Biochemical and Serological Studies of Pathogenesis-related Proteins of Nicotiana Species

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(Accepted 6 September 1984)

SUMMARY

Pathogenesis-related proteins (PR proteins), found in leaves of different Nicotiana spp. infected with tobacco mosaic virus (TMV) or treated with potassium salicylate, were compared biochemically and also serologically using an antibody to PR1a purified from TMV-infected leaves of Nicotiana tabacum cv. Samsun NN. The antibody preparation reacted with PR proteins, PR1a, PR1b and PR1c, from four cultivars of N. tabacum and with a PR protein, b1v, from TMV-infected leaves of Nicotiana glutinosa, but not with a PR protein, PR2, from cv. Samsun NN. The isoelectric points in 9 M-urea of PR1a and PR1b from N. tabacum cv. Samsun NN were pH 4.3 and 4.8, respectively. Partial proteolysis of PR1a and PR1b with Pronase E yielded peptides which, although similar in size to those from PR1b, differed from them serologically. When Staphylococcus aureus V8 protease was used, the peptides released from each protein differed both in size and serological reaction. The patterns of peptides released from b1v of N. glutinosa by V8 protease and Pronase E were different from those of peptides from PR1a and PR1b from N. tabacum. The antibody preparation reacted strongly with one of four peptides released by Pronase E and with two of the three peptides released by V8 protease. These results indicate that PR1a, PR1b and PR1c from N. tabacum and b1v from N. glutinosa contain some similar antigenic determinants and have similar structures, and confirm that PR1a and PR1b are very similar but not identical in their primary structures.

INTRODUCTION

When local lesions develop in tobacco mosaic virus (TMV)-infected leaves of Nicotiana tabacum cv. Xanthi-nc or cv. Samsun NN at least four novel soluble proteins appear in the leaves (Van Loon & Van Kammen, 1970; Van Loon, 1975). These new proteins have been called pathogenesis-related proteins (PR proteins) because they are host plant-coded and are induced in pathological or related situations (Antoniw et al., 1980).

The synthesis of the same proteins can also be induced in N. tabacum leaves by treatment with certain chemicals such as polyacrylic acid, aspirin, salicylic acid and benzoic acid (Gianinazzi & Kassanis, 1974; White, 1979; Antoniw & White, 1980). They have also been designated as PR proteins because they induce resistance to virus infection in tobacco leaves (Antoniw et al., 1981). Antoniw et al. (1980) reported that PR1a, PR1b and PR1c resemble each other in electrophoretic mobility in SDS-containing polyacrylamide gels and that PR1a and PR1b from N. tabacum cv. Samsun NN have similar amino acid compositions, but no other studies have been reported on the biochemical and serological properties of PR proteins.

We have compared biochemically and serologically PR proteins induced by TMV infection or potassium salicylate treatment using immunological blotting and peptide mapping by limited proteolysis.

METHODS

Plant materials. N. tabacum cv. Xanthi, Xanthi NN, Samsun and Samsun NN, and N. glutinosa were raised in a glasshouse kept at 20 to 32 °C for 2 to 3 months after sowing. Plants were trimmed of senescent and young (< 7 cm-long) leaves, and after 2 or 3 days, the remaining leaves were inoculated with purified preparations of TMV-OM at 10 μg/ml. Plants were transferred to a chamber controlled at 22 °C with a constant illumination of 6000 lx. About 300 lesions developed in each inoculated leaf. Leaves of control plants were inoculated with water.
Treatment with potassium salicylate. Fully expanded leaves were detached and floated on 0.01% potassium salicylate, pH 7.0, and incubated in the chamber described above.

Protein extraction. Samples of 1 g of leaves were ground in a chilled mortar with 0.1 g Polyclar AT (Goky San- gyo Co.) and 1.5 ml 84 mm-citric acid, 32 mm-NaHPO₄, pH 2.8, containing 14 mm-mercaptoethanol and 6 mm-sodium ascorbate (pH 2.8 buffer). The extract was centrifuged for 15 min at 8000 g and the supernatant fluid was dialysed against 5 mm-Tris–HCl pH 8.3. The solution was used as the crude protein solution.

Purification of PR₁a and PR₁b. Seven days after inoculation with TMV, leaves (200 g) of N. tabacum cv. Samsun NN were homogenized in 600 ml of pH 2.8 buffer containing 20 g Polyclar AT. After squeezing through two layers of cheesecloth, the extract was centrifuged at 20000 g for 20 min and the supernatant fluid was passed through a column of Sephadex G-25 (6 × 40 cm) previously equilibrated with pH 2.8 buffer. The void volume fraction was brought to 80% saturation with ammonium sulphate and stored at 0 °C for 30 min. Precipitated protein was collected by centrifugation, dissolved in 10 ml 5 mm-Tris–HCl pH 8.3, containing 6 mm-sodium ascorbate and dialysed against the same buffer. The solution was centrifuged at 20000 g for 30 min and the supernatant fluid (about 20 ml) was subjected to preparative polyacrylamide gel electrophoresis (Canal Industrial Co). Electrophoresis was performed using Davis’ (1964) buffer system, with 30 ml of running gel (10% polyacrylamide) and 20 ml of stacking gel (3% polyacrylamide) at 300 V/cm. The fractions comprising the first peak of u.v.-absorbing eluate and the dye front were discarded and the peak fractions containing the second (PR₁a) and third (PR₁b) peaks of u.v.-absorbing eluate were collected and used as the purified preparations.

When purified preparations of PR₁a and PR₁b were analysed by isoelectric focusing, only one protein band was detected (Fig. 1). The isoelectric points in 9 M-urea of PR₁a and PR₁b were pH 4.3 and 4.8, respectively.

A single polypeptide band was also detected when the purified preparations were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 4, lanes 1P, 2P). The molecular weights of PR₁a and PR₁b were almost identical, each being about 16000.

Immunological methods. Serum was collected from a rabbit following a course of six injections at intervals of 2 weeks with a mixture of 0.5 to 1 mg purified PR₁a and Freund’s complete adjuvant in 0.5 ml. An immunoglobulin G fraction was prepared from the antiserum by fractionation with 33% ammonium sulphate. A titre of 1/10 was obtained in Ouchterlony double-diffusion tests using purified PR₁a at 0.5 μg/well.

The electrophoretic blotting technique, used to characterize immunoreactive proteins in crude extracts or immunoreactive peptide-digested fragments of PR proteins, was as described by Matsuoka & Asahi (1983). After electrophoresis, the gel was covered with a sheet of nitrocellulose (pore size 0.45 μm; Schleicher & Schuell) which had been wetted with 25 mM-Tris, 192 mM-glycine, 20% methanol (pH 8.3). The polypeptides in the gel were transferred to nitrocellulose paper by electrophoresis at a constant current of 0.2 A for 1 h using a Toyo electrophoretic transblot apparatus. The nitrocellulose was then shaken in a saline solution (0.15 M-Tris–HCl pH 7.8, 0.9% NaCl) containing 2% bovine serum albumin, shaken in the same saline solution containing 1% anti-PR₁a IgG for 1 h, washed with saline solution, shaken in a 1/50 dilution in saline solution of fluorescein-labelled goat anti-rabbit IgG (Cappel Laboratories, Cochranville, Pa., U.S.A.) for 1 h and finally washed with saline solution. Serological reactions were detected as fluorescence in u.v. light.

Polyacrylamide gel electrophoresis. Non-denaturing gel electrophoresis in slabs of 10% polyacrylamide gel was in the buffer system described by Davis (1964). SDS-PAGE was in slabs of 15% polyacrylamide gel in a discontinuous buffer system (Laemmli, 1970). After electrophoresis, the gels were stained with Coomassie Brilliant Blue R (CBB).

Isoelectric focusing in slabs of 5% polyacrylamide gel was by the method of Finlayson & Chrambach (1971).

For electrophoresis in two dimensions, extracts of TMV-infected leaves of cv. Samsun NN were made in pH 2.8 buffer and subjected to non-denaturing gel electrophoresis (Davis, 1964). After electrophoresis, an 8 mm-wide strip of this gel was shaken in 2% (w/v) SDS and 5% (w/v) 2-mercaptoethanol at room temperature for 1 h. The strip of gel was then loaded on the stacking gel of an SDS-containing 15% polyacrylamide gel (Laemmli, 1970), and subjected to electrophoresis.

Limited proteolysis in SDS–polyacrylamide gel was as described by Cleveland et al. (1977). After SDS-gel electrophoresis of purified PR₁a, PR₁b or of extracts of TMV-infected leaves of N. glutinosa made in pH 2.8 buffer, the gels were stained with CBB and the stained bands of PR₁a, PR₁b or PR₁b were cut out. The gel slices were put in sample wells of another SDS-containing gel, overlaid with 10 μl of 20 μg/ml Pronase E or 20 μl of 0.1 mg/ml Staphylococcus aureus V8 protease in 0.125 m-Tris–HCl pH 6.8, 0.1% SDS. Digestion was performed in the stacking gel for 30 min, followed by electrophoresis of the products.

RESULTS

Biochemical properties of PR proteins from N. tabacum and N. glutinosa

Fig. 2 shows protein extracts of N. tabacum cv. Samsun NN made at intervals after inoculation with TMV or treatment with 0.01% potassium salicylate. Four new proteins (PR proteins)
Fig. 1. Isoelectric focusing of purified PR1a and PR1b of \textit{N. tabacum} cv. Samsun NN. Isoelectric focusing in 5\% polyacrylamide gel containing 9 M-urea and 2\% Ampholine, pH range 3-5 to 10 (LKB). Samples were focused at 200 V for 16 h and then at 500 V for 1 h. Lane 1, purified PR1a (10 \(\mu\)g); lane 2, purified PR1b (10 \(\mu\)g); lane 3, pH standard markers: myoglobin (7.35), human carbonic anhydrase B (6.55), bovine carbonic anhydrase B (5.85), soybean trypsin inhibitor (4.55) and amyloglucosidase (3.50).

Fig. 2. Appearance of PR proteins in \textit{N. tabacum} cv. Samsun NN leaves. Extracts made in pH 2.8 buffer of leaves following inoculation with TMV and the development of local lesions (left four tracks), or of leaves following transfer to potassium salicylate (right five tracks), were electrophoresed in 10\% acrylamide gel. The interval (days) between commencing treatments and making the extracts is shown above each track. The positions of the PR proteins are indicated by arrows.

were induced by either treatment, although more rapidly by treatment with 0.01\% potassium salicylate than by inoculation with TMV. These new proteins were designated PR1a, PR1b, PR1c and PR2 in order of decreasing mobility, using the nomenclature system proposed by Antoniw \emph{et al.} (1980).

In the immunological blotting test, antibody against PR1a purified from TMV-infected \textit{N. tabacum} cv. Samsun NN leaves reacted with PR1a, PR1b and PR1c, but not PR2 from the same leaves. The reaction with PR1b and PR1c seemed somewhat weaker than that with PR1a (Fig. 3, lanes 1). This antibody also reacted with PR1a and PR1b extracted from potassium salicylate-treated leaves of Samsun NN (Fig. 3, lanes 2). This result confirmed serologically that PR1a and PR1b in \textit{N. tabacum} cv. Samsun NN induced by treatment with potassium salicylate are related to those induced in Samsun NN by TMV infection. The antibody also reacted similarly with PR1a and PR1b induced in the other three cultivars of \textit{N. tabacum} by potassium salicylate (Fig. 3, lanes 3 to 5). These results suggest that the same proteins are produced in four cultivars.

When leaves of \textit{N. glutinosa} were infected with TMV or treated with potassium salicylate, one new protein, designated as b1- (see Ahl \emph{et al.}, 1982), accumulated. It was similar to the PR proteins of \textit{N. tabacum} in its solubility at low pH, but its mobility in non-denaturing polyacrylamide gels was not the same as those of the \textit{N. tabacum} proteins (Fig. 3, lanes 6). However, the new protein also reacted with antibodies against PR1a of \textit{N. tabacum} (Fig. 3, lanes 6). These results indicate that PR1a, PR1b and PR1c of \textit{N. tabacum} and b1- of \textit{N. glutinosa} contain some similar antigenic determinants.
Fig. 3. Immunological blotting with anti-PR1a antibodies of native polyacrylamide gels. Extracts made in pH 2.8 buffer were electrophoresed in 10% acrylamide gel and stained (P) or blotted and reacted with antiserum (B). Lanes 1, extract from leaves (0.05 g) of *N. tabacum* cv. Samsun NN 4 days after inoculation with TMV; lanes 2, extract from 0.05 g of cv. Samsun NN leaves following treatment with 0.01% potassium salicylate for 4 days; lanes 3, extract from 0.05 g of cv. Samsun leaves following treatment with 0.01% potassium salicylate for 4 days; lanes 4, extract from 0.05 g of cv. Xanthi NN leaves following treatment with 0.01% potassium salicylate for 4 days; lanes 5, extract from 0.05 g of *N. tabacum* cv. Xanthi leaves following treatment with 0.01% potassium salicylate for 4 days; lanes 6, extract from leaves (0.05 g) of *N. glutinosa* 4 days after inoculation with TMV.

Fig. 4. Immunological blotting with anti-PR1a antibodies of SDS polyacrylamide gels. Purified PR1a, PR1b or extracts made in pH 2.8 buffer were electrophoresed in SDS polyacrylamide gel and stained (P) or blotted and reacted with antibodies (B). Lanes 1, purified PR1a (10 µg); lanes 2, purified PR1b (10 µg); lanes 3, 4, 5 and 6 are the same as for lanes 1, 2, 5 and 6 in Fig. 3. Arrows on the left indicate positions of molecular weight (×10^{-3}) markers: albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20100) and lactalbumin (14400).

Purified PR1a and PR1b migrated similarly in SDS-PAGE and antibodies against PR1a reacted with these proteins dissociated with SDS in the immunological blotting test (Fig. 4, lanes 1, 2). The antibodies recognized one band in gels following electrophoresis of the crude extract from TMV-infected or potassium salicylate-treated *N. tabacum* leaves as well as from TMV-
Properties of pathogenesis-related proteins

Non-denaturing PAGE

Fig. 5. Immunological blotting of two-dimensional gel. Extracts made in pH 2.8 buffer of leaves (0.05 g) of *N. tabacum* cv. Samsun NN 4 days after inoculation with TMV were subjected to non-denaturing gel electrophoresis. After electrophoresis, a strip of gel was incubated with SDS and 2-mercaptoethanol and subjected to SDS-gel electrophoresis, then blotted and reacted with antibodies. Lane A is a sample similar to that analysed in the first dimension but stained with CBB. The positions of the PR protein bands are arrowed. Lane B is an extract similar to that analysed in two dimensions but applied direct to the second dimension gel and electrophoresed and immunologically blotted like the two-dimensional gel.

infected *N. glutinosa* leaves (Fig. 4, lanes 3 to 6). Thus, all immunoreactive proteins of *N. tabacum* cv. Samsun NN or Xanthi had molecular weights of around 16000. The immunoreactive protein of *N. glutinosa* migrated slightly faster than that of *N. tabacum*, and its molecular weight was estimated to be 14000. Two-dimensional electrophoresis of the crude extract of TMV-infected *N. tabacum* cv. Samsun NN leaves showed that the 16000 molecular weight polypeptide recognized by the antibodies was composed of three proteins (Fig. 5). No other reacting spots were detected, indicating that the antiserum was very specific to PR proteins.

Peptide mapping of PR1a, PR1b and b1-13

Maps of peptides released by limited proteolysis of PR1a and PR1b with Pronase E showed the same-sized cleavage fragments (Fig. 6, lanes 1P, 2P). However, although the largest fragment of PR1b reacted with the antibodies, the other fragments did not (Fig. 6, lane 2B). Protein b1-13 was also digested to give four fragments but of different sizes from those from PR1a and PR1b. As with the fragments of PR1b, only the largest of fragments from b1-13 reacted with the antibodies (Fig. 6, lanes 3P, 3B).

PR1a was digested by *S. aureus* V8 protease into three peptides which reacted with the antibodies but PR1b was digested into a peptide which co-migrated with the smallest peptide from PR1a (Fig. 7, lanes 1P, 2P) and a smaller peptide. Interestingly, the co-migrating peptides from each protein reacted with the antibodies whereas the smaller peptide from PR1b, which did not correspond to any of the peptides of PR1a, did not (Fig. 7, lanes 1B, 2B). Protein b1-13 was digested into four peptides; the second largest co-migrated with the smallest peptide from PR1a and reacted with the antiserum (Fig. 7, lanes 3P, 3B). The antibodies also reacted with the largest peptide, which did not correspond to any from PR1a.
Fig. 6. Peptide mapping of PR1a and PR1b of N. tabacum and b1- of N. glutinosa by Pronase E. After SDS–PAGE of purified PR1a and PR1b (Fig. 4, lanes 1P, 2P) or of extracts made in pH 2.8 buffer from N. glutinosa infected with TMV (Fig. 4, lane 6P), the bands of PR1a, PR1b and b1- of N. glutinosa were cut from the gel and subjected again to SDS–PAGE after the addition of 10 μl of 20 μg/ml Pronase E. Then the gel was stained (P) or blotted and reacted with antibodies (B). Lanes 1, PR1a (20 μg); lanes 2, PR1b (20 μg); lanes 3, b1- (20 μg). The positions of the undigested PR proteins are indicated by asterisks. Arrows on the left indicate positions of molecular weight markers: myoglobin (17200), myoglobin I and II (14600), myoglobin I (8240), myoglobin II (6380) and myoglobin III (2560).

Fig. 7. Peptide mapping of PR1a, PR1b and b1- by V8 protease. The bands of PR1a, PR1b and b1- were subjected to peptide mapping as described in Fig. 6, except for digestion by 20 μl of 0.1 mg/ml V8 protease. The gel was stained (P) or blotted and reacted with antibodies (B). Lanes 1, PR1a (20 μg); lanes 2, PR1b (20 μg); lanes 3, b1- (20 μg). The positions of the undigested PR proteins are indicated by asterisks. Arrows on the left indicate positions of molecular weight markers as for Fig. 6.

DISCUSSION

The present work shows that PR1a, PR1b and PR1c of all tested cultivars of N. tabacum and also b1- of N. glutinosa, induced by potassium salicylate or by TMV infection, have some common antigenic determinants. No other immunoreactive protein was detected in blots of two-dimensional gels (Fig. 5), which means that the antisera do not recognize PR2 of N. tabacum. These results suggest that PR1a, PR1b and PR1c of N. tabacum and b1- of N. glutinosa are similar and differ from PR2 of N. tabacum.

The isoelectric points in 9 M-urea of PR1a and PR1b were pH 4.3 and pH 4.8, respectively. These results support the suggestion that PR1a and PR1b are ‘charge isomers’ (Antoniw et al., 1980).
We also examined the structures of PR1a and PR1b of *N. tabacum* and br, of *N. glutinosa* by peptide mapping. The similarity between the patterns of peptides released from PR1a and PR1b by Pronase E indicates that these two proteins have similar primary structures (Fig. 6, lanes 1P, 2P). However, the primary structures are not identical because the immunoreactivities of cleavage peptides of PR1a differed from those of peptides from PR1b (Fig. 6, lanes 1B, 2B), the peptides released from PR1a and PR1b by V8 protease were distinct and these cleavage peptides had different immunoreactivities (Fig. 7, lanes 1, 2). The reason for V8 protease producing different cleavage peptides might be that PR1a and PR1b have similar primary structures but contain an aspartic or glutamic acid residue (or both) in different positions, as V8 protease cleaves at the COOH terminal side of aspartic and glutamic acid residues. The difference in isoelectric points of PR1a and PR1b might depend on this difference in position or amino acid residue content. The digestion patterns with both V8 protease and Pronase E of protein br, of *N. glutinosa* differed from those of PR1a and PR1b in *N. tabacum* (Fig. 6, 7), suggesting that the structures of the PR proteins of *N. glutinosa* and *N. tabacum* differ, although br, in *N. glutinosa* is serologically related to PR1a. Similarities between the structures of these PR proteins may reflect their involvement in specific function(s) in tobacco plants reacting to infection with TMV or treatment with certain chemicals.

**REFERENCES**


(Received 15 May 1984)