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THE PATHOGENESIS-RELATED PROTEINS OF TOBACCO LEAVES

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco mosaic virus; hypersensitive reaction; PR-proteins; chromatofocussing.

Abstract—Extracts from the leaves of tobacco plants (cv. Xanthi-nc.) which are reacting hypersensitively to infection with TMV contain the four well-known pathogenesis-related proteins (PR-Ia, b, c and II), and in addition other PR-proteins which can be distinguished by electrophoresis and chromatofocussing. These proteins are referred to as N, O, P, Q, R and O′, P′, Q′ and R′ which have electrophoretic mobilities similar to O, P, Q and R. Four of the proteins have been isolated and characterized in terms of subunits and amino acid composition; R′ contains small amounts of *N*-acetylglucosamine. None of the proteins appears to have lectin-like properties or to interact with concanavalin A, and only R′ weakly interacts with wheat germ agglutinin. Proteins N-R′, like PRs I and II, are present in appreciable amounts in leaves treated with salicylate and in old leaves from flowering tobacco plants.

INTRODUCTION

Plants reacting hypersensitively to infection by viruses [1–3] or to other pathogens [4], often make relatively large amounts of 'novel' plant-coded proteins that are present in uninfected tissue only at very low concentrations (~ ng/g fr. wt; ref. [5]). These 'pathogenesis-related' proteins, PR-proteins [6], received attention following the suggestion that they may be involved in the mechanisms that lead to the localization of viruses or that confer some degree of resistance to subsequent infections on neighbouring tissue [see 1, 2]. However, no specific role can yet be assigned to these proteins, nor has any biological activity been reported for those which have been isolated. And because these proteins can also be induced by exogenous chemicals such as salicylic acid [7], by osmotic stress [8] and by flowering [9], it has been suggested that they are part of a general, unspecific response to stress conditions. This suggestion does not necessarily make them less interesting: they are unlikely to be the products of metabolic derangement and autolysis [see 10] and they may still play a role in minimizing or restricting some aspect of the stresses that are imposed by the conditions which induce them [1]. Although their description as 'pathogenesis-related' proteins has been criticized as implying too much [11], it will be retained here; alternative suggestions such as 'resistance-associated proteins' [12] seem to imply even more.

Typically the PR-proteins described for a number of plant species are of low molecular weight, are acid-soluble, proteolysis-resistant and have a high mobility in polyacrylamide gel electrophoresis (PAGE). Where there is more than one of them, as in cultivars of tobacco [6] or beans [13] some of them are charge-isomers which, in tobacco at least, have related structures and antigenicity [14]. But in tobacco leaves infected with TMV there is a further group of proteins of lower electrophoretic mobility that are also acid-soluble, proteolysis-resistant and which are not detectable or are present in much smaller amounts in uninfected leaves. Van Loon [15] separated these proteins from Samsun NN tobacco into six components by electrophoresis and provisionally designated them PRs N, O, P, Q, R and S in order of decreasing mobility: we [10] resolved those of cv. Xanthi-nc. tobacco into 7 components by chromatofocussing and labelled them N, O, P, P′, Q, R and R′, as in the conditions used, P′ and R′ had virtually the same electrophoretic mobility as P and R. From what little is known about these proteins, they have no obvious relationship to PR-I. It was recently reported in brief that N is a charge isomer of PR-II [16], and when the evidence is presented these proteins ought to be renamed PR-IIb and PR-IIa respectively, in line with the suggested nomenclature [6]. Three of the proteins, probably P, Q and R′, can be absorbed onto columns of chitin, and it was suggested that they may be lectins with affinity for *N*-acetyl glucosamine derivatives [10]: such lectins have been reported in tobacco leaves and they may help confer resistance to bacterial pathogens [17]. The present work provides further information on these slower migrating PR-proteins. It described the isolation and composition of some of them, their examination for lectin-like properties, and it reports their presence, along with the faster-migrating proteins, in leaves treated with salicylic acid and the leaves of plants in flower.

RESULTS

Separation of proteins by chromatofocussing

Eleven or twelve major protein components were routinely detected by electrophoretic analysis of the fractions produced by passing extracts from virus-infected leaves down a column of ion-exchanger under chromatofocussing conditions. Although the sequence of separated proteins resembled that previously described [10] differences were observed from one experiment to another depending upon the proportions of proteins in each leaf extract and the pH gradient developed during the separation. In the experiment shown in the upper part of

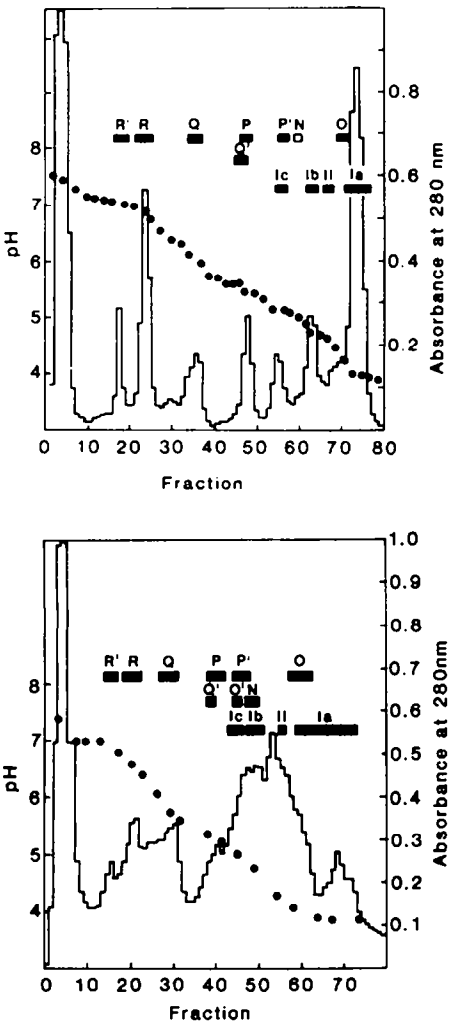

Fig. 1. Separation of leaf proteins by chromatofocussing. Upper, 43 mg of a protein preparation from TMV-infected tobacco leaves (26 g fr. wt) was passed down a column of ion-exchange material (PBE 94) under chromatofocussing conditions, and fractions of the eluate examined for pH (●), absorbance at 280 nm (histogram), and by electrophoresis for specific proteins (■): the identity of proteins was assigned from their electrophoretic mobility. Although N was not detected, its usual position is indicated (□). Lower, 60 mg of a protein preparation from yellowing leaves (70 g fresh weight) of flowering tobacco plants, was passed down a similar electrofocussing column, and fractions of eluate examined in the same way.

Fig. 1, the preparation contained only small amounts of component N and this protein could not be detected in the eluted fractions. However, small amounts of a new component Q', which had the same electrophoretic mobility as Q but 'chromatofocussed' like P near pH 5.5 was detected. Similarly, in another experiment O was resolved into two components, the new O' being eluted at pH 5–5.2, almost 1 pH unit higher than the other; in most experiments however only one or the other form of O was detected. Sometimes PR-Ia required pHs below 4 before it was eluted from the column. With the pH gradients used, R', R, Q, P, Ic and Ia were separated and purified sufficiently to be examined further.

Most of the proteins in an extract of healthy leaves (96 mg from 70 g fr. wt) passed straight through the chromatofocussing column, as if they had pIs greater than 7. However, trace amounts of proteins whose pH of elution and whose electrophoretic mobility suggested that they were R' and R were detected. Slightly larger amounts of other components were eluted at pHs near 6.2, 4.9 and 4.5, but on electrophoresis they gave diffuse bands of mobilities 0.39, 0.4 and 0.35 respectively, and could not be identified with any of the recognized PR-proteins.

Properties of purified proteins

Separation of PRs Ia, Ic, P and Q from leaf extracts by chromatofocussing had no detectable effect on their electrophoretic mobility, and indeed this is the criteria by which they are identified. The mobilities of R and R' were slightly but consistently modified, so that on co-electrophoresis they could be convincingly separated with R migrating fastest. This may indicate some modification of tertiary structure during separation, or that the proteins interact in leaf extracts and the interaction is broken and not readily restored.

On electrophoresis in dissociating conditions (SDS-PAGE), Ia and Ic each gave a single component whose molecular weight was judged to be near 16 500, rather higher than the expected 14 300 [6]: these SDS-derivatives could not be resolved on co-electrophoresis. R' in the same conditions gave two smaller, faster-moving components in approximately equal amounts whose apparent molecular weights were 14 500 and 15 300. P, Q and R each gave rise to a single main component with only traces of others or of material that ran with the electrophoretic front: the subunit from R was the faster migrating, with an apparent molecular weight near 23 500, and it separated from the others during co-electrophoresis. Those from P and Q both have apparent molecular weights near 27 500, as Jamet *et al.* [16] also recently reported, and were not separated from each other in the conditions used. They may be charge isomers, but a more extensive comparison of their electrophoretic mobilities is required to test this.

Amino acid composition of purified proteins

The amino acid content of the proteins P, Q, R and R', expressed as residues/100 residues recovered, are generally similar (Table 1). The content of glycine recovered was variable, the larger values possibly indicating incomplete removal of glycine-containing ampholines from the chromatofocussing buffers. Accepting the smallest glycine values, the proteins contain 25–30% of their residues as Asp plus Gly. R contained a high proportion of Cys residues; the 8% reported in Table 1 probably being an underestimate. R' contained small amounts, approximately 1% of recovered residues, of a substance that co-eluted with glucosamine. No galactosamine or hydroxyproline was detected in the samples.

Previous studies [6, 18] indicate a composition for PR-Ia which contains, for instance, more Glu, Val, Tyr and His, but less Ile and Thr than do the slower-migrating PRs (Table 1). These differences are also noticeable in more recent analyses (Table 2) which also show the expected similarities between the compositions of the charge isomers Ia, Ib and Ic. PR-II, which resembles Ib in its affinity for DEAE-cellulose and its mobility during chromatofocussing, differs from the PR-I charge-isomers

Table 1. Amino acid composition of 'slow migrating' PR proteins (residues/100 residues recovered)

	P	Q	R	R'	PR-Ia
Asp	14.9	14.2–17.0	15.6	15.0–18.2	15.5
Thr	6.1	6.7	8.0	8.6	4.1
Ser	6.1	5.4–7.9	4.3	5.1	6.6
Glu	3.2	6.7	6.4	7.0	13.4
Pro	7.5	6.0	8.7	5.6	3.5
Gly	18.0	13.7–18.3	13.0–19.0	12.0–17.0	9.0
Ala	9.6	9.2	5.9	8.4	11.2
Val	5.2	3.9	4.2	6.5	9.4
Cys/2	2.0	2.1	8.0	1.0–4.0	nd
Met	tr	0.5	0.7	0	0.9
Ile	5.4	5.0	3.4	2.7	0.6
Leu	5.0	4.3	4.8	7.1	4.2
Tyr	3.5	3.8	2.5	3.2	8.0
Phe	4.2	4.2	4.9	3.5	0.9
Lys	4.0	2.9	2.8	2.8	2.1
His	1.3	1.2	0.3	1.7	4.0
Try	nd	nd	nd	nd	3.0
Arg	4.0	5.3	4.6	5.2	3.6
Glucosamine	0	0	0	1.0	0
	100	95–105	98–104	97–107	100

Two samples of each protein were hydrolysed and analysed in duplicate. Results were averaged, except where the scatter made this inappropriate. Values for PR-Ia are taken from Antoniw and Pierpoint [18].

Table 2. Amino acid composition of some 'fast-migrating' PR-proteins (residues/100 residues)

	PR-Ia	PR-Ib	PR-Ic	PR-II
Asp	14.5	14.9	13.7	11.5
Thr	3.0	3.8	4.2	3.9
Ser	5.1	4.4	5.4	4.8
Glu	15.6	13.5	13.2	9.7
Pro	2.8	4.0	5.3	8.2
Gly	9.4	10.6	9.2	7.6
Ala	11.8	11.1	12.7	9.5
Val	11.6	12.7	10.1	5.6
Cys	0.1	0.2	0.4	0
Met	1.7	1.3	1.2	1.4
Ile	0.2	1.0	1.8	7.0
Leu	4.0	3.5	4.1	7.0
Tyr	9.7	8.7	8.4	7.5
Phe	0.8	0.7	0.9	6.7
Lys	0.9	2.1	2.5	5.4
His	4.5	3.9	3.7	2.0
Try	nd	nd	nd	nd
Arg	4.3	3.1	3.3	2.4

Proteins were hydrolysed and duplicate samples analysed as described in the Experimental.

in containing relatively more Pro, Ile and Phe (Table 2); these differences were confirmed by GC analyses of the hydrolysates as well as by ion-exchange analyses.

No phosphate component could be detected in the PR-proteins using methods that were expected to detect a few atoms per molecule of the most abundant proteins. When infected leaves with expanding lesions were fed high levels of ^{32}P, the protein extracts made at low pH were radioactive but when they were subjected to electrophoresis, none of the radioactivity detected in the gels could be attributed to any of the identifiable proteins.

Reaction of proteins as glycoproteins, lectins or agglutinins

When extracts of infected leaves were allowed to trickle through a column of colloidal or non-colloidal chitin, three proteins, probably R', P and a component of Q, were absorbed [10]. They were recovered free from other proteins by washing the column with phosphate–citrate buffer, pH 5.4, or more quickly by using Tris buffer at pH 7.4. The absorption is not likely to be an ion-exchange reaction involving free, deacetylated amino groups in the chitin; for neither these proteins nor any of the others was absorbed onto a column of chitosan in which over 90% of the amino groups are unacetylated.

One of the proteins, either R or R', weakly but perceptibly reacts with immobilized wheat germ lectin: when leaf extracts (1 ml containing about 5 mg protein) were passed through a column (2.5 ml) of wheat germ lectin-Sepharose 6MB, the first fractions of eluate are depleted of about half their [R + R'], while latter fractions contained twice as much. A weak interaction of protein R' with the lectin would be consistent with its small content of *N*-acetylglucosamine (Table 1). None of the major proteins was absorbed or retarded when extracts were passed down columns of immobilized *N*-acetylglucosamine or concanavalin A, making it unlikely that any of them are lectins specific for *N*-acetylglucosamine or are α-D-glucosyl-containing glycoproteins. Although traces of two proteins with electrophoretic mobility similar to PRs O and P were recovered when the concanavalin A column was washed

with borate, the amounts detected seemed too small for the proteins to be any of the recognized PR- constituents. No lectin-like haemagglutinating activity [19] could be detected in extracts of healthy or virus-infected leaves using trypsin-treated erythrocytes [Dr. D. Bowles, personal communication]. Both types of extracts had a weak agglutinating activity against *Pseudomonas solanacearum* at concentrations above 63 μg/ml [Drs Young and L. Sequeira, personal communication] but it was absent from purified preparations of proteins R, R′ and Q. Similarly, only a weak agglutinating activity was detected against *Erwinia amylovora* and it was present in extracts of healthy and infected leaves [R. Goodman, personal communication].

Extracts of leaves from flowering plants

Three batches of plants were allowed to grow until flowering by which time they were about 100 cm tall and the bottom leaves were brown and withered. Extracts were made from the yellowing leaves between 40 and 80 cm above soil level, excluding any that had necrotic blotches. Following electrophoresis the extracts produced a pattern of proteins which resembled that derived from virus-infected leaves (Fig. 2A) indicating the presence of the slower migrating PR-proteins as well as the faster-moving ones [9]. Co-electrophoresis emphasized the similarities of the two types of extracts (Fig. 2B). Moreover both sets of proteins were relatively resistant to proteolysis, being little affected by incubation with a mixture of trypsin and chymotrypsin [20] that almost completely digested the proteins in extracts of healthy leaves. The proportions in which the proteins appeared in extracts of old leaves differed from that in extracts of virus-infected leaves.

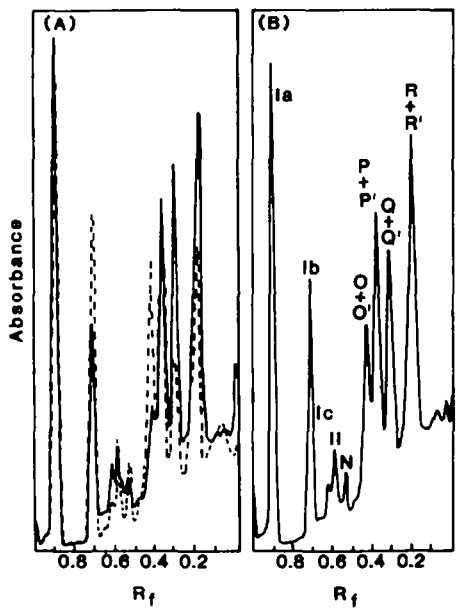

Fig. 2. Electrophoresis of proteins in leaf extracts. (A) Extracts of the proteins (320 μg/40 μl) of TMV-infected (——) and of flowering (– – –) tobacco plants were electrophoresed separately in 10% polyacrylamide gels which were stained and scanned. (B) The same extracts (160 μg/20 μl each) were co-electrophoresed in the same conditions.

Referring to the proteins by the symbols used for the corresponding protein components of virus-infected leaves, differences that were consistently observed were that R was better resolved from the fast moving minor R′, that Q was present in smaller amounts and sometimes resolved into two components, and that O was very prominent: PR-Ic was not detected in two of the extracts and only present in small amounts in the third. Frazer [9] noted that this protein was less evident in extracts of the higher leaves of flowering plants.

When extracts of the leaves of flowering plants were passed through the chromatofocussing column, the main proteins were retained and eluted in the same order, and at similar pHs as were those in extracts of virus-injected leaves. In the experiment summarized in the lower section of Fig. 1, both components of O and of Q were detected as well as those of R and P. When R and R′, recovered from the eluates, were electrophoresed in denaturing conditions, they gave SDS-products which migrated similarly to those derived from the R and R′ of virus-infected leaves, those from R′ forming a duplet of fast-moving bands.

The extracts of leaves from these plants, again like those of virus-infected leaves, contain three proteins which can be absorbed onto chitin at pH 5.3 and eluted at pH 7.4: their electrophoretic mobilities are indistinguishable from P, Q and R′. The amounts in which they are present, judged from spectrophotometric scans of the gels and expressed per dry weight of extract, are only about 2% of the amounts present in extracts of virus-infected leaves. These amounts are at least ten times higher than those of the questionable traces of similar proteins extracted from the healthy leaves of non-flowering plants.

Extracts of salicylate-treated leaves

Expanded leaves of 20 cm high tobacco plants were sprayed with 2 mM salicylate as described by van Loon and Antoniw [21], and extracts made from them 7 days later. Electrophoresis demonstrated the presence of eight major bands of protein which resembled those of the proteins PR-Ia to R, but without Ic (Fig. 3A). Co-electrophoresis with an extract of virus-infected leaves confirmed their similarity (Fig. 3B). Most of the proteins were absorbed by the chromatofocussing column and eluted similarly to those of virus-infected or old leaves as in Fig. 1, except that the protein Q′ was eluted at a lower pH (4.6) and no Ic could be detected. Three proteins of the extracts, whose R_f s correspond to those of P, Q and R′, were absorbed onto chitin at pH 5.3 and eluted at pH 7.4; their amounts, per dry weight of extract were about half that of extracts of virus-infected leaves.

DISCUSSION

Some of the properties of the 13 major proteins detected in extracts of infected leaves are summarized in Table 3. At least two of the slower-migrating proteins, R and R′, can be detected by electrophoretic techniques in healthy leaves of non-flowering tobacco plants. They increase about 500-fold following infection, a much smaller increase than the 20 000-fold one reported for PR-Ia [5]. They may not therefore be strictly 'novel' proteins in infected plants, but they have enough properties in common with the PR-I proteins, such as acid-solubility and proteolysis-resistance [15, 20], to be considered with them as part of a concerted response to infection. The

Table 3. Summary of properties of PR-proteins from *N. tabacum* cv. Xanthi-nc.

PR-protein	Number of subunits	M_r*	Charge isomers	Elution-pH on chromato-focussing (~ pI)	Composition	
Ia	1	14200	}	4.0	} Relatively rich in	
Ib	1	14220	}	4.7	} potentially acidic	(~ 29%)
Ic	1	14100	}	5.2	} and aromatic AAs	(~ 12%)
II	2	{29000	}	4.5		
		{23000	}			
N				5.0		
O				4.3		
O'				5.2		
P	1	~27500		5.5		
P'				5.2		
Q	1	~27500		6.0		
Q'				5.5		
R	1	~23500		6.8	Relatively rich in Cys/2	(~ 8%)
R'	2	{~14500		7.0	Some *N*-acetyl	
		{~15300			glucosamine	(~ 1%)

*Molecular weights quoted from the text are not strictly comparable with those taken from refs [6, 23].

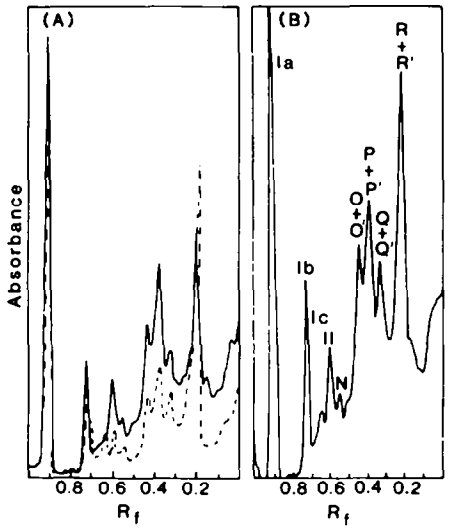

Fig. 3. Electrophoresis of proteins in leaf extracts. (A) Extract of proteins of TMV-infected plants (----; 130 μg/30 μl) and of salicylate-treated plants (——; 215 μg/30 μl) were electrophoresed separately in 10% polyacrylamide gels which were then stained and scanned. (B) The same extracts were mixed and co-electrophoresed in the same conditions.

presence of most of the proteins in large amounts in the leaves of flowering plants and also in salicylate-treated leaves indicates that they are part of a concerted response to other stimuli even when the stimuli may trigger protein synthesis by different mechanisms [15]. This does not, however, imply that all conditions which stimulate the synthesis of PR-I and II stimulate the synthesis of the others; extracts of tobacco crown gall tissue which had been transformed by *Agrobacteria tumefaciens* appear to contain PRs Ia, Ia' and Ib and little if any of the slower-migrating proteins [22].

Known properties of the 13 proteins (Table 3), suggest that they are a diverse collection even though they contain at least two families of charge isomers. Members of the first family are known to have common structural [14] and antigenic [5, 14] properties which are absent from the other proteins, and certainly the other proteins that we have now examined by SDS-PAGE do not contain the subunit characteristic of PR-Is. The second family of charge isomers is briefly reported to include PR-II and N [16], although other reports, not yet presented in detail, suggest that PR-II contains, besides the major component, smaller amounts of one with an M_r about 23000 [23]. Of the 13 proteins, only R' has so far been found definitely to contain a sugar component.

The main reasons for our previous suggestion that some PRs might be carbohydrate-containing lectins [10] was their interaction with chitin, and their apparent reaction in gels with the periodate–Schiff reagent. Moreover lectins resembling potato lectin and with an affinity for *N*-acetylglucosamine derivatives have been reported to occur in tobacco leaves [17], and similar compounds in other leaves increase following infection [24]. However, we can now find no evidence to support the previous suggestion: none of the proteins is absorbed by lectin absorbants, and there is no detectable haemagglutinating activity in crude PR-extracts or in purified samples of R and R'. Moreover the periodate–Schiff stain has since proved an unreliable indicator of glycoproteins in the conditions we previously employed. Because the interaction of P, Q and R' with chitin is now shown to a weak one, especially at neutral pHs, it seems most likely that it is a relatively unspecific interaction involving the secondary valency forces that are thought to bind reversibly a variety of enzymes to chitin [25].

The demonstration of a functional role for the PR-proteins remains elusive. We found no evidence that extracts containing PR-proteins would agglutinate some bacterial pathogens and so were unlikely to contain proteins such as the glycoprotein from potato which is active against virulent strains of *Pseudomonas* sp. [26],

nor the thiol-rich protein from apple which agglutinates *Erwinia amylovora* [27]. Nor could we detect in these extracts arabinogalactan-proteins (AGP) of the type described by Akiyama and Kato [28]: a purified sample of AGP isolated from tobacco leaves and kindly supplied by Dr. Akiyama, gave a very diffuse slow moving band on electrophoresis, unlike any of the proteins in Table 3. Our extracts of PR-proteins as well as purified proteins were unable, when tested by Dr. Wieringa-Brants, to restrict the spread of developing TMV-induced lesions in tobacco (cv. Xanthi-nc.) leaves, a test most relevant to ideas of the function of these proteins [29]. Although extracts from TMV-infected leaves, which have some resistance to further virus infection, do indeed restrict the spread of lesions in freshly inoculated plants, the active components may well be carbohydrates derived from degraded cell walls rather than induced proteins [30]: the effect of such carbohydrates in producing some of the physiological responses of plants to infection and to cell-injury is well known [31].

NOTE ADDED IN PROOF

In a recent paper (1985: *Can. J. Botany* **63**, 928) Parent *et al.* show that two of the PR-proteins present in the intracellular fluid of TMV-infected tobacco leaves, probably corresponding to components of PRs O and P in our nomenclature, are absorbed onto concanavalin A and eluted with methyl-D-mannoside. They suggest that these PR-proteins are glycoproteins and may also be peroxidases. The relationship of these proteins to the traces of proteins that we recovered from concanavalin A with borate is being investigated.

EXPERIMENTAL

Nicotiana tabacum cv. Xanthi-nc. was grown in the glasshouse [10] and when plants were about 30 cm high, expanded leaves were inoculated with a suspension of purified type-strain TMV (4 μg/ml) and carborundum. Control leaves were 'sham-inoculated' with H_2O or left untouched. Salicylic acid-treated leaves were prepared by spraying uninfected leaves to run-off with a soln of 2 mM salicylic acid whose pH was adjusted to 6.5. Leaves were harvested between 6 and 8 days after these treatments when any virus-induced lesions were well developed. Leaves were also taken from untreated, flowering plants when they were 90–95 cm tall and when their lower leaves were brown and withered [9].

Extraction of leaf proteins. Leaves, usually 20–80 g, were extracted into 2–3 times their weight of citrate–Pi buffer, pH 2.8, containing 0.1% (w/v) ascorbic acid and 0.1% β-mercaptoethanol [6]. Extracts were filtered through muslin and clarified by centrifugation at 15000 rpm for 15 min. Supernatants were dialysed for 16 hr against half-strength extraction buffer, then for 8–16 hr against 0.05% β-mercaptoethanol and finally for 20 hr against H_2O. They were freeze-dried and stored at −20° until used. Protein estimations using the Folin–Ciocalteau reagent [32] suggested that the powders contained 60–70% of their weight as protein.

Proteolytic digestion of protein samples. Freeze-dried extracts of leaves were redissolved in 50 mM Tris–HCl (pH 8), clarified if necessary, and their protein content estimated spectrophotometrically [20]. They were then incubated overnight with 1/50 of this amount of trypsin and also of chymotrypsin, in the presence of 1 mM $CaCl_2$ [20]. Usually it was necessary to repeat the incubation in order to destroy completely the protein extracted from healthy, untreated leaves: proteins previously extracted from such leaves by a slightly different procedure were destroyed by a single incubation.

Chromatofocussing of protein extracts. Freeze dried samples of leaf extracts, usually 40–100 mg, were dissolved in 2 ml imidazole (0.025 M)–HCl buffer, pH 7.4, dialysed against the same buffer, clarified by centrifugation, and 2–4 ml samples applied to a column (1 × 22 cm) of Polybuffer exchanger (PBE 94; Pharmacia) equilibrated with imidazole buffer. The columns were developed with Polybuffer 74 diluted eight-fold as described in the technical literature of Pharmacia Fine Chemicals. Fractions, 1–2 ml, were examined for pH and absorbance at 280 nm and then freeze-dried. They were dissolved in 0.2–0.3 ml H_2O and dialysed against Tris–glycine buffer, pH 8.3, and samples taken for electrophoretic examination. Proteins were recognized by their mobility and by migration during co-electrophoresis with small amounts of original extract.

Purification of proteins from leaf extracts. PRs Ia, Ib, Ic and II were purified from extracts of infected leaves by adsorption onto DEAE-cellulose, elution with a salt gradient and a final chromatography on Sephadex G-50 [6]. PR-II was eluted from DEAE-cellulose along with PR-Ib, but separated satisfactorily from it on the G-50. Preparations taken for analysis were essentially pure as judged by PAGE.

Polyacrylamide gel electrophoresis (PAGE). Proteins were separated in rod gels containing 10% (w/v) polyacrylamide cross-linked with 0.2% *N,N*-methylene-bisacrylamide (MEB) which were overlaid with a stacking gel (2.5% w/v polyacrylamide; 0.23%, w/v MEB) as described previously [10]. They were stained with Coomassie Brilliant Blue G 250 in $HClO_4$ for 1 hr, destained in 5% (v/v) HOAc overnight and scanned at 610 nm. Electrophoresis in rod gels containing SDS (SDS-PAGE) was as described by Hames [33], using standard proteins (Dalton Mark VI, Sigma Chemical Co.) as M_r markers. Protein samples were dissolved in the sample buffer and heated on a boiling water bath for 3 min immediately before electrophoresis. Proteins from the older leaves of flowering tobacco plants required a larger (5% w/v) concn of SDS in the sample buffer to ensure complete denaturation and absence of spurious bands.

Absorption of proteins onto chitin and other reagents. Purified uncolloidal chitin (Crab shells, Sigma Chemical Co.) was packed into the barrels of syringes to make small (0.5–1.0 ml) columns, which were equilibrated with citrate (0.075 M)–Pi (0.15 M) buffer at pH 5.3 or Tris buffer (50 mM) at pH 7. Proteins extracted from infected leaves were dissolved and dialysed against the appropriate buffer, trickled through the columns, and washed through with the same buffer or Tris at pH 7. Fractions of the eluates were collected, freeze-dried, redissolved and dialysed against Tris–glycine buffer, pH 8.3, and examined by PAGE. Columns (2.5 ml) of chitosan were made and used in the same way. The chitosan, from shrimp shells (Hercules Inc., Wilmington) was generously given by Drs. Stossel and Leuba who estimated [34] that 91% of the glucosamine residues were unacetylated. Columns of other materials were made and used similarly, but with different buffers and eluting solns. Those of Sepharose 6MB-bound wheat germ lectin (Pharmacia Fine Chemicals) were used in Pi buffer (0.05 M; pH 7) containing 0.2 M NaCl. Columns (1 ml) of Sepharose-bound concanavalin A (Con A-Sepharose; Pharmacia) were equilibrated with Tris buffer (0.05 M, pH 7.3) containing 0.5 N NaCl, and protein eluted with this buffer followed by borate (0.1 M, pH 6.5). Columns (0.5 ml) of agarose bound-*N*-acetylglucosamine (Pharmacia) were equilibrated and washed either in Pi–citrate buffer, pH 5, or Tris buffer pH 7, and further washed with the same buffer containing either 1 N NaCl or 0.2 N *N*-acetylglucosamine, and finally with 0.1 N HOAc [35].

Protein hydrolysis and analysis. Well dialysed protein samples were lyophilized and hydrolysed in 6 N HCl at 110° for 24 hr

either in evacuated tubes (Table 1) or, after the addition of β-mercaptoethanol (0.1 %), under nitrogen (Table 2). Samples were dried, freed from HCl and analysed in a Technicon amino acid analyser by Mrs. Susan Smith. Some samples were also examined, after derivitization, by GLC [36].

Radioactive experiments. Infected leaves were detached 4 days after inoculation, and their petioles immersed [10] in 0.4–0.5 ml MES buffer (10 mM; pH 6.2) containing 0.1 mCi of carrier-free ^{32}P-phosphate (PBS 11; Amersham International plc) and 40–100 Mμmol of unlabelled phosphate. These solns were usually absorbed, under illumination, in an hour, and were replaced by H_2O. The leaves were kept illuminated [10] for various times (6–20 hr), ground in extraction buffer and the proteins extracted, separated on a column of Sephadex G-50, concd and electrophoresed on rod gels. Gels were stained, scanned and sliced; radioactivity in the slices were examined either in a Beckman, LS 250 scintillation counter, or by autoradiography [10].

Under the conditions used, radioactivity was incorporated into the protein fraction; it was judged that the techniques used would detect a few atoms of phosphorus per molecule of the major PR-proteins, even if the absorbed radioactivity was equilibrated with total leaf phosphate.

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REFERENCES

1. van Loon, L. C. (1985) *Plant Mol. Biol.* **4**, 111.
2. Gianinazzi, S. (1982) in *Active Defence Mechanisms in Plants* (Wood, R. K. S., ed) p. 272. Plenum Press, New York.
3. Pierpoint, W. S. (1983) *Trends Biochem. Sci.* **8**, 5.
4. Gianinazzi, S., Ahl, P., Cornu, R. and Scalla, R. (1980) *Physiol Plant Pathol.* **19**, 85.
5. Antoniw, J. F., White, R. F., Barbara, D. J., Jones, P. and Longley, A. (1985) *Plant Mol. Biol.* **4**, 55.
6. Antoniw, J. F. Ritter, C. E., Pierpoint, W. S. and van Loon, L. C. (1980) *J. Gen. Virol.* **47**, 79.
7. White, R. F. (1979) *Virology* **99**, 410.
8. Wagih, E. E., Raflopoulos, A. E., Archer, S. A. and Coutts, R. H. S. (1983) *Phytopathol. Z.* **107**, 233.
9. Fraser R. S. S. (1981) *Physiol. Plant Pathol.* **19**, 69.
10. Pierpoint, W. S. (1983) *Phytochemistry* **22**, 2691.
11. Wagih, E. E. and Coutts, R. H. A. (1982) *Phytopathol. Z.* **104**, 364.
12. Kassanis, B. (1981) *Phytopathol. Z.* **102**, 277
13. Redolfi, P. and Contisani, A. (1984) in *Physiol. Plant Pathol.* **25**, 9.
14. Matsuoka, M. and Ohashi, Y. (1984) *J. Gen. Virol.* **65**, 2209.
15. van Loon, L. C. (1982) in *Active Defence Mechanisms in Plants* (Wood, R. K. S., ed.) p. 247. Plenum Press, New York.
16. Jamet, E., Kopp, M. and Fritig, B. (1985) *Physiol. Plant Pathol.* **27**, 29.
17. Sequeira, L. and Graham, T. L. (1977) *Physiol. Plant Pathol.* **11**, 43.
18. Antoniw, J. F. and Pierpoint, W. S. (1978) *J. Gen. Virol.* **39**, 343.
19. Bowles, D. J. and Marcus, S. E. (1981) *FEBS Letters* **129**, 135.
20. Pierpoint, W. S., Robinson, N. P. and Leason, M. B. (1981) *Physiol. Plant Pathol.* **19**, 85.
21. van Loon, L. C. and Antoniw, J. F. (1982) *Neth. J. Plant Pathol.* **88**, 237.
22. Antoniw, J. F., Ooms, G., White, R. F., Wullums, G. J., Vloten-Doting, L. V. (1983) *Plant Mol. Biol.* **2**, 317.
23. Carr, J. P., Antoniw, J. F., White, R. F. and Wilson, T. M. A. (1982) *Biochem. Soc. Trans.* **10**, 353.
24. Touze, A. and Esquerre-Tuguye, M. T. (1982) in *Active Defence Mechanisms in Plants* (Wood, R. K. S., ed.) p. 103. Plenum Press, New York.
25. Synowtecki, J., Sikorski, Z. E., Naczk, M. and Piotrzkowska, H. (1982) *Biothechol Bioengng.* **24**, 1871.
26. Leach, J. E., Cantrell, M. A. and Sequeira, L. (1982) *Plant Physiol.* **70**, 1353.
27. Romeiro, R., Karr, A. and Goodman, R. (1981) *Plant Physiol.* **68**, 772.
28. Akiyama, Y. and Kato, K. (1981) *Phytochemistry* **20**, 2507.
29. Wieringa-Brants, D. H. (1983) *Phytopathol Z.* **106**, 369.
30. Modderman, P. W., Schot, C. P., Klis, F. M. and Wieringa-Brants, D. H. (1985) *Phytopathol. Z.* **113**, 165.
31. Ryan, C. A., Bishop, P., Pearce, G., Darvill, A. G., McNeil, M. and Albersheim, P. (1981) *Plant Physiol.* **68**, 616.
32. Wu, A. M., Wu, J. C. and Herp, A. (1978) *Biochem. J.* **175**, 47.
33. Hames, B. D. (1981) in *Gel Electrophoresis of Proteins* (Hames, B. D. and Rickwood, D., eds) p. 1. IRL Press, London.
34. Stossel, P. and Leuba, J. L. (1984) *Phytopathol. Z.* **111**, 82.
35. Delmotte, F., Keida, C. and Monsigny, M. (1975) *FEBS Letters* **53**, 324.
36. Kirkman, M. A., Burrell, M. M., Lea, P. J. and Mills, W. R. (1980) *Analyt. Biochem.* **101**, 364.