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Biochemical properties of the pathogenesis-related proteins from tobacco

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Abstract

This review describes the discovery and identification of the pathogenesis-related proteins (PRs) from tobacco. In crude leaf extracts the PRs are distinguished from the proteins in uninfected plants by their solubility at pH 3, resistance to a range of proteases, and mobility in polyacrylamide gels upon electrophoresis (PAGE) in non-denaturing conditions. PAGE has been used as a qualitative and semi-quantitative assay for PRs, and their migration in gels made from different acrylamide concentrations has been used to identify charge and size isomers and electrophoretically identical PRs in different tobacco cultivars. The subunit composition and molecular weight (mol. wt) of the four PRs identified first in 'Xanthi-nc' were determined by SDS-PAGE; staining the gels has shown that these same four proteins in 'Samsun NN' did not contain carbohydrate, lipid or nucleic acid, nor were they isozymic forms of twenty five enzymes known to increase in activity following infection with TMV. Evidence suggests that most of the PRs in 'Xanthi-nc' and 'Samsun NN' are extracellular.

The purification of several PRs from 'Xanthi-nc', 'Samsun NN' and other tobaccos is described, as well as their mol. wt, subunit and amino acid composition. PRs 1a, b and c consist of a single polypeptide and have similar mol. wt and amino acid compositions. Antisera prepared against purified 'Xanthi-nc' b_1 protein have been used to determine serological relationships between PRs and form the basis of a very sensitive quantitative assay using ELISA. The regulation of synthesis of some PRs has been shown to involve translational control.

Additional keywords: Nicotiana tabacum, tobacco mosaic virus, polyacrylamide gel electrophoresis, enzyme-linked immunosorbent assay.

Introduction

Pathogenesis-related proteins (PRs) (Antoniw et al., 1980a) are host plant proteins induced by a wide range of pathogens including viruses, bacteria and fungi. They are found in several plant species but the *Nicotiana tabacum* proteins were the first to be identified and most has been published on their biochemical properties. The description of the properties of PRs is divided into two sections, one dealing with those properties which were determined from the proteins in leaf extracts and another dealing with the properties determined from the purified proteins. This approach shows how much information can be obtained from leaf extracts and how this is confirmed and extended once the proteins are purified. We believe that the knowledge of the tobacco PRs will provide a useful model for the characterisation of PRs from other plants and will enable some interesting comparisons to be made.

Properties of PRs determined in leaf extracts

Discovery and identification. The PRs were discovered in tobacco 'Xanthi-nc' (Gianinazzi et al., 1970) and 'Samsun NN' (Van Loon and Van Kammen, 1970) by comparing extracts, made at pH 8, of healthy and TMV-infected leaves using polyacrylamide gel electrophoresis (PAGE). Initially four PRs in each cultivar were identified by electrophoresis of leaf extracts in gels made from 10% acrylamide using the non-denaturing procedure of Ornstein (1964) and Davis (1964). These PRs, when observed in 'Xanthi-nc', were called b_1 to b_4 in order of decreasing electrophoretic mobility (Gianinazzi et al., 1970), and those in extracts from 'Samsun NN' were called I to IV in order of increasing mobility (Van Loon and Van Kammen, 1970). The protein-stained bands were also designated by their mobility relative (Rf) to a suitable dye e.g. bromophenol blue (Table 1).

Table 1. Relative mobilities and amounts of the first four pathogenesis-related proteins discovered in tobacco 'Xanthi-nc' and 'Samsun NN'.

'Xanthi-nc'			'Samsun NN'		
	Rf	relative amount		Rf	relative amount
b_1	0.85	100	IV	0.84	100
b_2	0.67	43	III	0.68	130
b_3	0.60	9	II	0.60	67
b_4	0.55	14	I	0.55	53

Data taken from Pierpoint et al. (1981) and Van Loon (1982). Rf - mobility of proteins relative to bromophenol blue on electrophoresis in non-denaturing gels made from 10% acrylamide using the procedure of Ornstein (1964) and Davis (1964). Relative amounts of the proteins were estimated from spectrophotometric scans of the protein-stained gels and determined as peak heights.

Extraction of tobacco leaves at pH 3 (Van Loon, 1976) or pH 2.8 (Gianinazzi et al., 1977) was found to solubilise the PRs but less than 30% of the pH 8-soluble proteins. This partial purification made easier the subsequent identification of PRs with PAGE by reducing the background of 'healthy' proteins with similar mobilities. PRs similar but not identical to those in 'Xanthi-nc' and 'Samsun NN' have since been identified in other cultivars using low pH extraction followed by PAGE (Antoniw and White, 1980; Ahl et al., 1982).

A second remarkable property which distinguishes tobacco PRs from normal plant proteins is their ability to withstand digestion by a range of proteases. Incubation of pH 8-extracts at room temperature under conditions in which the endogenous proteases were active, completely degraded the pH 3-soluble proteins from healthy leaves but not those of TMV-infected leaves (Van Loon, 1982). PRs were also resistant to digestion with trypsin, chymotrypsin (Van Loon and Ritter, 1978; Pierpoint et al., 1981), papain, pronase and proteinase K (L.C. van Loon, personal communication).

Resistance to proteolysis enabled ten PRs to be identified in TMV-infected leaves of 'Samsun NN' (Van Loon, 1982), but only the first four to be identified have been studied in any detail.

Quantitative assay. The relative amounts of the different PRs in an extract can be estimated after PAGE from spectrophotometric scans of the protein-stained gels by measuring the areas under the peaks (Van Loon, 1973) or the peak heights (Pierpoint et al., 1981). The relative amounts of PRs are different in different cultivars (Table 1) and can vary within one cultivar depending on the time between induction and extraction (Van Loon and Van Kammen, 1970), on the temperature (Van Loon & Antoniw, 1982; White et al., 1983) and on the nature of the PR inducer (Antoniw & White, 1980). This procedure has been used as a semi-quantitative assay for individual PRs (Van Loon, 1973; Pierpoint et al., 1981; Fraser, 1981), but there may be problems associated with the choice of stain, non-linearity of staining intensity with amount of protein, differential binding of stain by different proteins and reproducibility. Nevertheless, it is possible to detect as little as 0.5 μg 'Xanthi-nc' PR b_1 per g fresh wt of leaf; the lower limit is set by the problem of distinguishing between small amounts of genuine PRs and 'healthy' proteins with similar Rfs.

Comparison of PRs. The 'Xanthi-nc' PRs b_1 to b_4 and 'Samsun NN' PRs IV to I, respectively, have similar Rfs and comigrate when a mixture of leaf extracts from these two cultivars is subjected to electrophoresis in 10% polyacrylamide gels (Antoniw et al., 1980a), suggesting that these proteins are very similar in the two cultivars. However, two different proteins may comigrate in gels of one acrylamide concentration but separate in gels of another concentration. The migration of proteins during electrophoresis in non-denaturing conditions is a function of both the size and charge of the protein and the acrylamide concentration used to make the gel. When a protein is subjected to electrophoresis in gels made from different acrylamide concentrations and the log Rf values of the bands plotted against acrylamide concentration (Hedrick and Smith, 1968), the data are represented by a straight line; the slope and intercept of the line are related to the size and charge of the protein. A computer program has been written to find the best fit straight line by the least squares method. The program also tests statistically for lines of similar slope (indicating proteins of similar molecular weight but different charge) or similar intercept (indicating proteins of similar charge but different molecular weights) or identical lines (suggesting proteins of similar size and charge) (Antoniw et al., 1980a, b; Antoniw and White, 1980). This procedure has been used to show that the b_1, b_2 and b_3 proteins of 'Xanthi-nc' are charge isomers, having similar size but different charge, and that b_4 has a different size and charge. Similarly, PRs IV, III and II of 'Samsun NN' are charge isomers and different from protein I. Furthermore, comparison of the 'Xanthi-nc' proteins with those of 'Samsun NN' shows that b_1 and IV, b_2 and III and b_3 and II are electrophoretically identical pairs of proteins; but that b_4 and I are distinguishable as different proteins. In this way other proteins can be compared with any already characterized (Antoniw et al., 1980a, b; Ahl et al., 1982).

Composition and size of PRs. After separation of PRs by PAGE in non-denaturing conditions the region of gel containing an individual PR can be cut out and extracted

with SDS and the sample analysed on denaturing gels such as those described by Weber and Osborne (1969). Gianinazzi et al. (1977) used this procedure to show that 'Xanthi-nc' PRs b_1, b_2 and b_3 each had a mol. wt of 16 000 whilst b_4 had a larger mol.wt of 29 500. However, this method cannot be used when there are other proteins with similar Rfs to that of the PR under investigation. Two-dimensional gel electrophoresis, using the non-denaturing method of Ornstein (1964) and Davis (1964) in the first dimension followed by SDS-PAGE (Laemmli, 1970) in the second, overcomes this problem but is technically more difficult. Although the leaf extracts are normally stained to identify protein after separation by electrophoresis in non-denaturing conditions, there are also stains available to identify other materials. Van Loon (1972) showed that the PRs I to IV of TMV-infected 'Samsun NN' leaves do not stain for carbohydrate, lipid or nucleic acid. In 'Xanthi-nc' PRs b_1 to b_4 do not stain for carbohydrate but there is evidence that some of the PRs with lower Rfs may contain carbohydrate (W.S. Pierpoint, personal communication).

Location and function of PRs. The PRs are not evenly distributed in the inoculated leaves but are more concentrated in the areas closest to the lesions (Rohloff and Lerch, 1977). Furthermore, L.C. van Loon (personal communication) has shown that all ten PRs can be extracted from leaves by infiltration of the intercellular spaces with buffer, suggesting that the PRs are extracellular. This is supported by the observation that only 25% of b_1 and 45% of b_2 was present in protoplasts extracted from aspirin-treated 'Xanthi-nc' leaves, the major proportion of these two PRs being found in the pectinase and cellulase solution used to digest the cell walls (J.F. Antoniw, J.S.H. Kueh and R.F. White, unpublished results). Furthermore, 'Xanthi-nc' callus tissue grown in liquid culture released PRs into the medium (J.F. Antoniw, J.S.H. Kueh and R.F. White, unpublished results). The presence of PRs on the outside of the cell membrane or in the cell wall matrix may be relevant to the role that these proteins have in localising infection by pathogens.

Leaf extracts separated by electrophoresis in non-denaturing conditions can be

Table 2. Enzyme activities not associated with 'Samsun NN' pathogenesis-related proteins I-IV. Data taken from Van Loon (1972).

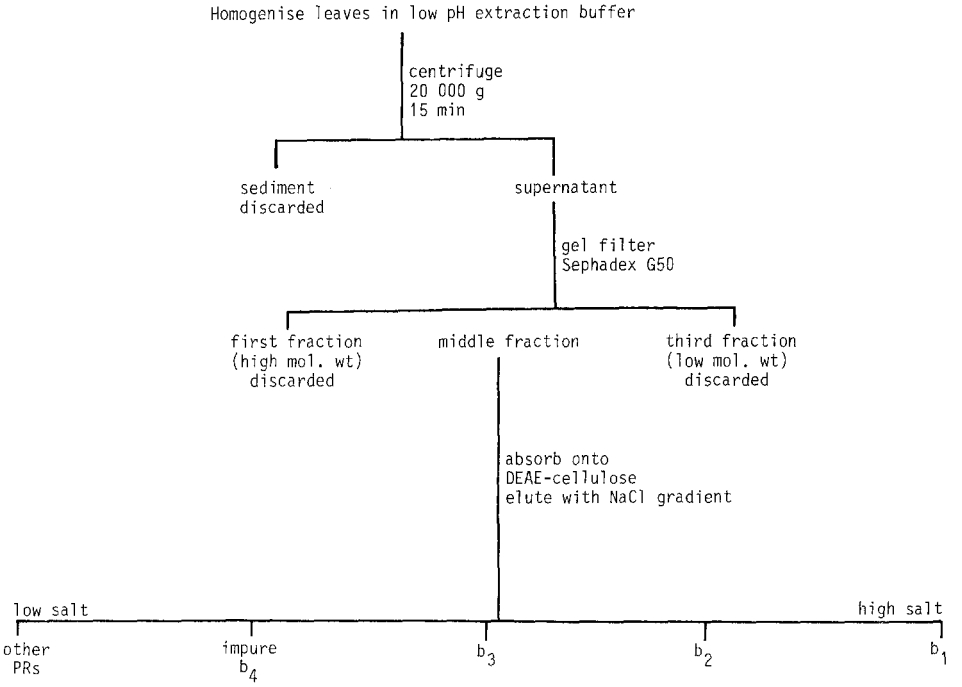
1. alcohol dehydrogenase
2. shikimate dehydrogenase
3. lactate dehydrogenase
4. NAD-dependent malate dehydrogenase
5. NADP-dependent malate dehydrogenase
6. NADP-dependent isocitrate dehydrogenase
7. 6-phosphogluconate dehydrogenase
8. glucose-6-phosphate dehydrogenase
9. glutamate dehydrogenase
10. diaphorase
11. oxidases
12. o-diphenol oxidase
13. catalase
14. peroxidase
15. phosphorylase
16. polynucleotide phosphorylase
17. ribonuclease
18. esterase
19. acid phosphatase
20. phosphodiesterase
21. deoxyribonuclease
22. amylase
23. leucine aminopeptidase
24. protease
25. polyphenoloxidase

stained to show the position of different enzyme activities. Comparison of the histochemical staining patterns of specific enzyme activities after PAGE of extracts of healthy and TMV-infected 'Samsun NN' leaves showed that PRs I to IV are not isozymic forms of those enzymes (Table 2) known to increase in activity following infection with TMV (Van Loon, 1972).

Purification of PRs

It is not possible to characterise a protein fully until it is pure. Antoniw and Pierpoint (1978a, b) developed a rapid purification procedure for three PRs of 'Xanthi-nc' (Table 3). The pH 2.8 extraction provides a useful initial enrichment step by removing most of the normal proteins whilst quantitatively solubilising the PRs. Although Gianinazzi et al. (1977) used gel filtration on a Sephadex G10 or G25 column to remove low mol. wt materials, Antoniw and Pierpoint (1978a, b) found that on Sephadex G50 the PRs were also retarded sufficiently to separate them from some higher mol. wt material eluting in the void volume. This step also exchanged the low pH extraction buffer for one more suitable for binding the PRs to a DEAE-cellulose column. The first three PRs bind to Whatman DE52 equilibrated in 50 mM Tris-HCl pH 8, 1 mM EDTA. Using a 0-300 mM NaCl linear gradient, almost pure b_3, b_2 and b_1 PRs can be eluted at increasing salt concentrations. These can be further purified by gel filtration on an analytical column of Sephadex G50 (fine). The same procedure has been used to purify the PRs IV, III and II from 'Samsun NN' (Antoniw et al., 1980a), b_0 from *N.*

Table 3. Purification of pathogenesis-related proteins from 'Xanthi-nc' tobacco.



sylvestris, b_1 from *N. glutinosa* and b_2 from *N. tomentosiformis* (P. Ahl, J.F. Antoniw, R.F. White and S. Gianinazzi, unpublished results).

Properties determined from purified PRs

Composition and size. The three PRs purified from 'Xanthi-nc' each gave a single band on PAGE both in the absence or presence of SDS (Antoniw et al., 1980a). Although these proteins have different Rfs during electrophoresis in non-denaturing gels, they comigrated in gels containing SDS and their mol.wts were estimated to be 15 000 (Antoniw et al., 1980a), confirming the observations made on the impure proteins (Gianinazzi et al., 1977). Furthermore, the three proteins eluted similarly from gel filtration columns with K_{av}'s of 0.25 ± 0.01 on Sephadex G50 (fine), and low-speed sedimentation equilibrium analysis also showed that the purified native proteins had similar mol. wts close to 14 200 (Antoniw et al., 1980a). These data together with the results obtained from Hedrick-Smith plots of the impure proteins show that these proteins each consist of a single polypeptide chain of mol. wt about 15 000 but differ in charge. Similar results were obtained with the analysis of the three PRs IV to II purified from 'Samsun NN' (Antoniw et al., 1980a).

Amino acid analysis has been carried out on five purified PRs (Table 4). There is a remarkable similarity between all five amino acid compositions; PR b_1 from 'Xanthi-nc' is typical with a high proportion of potentially acidic (29%) and aromatic residues (12%) but comparatively few basic residues (10%). The high content of acidic residues may account for the solubility of these proteins at low pH. The behaviour of

Table 4. Amino acid composition of pathogenesis-related proteins (mol/100 mol).

	b_0	b_1	b_2	IV	III
Asp	15.2	15.5	15.5	15.0	15.8
Thr	4.6	4.1	4.1	3.6	4.0
Ser	7.7	6.6	7.5	4.7	4.8
Glu	14.2	13.4	13.1	14.6	13.0
Pro	5.4	3.5	4.3	3.2	4.2
Gly	9.7	9.0	9.8	9.0	9.6
Ala	9.9	11.2	11.1	11.3	10.0
Val	8.3	9.4	11.6	12.0	13.1
Met	1.2	0.9	1.2	0.9	1.0
Ile	1.8	0.6	0.8	0	0.9
Leu	4.8	4.2	3.8	4.4	4.4
Tyr	6.3	8.1	7.0	8.8	7.9
Phe	1.8	0.9	0.9	0.7	0.8
His	3.2	4.0	3.4	5.2	4.6
Lys	3.0	2.1	2.7	1.7	2.6
Arg	3.0	3.6	3.1	3.7	3.2

Sources of PR proteins: b_0 = *N. sylvestris;* b_1 = 'Xanthi-nc'; b_2 = *N. tomentosiformis;* IV = 'Samsun NN'; III = 'Samsun NN'.

the 'Xanthi-nc' PRs b_1, b_2 and b_3 on PAGE in non-denaturing conditions and on elution from DEAE-cellulose with a salt gradient shows that b_1 is the most negatively charged at pH 8-9 and b_3 the least, but there is no clear correlation of charge with the content of acidic and basic residues. Possibly the aspartic and glutamic residues are amidated to different extents, thus producing the charge differences.

In 'Xanthi-nc' PR b_1 is present in the largest amounts, and was the first PR to be purified: thus its biochemical properties have been most studied. The UV absorption spectrum of b_1 is typical of a protein and its absorbance ratio $A_{280} : A_{260}$ is 1.84, confirming that there is no nucleic acid component. The absorption coefficient ($A^{1\%}_{280\,nm}$) of b_1 determined by interference optics is 18.9, which is high relative to most proteins, probably reflecting the high content of tyrosine and tryptophan residues. As in crude preparations, the purified PR b_1 from 'Xanthi-nc' did not stain for carbohydrate, nor did it bind to a concanavalin-A sepharose column which specifically binds α-D-mannosyl and α-D-glucosyl residues (J.F. Antoniw and R.F. White, unpublished work).

Although the 'Xanthi-nc' PRs b_1 to b_4 are resistant to trypsin, they are not trypsin inhibitors since the purified proteins have little inhibitory activity compared with commercial soybean trypsin inhibitor (Pierpoint et al., 1981). These purified PRs are also resistant to trypsin showing that the resistance is a consequence of the molecular structure of the PRs and not due to the induction of trypsin inhibitors in infected leaves.

Antigenic properties. An antiserum has been prepared by injecting purified 'Xanthi-nc' PR b_1 into a rabbit, and a titre of 1/8 was obtained in the Ouchterlony double diffusion test (J.F. Antoniw and R.F. White, unpublished work). Cross-linking the protein to itself using glutaraldehyde before injection into the rabbit did not increase the titre, so the relatively low titre is probably due to the acidic nature of the protein (J.P. Carr, T.M.A. Wilson, J.F. Antoniw and R.F. White, unpublished work). No precipitin lines were obtained with concentrated extracts of healthy leaves in Ouchterlony tests indicating that the antiserum was specific; and Ouchterlony tests also showed that the purified PRs b_2 and b_3 from 'Xanthi-nc' are serologically closely related to b_1. The b_1 protein is also serologically related to b_0 from N. sylvestris and b_2 from *N. tomentosiformis* (P. Ahl, J.F. Antoniw, R.F. White and S. Gianinazzi, unpublished work).

The antiserum to 'Xanthi-nc' PR b_1 has been used as the basis for an indirect enzyme-linked immunosorbent assay (ELISA) for PR b_1 (J.F. Antoniw, R.F. White and D.J. Barbara, unpublished work) using the method described by Barbara and Clark (1982). In this procedure the protein is trapped by $F(ab')_2$ fragments of PR b_1-specific immunoglobulin (IgG) immobilised in polystyrene microtitre plate wells, the protein detected by intact specific IgG and positive reaction identified using a protein A peroxidase conjugate. This indirect ELISA takes no longer than the semi-quantitative PAGE assay but can detect as little as 50 pg of PR b_1, i.e. it is ten thousand times more sensitive than the PAGE assay. This assay has shown that apparently healthy leaves contain as much as 20 ng of PR b_1 per g fresh wt of leaf. As more PRs are purified, and antisera prepared, the specificity and sensitivity of the indirect ELISA should enable more effective analysis of PR induction.

Regulation of PR protein synthesis. Carr et al. (1982) have prepared poly(A) mRNA from young healthy and TMV-infected 'Xanthi-nc' plants and have translated it both

in a wheat-germ cell free system and in messenger-dependent rabbit reticulocyte lysates. The radiolabelled translation products analysed by PAGE and visualised by photofluorography showed bands with the same Rfs as 'Xanthi-nc' PRs b_1 to b_4, indicating that each of these PRs has its own mRNA. The fact that these proteins produced in both translation systems had the same Rfs as those produced in vivo indicated that there was no post-translational processing of the newly-synthesised proteins. Immuno-precipitation of the translation products with the antiserum to 'Xanthi-nc' PR b_1 and analysis by PAGE in the presence of SDS confirmed that these were PRs.

Microdensitometer scans of the photofluorographs showed that PR b_1 constituted about 3% of the translation products of both healthy and infected plant mRNA. This demonstrated that the mRNA coding for these PRs is present in the same amounts in leaves of 'Xanthi-nc' whether healthy or TMV-infected. Since there is about 20 ng PR b_1 per g fresh wt of healthy leaf and as much as 20 μg PR b_1 per g fresh wt of TMV-infected leaf, it was proposed that most of the mRNA for the PRs is sequestered in a latent, untranslatable form in healthy leaves until the stimulus of chemical treatment or infection by a pathogen causes its conversion to a translatable state. This suggested that the synthesis of the PRs was under translational and not transcriptional control. An interesting observation which supports this suggestion is that actinomycin D, which blocks transcription, induces PR proteins when injected into leaves of 'Xanthi-nc' (J.P. Carr, personal communication).

The mRNA for the PRs is present in polyribosomes of TMV-infected and aspirin-treated leaves but not healthy leaves, indicating that in the latter the mRNA may be complexed in messenger ribonucleoprotein (mRNP) particles which are not translated until their protein components are modified (J.P. Carr, personal communication). Four new proteins have been identified which are bound to non-polysomal mRNA in healthy plants but not in aspirin-treated plants and which may be mRNA-masking proteins involved in the proposed mRNPs (J.P. Carr, personal communication). When its nucleic acid metabolism is disrupted by an infecting pathogen, translational control of protein synthesis would confer benefit on a plant by allowing rapid response without the need for de novo synthesis of mRNA.

Conclusions

Since their discovery in tobacco more than ten PRs have been identified, and several have been purified and characterised to a limited extent. In order to fully characterise the PRs in tobacco it will be necessary to identify all potential PRs perhaps by two-dimensional PAGE. The present nomenclature is confusing and a unified system for similar proteins in different cultivars is desirable, but the system should be flexible enough to accommodate other proteins of the same type or with intermediate properties. The next step in the analysis of the purified PRs is amino acid sequence determination either by conventional protein sequencing or by sequencing of cDNA prepared by molecular cloning of mRNA coding for PRs.

Although PRs are induced by different pathogens, their role in the plant is unclear. Recent research on the regulation of PR synthesis may eventually lead to understanding the molecular basis of PR induction. Furthermore, the very sensitive detection procedure provided by ELISA should help in the analysis of PR induction and help to determine their role.

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