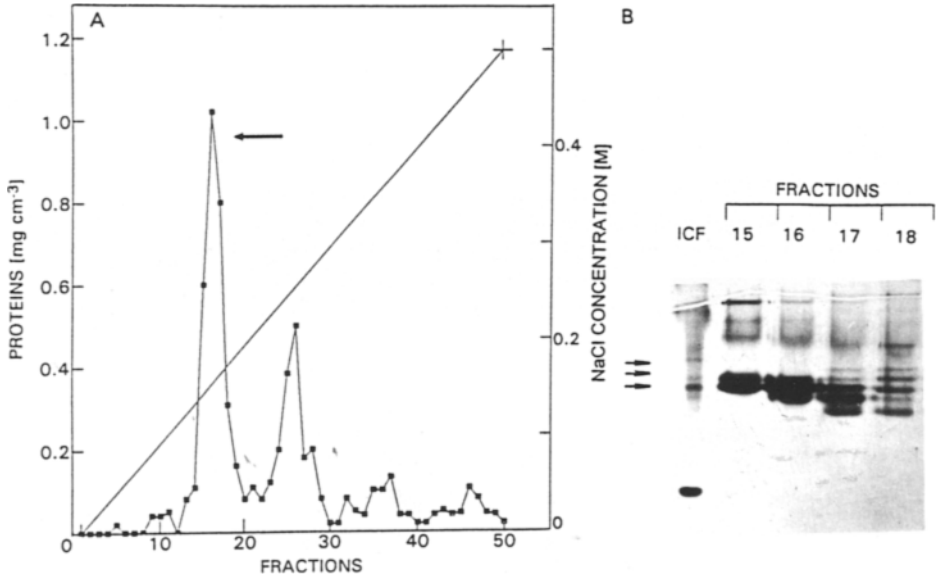

Fig. 3. Chromatographic profile of intercellular fluids extracted from TNV-infected cucumber cotyledons after ion-exchange chromatography on DEAE-cellulose. Insert shows PAGE profiles (50 μg of respective fractions were applied per lane) of peroxidase active fractions. The main peak of peroxidase activity is marked by a horizontal arrow. Small arrows denote position of peroxidases Prx1, 2 and 3.

Characterization of the purified peroxidases by PAGE: At the final stage of purification each peroxidase appeared homogeneous under native conditions (Fig. 5a). SDS-PAGE of the same samples confirmed the protein purity (Fig.5b). The molecular masses of these anionic peroxidases, estimated by SDS-PAGE, were 40.7, 38.0 and 37.1 kD, respectively. Isoelectric focusing revealed that most of the proteins present in ICF originating from the virus-infected cotyledons were acidic. The purified peroxidases appeared to be very acidic; their pI values were estimated at about 4.3 (Fig.5c).

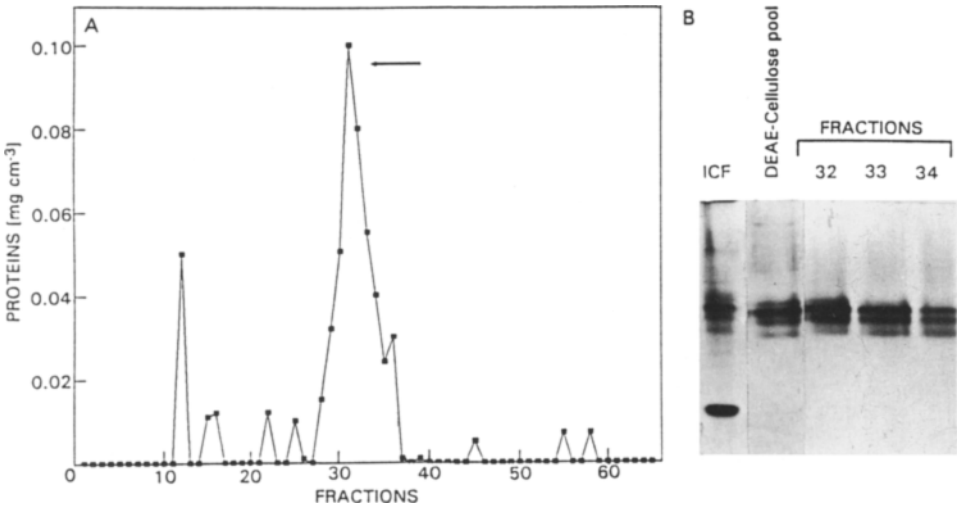

Fig. 4. Chromatogram of peroxidase peak from the experiment described in Fig. 3. (ion-exchange chromatography of ICF on DEAE-cellulose) obtained by gel filtration on a *Sephadex G-75* column. Insert shows PAGE profiles of peroxidase active fractions. The main peak of peroxidase activity is marked by a horizontal arrow.

Table 1. Effects of pH and temperature on stability and activity of purified cucumber peroxidases.

Parameters	Prx1	Prx2	Prx3
Optimum pH[a]	6.4	5.7	6.6
pH stability range[b]	6.1-12.0	6.1-12.0	6.1-12.0
Temperature stability[c] [°C]	35	30	30
Activity remaining [%]			
After 10 min at 30 °C	98.4	100.0	91.0
After 30 min at 30 °C	93.4	87.0	85.0
After 60 min at 30 °C	90.2	57.0	66.0
After 30 min at 55 °C	66.0	11.0	63.0

[a] pH with maximum activity under the conditions described in Materials and methods.
[b] pH range in which more than 90 % of the residual activity remained after being treated as described in Materials and methods.
[c] temperature limit within which more than 90 % of the original activity remained after being treated as described in Materials and methods.

Effects of pH and temperature on the stability and/or activity: Two peroxidase preparations were maximally active around pH 6.5 and one at pH 5.7. All purified peroxidases were active over a broad range of pH values (Table 1). More than 90 % of the original peroxidase activity remained after incubation at 25 °C for 24 h between pH 6.1 - 12.0 for Prx1, Prx2 and Prx3 (Table 1). The optimum temperatures for enzyme activity were 35 °C for Prx1 and up to 30 °C for Prx2 and 3 (Table 1). Prx2 had the lowest thermal stability and completely lost its activity after 30 min at 60 °C. In contrast, Prx1 and Prx3 were much more resistant to high temperature, and completely lost their activity after 30 min at 70 °C.

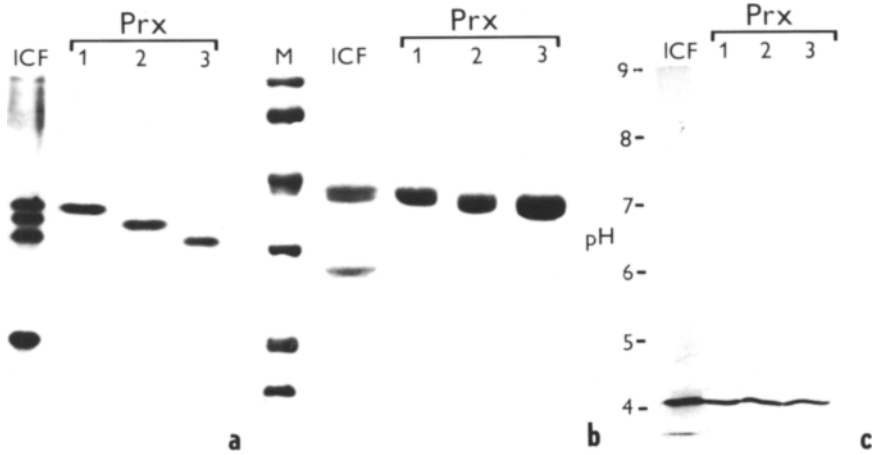

Fig. 5. PAGE of the purified peroxidases on native gel (*a*), SDS gel (*b*) and IEF gel (*c*). Besides the original intercellular fluid (lane ICF) loaded with 50 μg of protein, samples of 3, 5 and 10 μg of the purified peroxidases were loaded to the native, SDS and IEF gel, respectively. Lane marked as *M* in Fig. 5b contains molecular mass markers (Rabbit muscle phosphorylase b - 97 400, bovine serum albumin - 66 200, hen egg white ovalbumin - 42 699, bovine carbonic anhydrase - 31 000, soybean trypsin inhibitor - 21 500 and hen egg white lysozyme - 14 400).

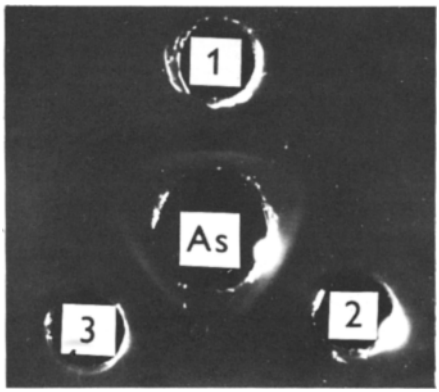

Fig. 6. Ouchterlony double immunodiffusion analysis of cross-reactivity of the purified peroxidases with anti-Prx1 antiserum. Center well contained 50 μl of whole serum (As). Outer wells were loaded with 1 μg of each cucumber peroxidase (wells 1, 2, 3 correspond to Prx1, Prx2 and Prx3, respectively).

Antibody production and immunological characterization of the purified anionic peroxidases: The immunoprecipitin lines for Prx1, Prx2 and Prx3 were completely fused (Fig.6), indicating that they were immunologically identical. Following Ouchterlony immunodiffusion tests, the agarose plates were activity-stained. All precipitin lines turned purple, which suggest that precipitated proteins have peroxidase activity.

Prepared antisera were also used to probe a Western blot of native gel loaded either with proteins from the ICF extracted from virus-infected cotyledons or with purified peroxidases. Antibodies raised against Prx1, Prx2 and Prx3 detected only Prx1, Prx2 and Prx3, respectively, while no cross-reaction with other proteins present in gel occurs (Fig.7).

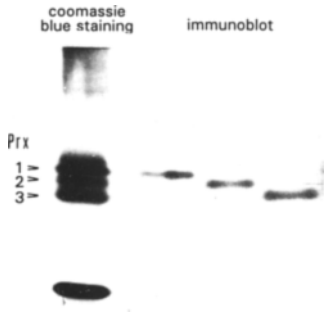

Fig. 7. Immunoblots of purified cucumber peroxidases (Prx1, 2 and 3) electrophoresed on native gel. Immunodecorated lanes contain 1 μg of individual peroxidases. The lane on the left represents Coomassie Brilliant Blue staining of total proteins present in ICF from TNV-infected cucumber cotyledons.

Discussion

This study has been undertaken to give additional information on our recent report on cucumber PR-proteins (Repka *et al.* 1993). We have shown that TNV infection of cucumber cotyledon tissues leads to the induction of peroxidase accumulation. Enzyme activity increased dramatically up to 7 d of infection after which there were only small changes.

Even though stress-induced peroxidases seem to be a general phenomenon there is only one report of purification and characterization of these anionic peroxidases in cucumber (Smith and Hammerschmidt 1988). They have characterized the accumulation of anionic peroxidases which are correlated with induced systemic resistance in cucumber, watermelon and muskmelon. Similar to the results of the present study, Smith and Hammerschmidt (1988) found that in native PAGE, extracellular peroxidase migrated as a cluster of three bands even though an entirely different agent of stress was involved.

However our results differ in important ways from those reported previously. We

have identified a similar cluster of anionic peroxidases and purified them to electrophoretic homogeneity. But surprisingly, when these purified proteins were subjected to the SDS-PAGE they had a much higher molecular masses than the 30 kD reported by Smith and Hammerschmidt (1988).

Although the anionic peroxidases extracted from cucumber cotyledons migrate as three bands on polyacrylamide gel, to date it has not been established whether they are the products of different genes or result from post-translational modifications of a single gene product. According to Van Huystee (1987), the modifications of a single gene product due to differential glycosylation or interactions with phenolics may result in multiple bands on gels.

Furthermore, Abeles *et al.*(1988) have identified in extracts from senescent cucumber cotyledons one ethylene-induced anionic peroxidase with a molecular mass of 60 kD. In contrast to this observation, we did not detect any counterpart to this anionic peroxidase either in ICF or in total leaf extracts from virus infected or healthy cucumber cotyledons. Different peroxidase isozymes may be associated with a local infection/wounding and a systemic reaction. Alternatively, the differences described above may be a consequence of the different cultivars used. In the light of our present knowledge, however, it seems more likely that plant-specific peroxidase isozyme accumulation is cultivar- rather than response-dependent.

Measurement of pH and temperature stability indicated that Prx1, Prx2 and Prx3 were stable at a wide range of pH and relatively sensitive to high temperature. Although the pH stabilities of these three anionic peroxidases were almost the same, the difference in the optimum pH and temperature stability suggest that these enzymes may have different physiological roles. Since the activity of these peroxidases was strongly enhanced during the hypersensitive response, different isozymes might control successive steps in defence mechanisms against pathogens (*e.g.* peroxidase-mediated lignification, Van Loon 1986).

Immunological analysis showed that the three purified peroxidase components are immunologically identical. The active sites of these peroxidases may differ from the reactive site of antigen-antibody reaction.

We have previously shown that three major anionic peroxidase isozymes were extractable by the different extraction procedures used (Repka *et al.*1993). Therefore, we suggest that the enhanced peroxidase isozymes purified from cucumber cotyledons in the present study may be refered to as "pathogenesis-related" or PRs, as has been reported after infestation by virus (Parent *et al.*1985), fungus (Ye *et al.* 1990) or mite (Bronner *et al.* 1991).

In spite of the fact that plant peroxidases have been studied by many authors, their physiological functions are only partially understood. The role most frequently cited for peroxidases is the catalysis of the terminal steps in lignin biosynthesis (Grisebach 1981). According to a working hypothesis that is fully consistent with the available information, anionic peroxidases are most probably involved in polymerizing phenolic monomers to generate the aromatic matrix of suberin (Kolattukudy 1987). Because the level of this enzyme correlates with progression of suberization induced by different factors (*e.g.* wounding, ABA, *etc.*), the anionic peroxidase appears to be a key component in the regulation of suberization. Thus it is likely that timely

elicitation of this enzyme could be an important defence mechanism against stress and pathogens. If such a defence role is played by anionic peroxidase(s), the peroxidase gene expression might be a succesful target for improvement of plant defence mechanisms.

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