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Note

Separation and quantitative assay of three pathogenesis-related (b)proteins from tobacco mosaic virus hypersensitive *Nicotiana tabacum* by reversed-phase high-performance liquid chromatography

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Evidence has been accumulated that plants, like animals, are endowed with active defence mechanisms induced by virus infection. In the hypersensitive tissues of *Nicotiana tabacum* cv. *xanthi* inoculated with tobacco mosaic virus (TMV), and also in the tobacco tissues showing resistance to secondary infection by TMV, accumulation of at least four host proteins called "b" proteins (b1–b4)[1–4] or pathogenesis-related (PR) proteins[5] were found in important amounts. Three of these b proteins were similar in molecular weight (15,000 ± 200 daltons) but differed in charge[6]. These proteins were acidic, stable at low pH and survived sodium dodecyl sulphate (SDS) electrophoresis; according to these physico-chemical similarities, they could be compared with interferon found in animal cells[7].

This kind of protein has also been found in plants that become resistant to infection after pre-inoculation with other viruses (potato virus Y, tobacco ringspot virus) or after treatment with fungi[8], polyacrylic acid[9], aspirin[10] or ethephon[11]. However, induced resistance could be obtained without b protein accumulation when tissues were sprayed with $HgCl_2$[12] or after yeast RNA injection[13]. Further, it has recently been shown that b proteins were accumulated during the flowering process of healthy plants[14]. Previously reported procedures relating to a biochemical approach to b proteins such as ion-exchange chromatography and electrophoresis were not suitable for rapid assays because of their low resolution, long duration and lack of sensitivity[15]. In this study, we developed a rapid and convenient method for the separation and the quantification of three b proteins using reversed-phase high-performance liquid chromatography (HPLC), which could be helpful in obtaining a better understanding of their precise relationship with induced resistance and to elucidate the molecular mechanism of their action in hypersensitive reactions of tobacco during TMV infection.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a Model 200/6/4GM solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) with a U6K injector (Waters Assoc.).

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Samples were chromatographed on two reversed-phase (RP) silica supports: analytical, LiChrosorb RP-8 (particle size 10 μm; 25 cm × 4 mm I.D.; Merck, Darmstadt, F.R.G.) with a LiChroprep RP-8 (particle size 25–40 μm) pre-column;preparative, LiChrosorb RP-8 (particle size 7 μm; 25 cm × 10 mm I.D.; Merck) with a LiChroprep RP-8 (particle size 25–40 μm) pre-column. A further analytical column used contained μBondapak C_{18} (particle size 9 μm; 30 cm × 3.9 mm I.D.; Waters Assoc.) with a μBondapak C_{18} Porasil pre-column (2.3 cm × 4 mm I.D.).

Recording and integration were carried out on an ICAP 50 EI 510 instrument (Delsi, France). The chromatography was performed at ambient temperature and at a flow-rate of 1 ml/min under analytical conditions and 2 ml/min under preparative conditions. The b proteins were detected on a Schoeffel spectrophotometer set to 280 nm.

Reagents

2-Propanol (R.P. Normapur) was obtained from Prolabo (Paris, France) and formic acid (analytical-reagent grade) from Merck. The solvents were filtered under vacuum through a 1-μm Millipore Celotate-type filter and degassed ultrasonically. The water was deionized, doubly distilled and stored in dark containers.

HPLC procedure

Under analytical conditions, 25-μl samples were eluted with a gradient of 2-propanol–5% formic acid (solvent A) in 5% aqueous formic acid (pH 2.1) (solvent B). The gradient started immediately after sample injection and proceeded for 35 min.

Under preparative conditions, fractions of roughly purified samples (1 ml) were eluted with a gradient of 2-propanol–15% formic acid (solvent A) in 15% aqueous formic acid (pH 1.3) (solvent B) and peaks were collected on the basis of the detector signal, then lyophilized and prepared for re-chromatography or gel electrophoresis.

Linearity and reproducibility

These were determined by injecting various calibrated volumes of a b_1 standard protein.

Plant material

Nicotiana tabacum cv. *xanthi* nc plants were grown at 23°C (16 h at 16,000 lux). The leaves of 50-day-old tobacco were inoculated with purified TMV common strain (25 μg/ml) and collected 7 days after inoculation.

Sample preparation

The tobacco leaves were homogenized in Tris–HCl (pH 2.8) containing 0.3% (w/v) of 2-mercaptoethanol. The homogenates were filtered through muslin and centrifuged at 20,000 *g* for 20 min. The supernatant was precipitated with 50% (w/v) of ammonium sulphate (73% saturated) during 30 min at 4°C and centrifugated at 10,000 *g* for 10 min. The pellet was extracted with Tris–HCl buffer (pH 8) (5 g of fresh material per millilitre of buffer for analytical chromatography and 15 g of fresh material per millilitre of buffer for preparative chromatography).

After centrifugation at 5000 *g* for 10 min, the final supernatant was stored at

4°C, and constituted the roughly purified sample which was injected directly for assay of b protein.

Preparation of standards

The b proteins were purified from the roughly purified sample by gel filtration on a Sephadex G-50 column (60 × 4 cm I.D.) followed by ion-exchange chromatography on DEAE-cellulose[14].

Proteins b_2 and b_3 were eluted from DEAE-cellulose (Cellex-D, Bio-Rad Labs., Richmond, CA, U.S.A.) (15 × 2.5 cm I.D.) in Tris–HCl buffer (pH 8) with 100 m*M* sodium chloride and further purified with a linear gradient of 0–150 m*M* sodium chloride. Protein b_1 was specifically eluted from DEAE-cellulose with 200 m*M* sodium chloride; the corresponding peak was further purified with a linear gradient of 100–400 m*M* sodium chloride and then re-chromatographed on a Sephadex G-50 analytical column (70 × 2 cm I.D.).

The standard purity was determined by gel electrophoresis and the final concentration was obtained according to the UV absorption coefficient for protein b_1 of

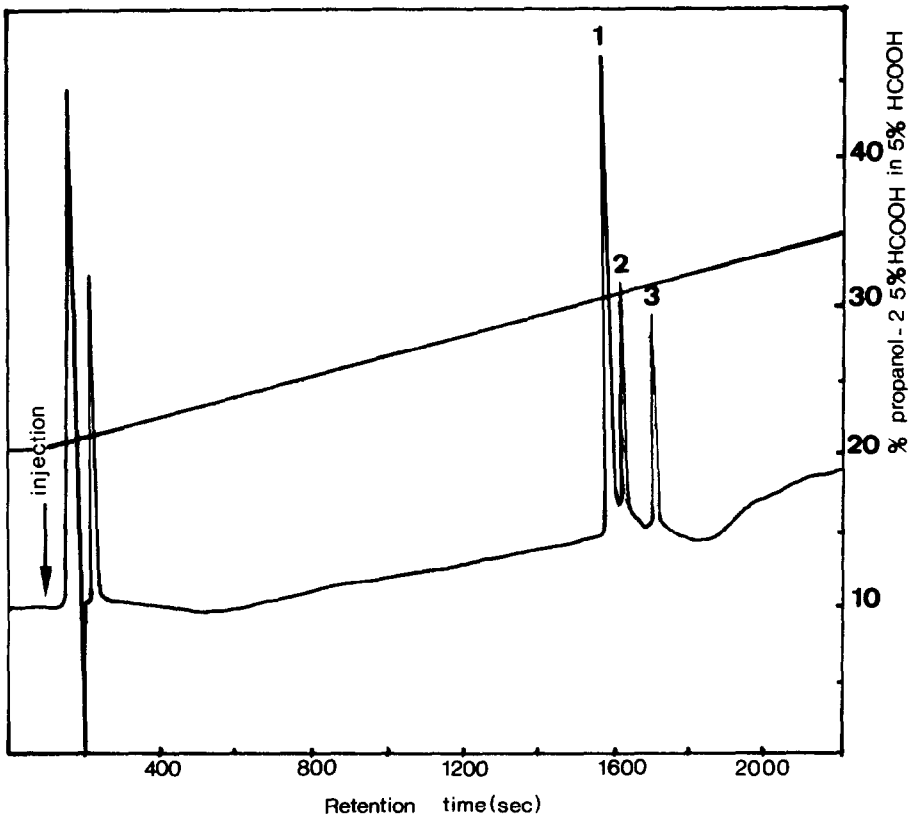

Fig. 1. Chromatogram of a 25-μl mixture of the three standard proteins. Column: LiChrosorb RP-8, 10 μm (25 cm × 4 mm I.D.). Solvent A, propanol–5% formic acid; solvent B, aqueous 5% formic acid (pH 2.1); linear gradient of A–B from 20:80 to 35:65 in 35 min. Flow-rate, 1 ml/min; room temperature; peak detection, 280 nm; pressure, 2000–2500 p.s.i. Peaks: 1 = protein b; 2 = protein b_2; 3 = protein b_3.

1 mg/ml ($A^{1\%}_{280\ nm}$ is estimated to be 18.9) and for the other b proteins of 0.5 mg/ml as in ref. 16.

All the samples were filtered through a 0.5-mm Millipore Celotate-type filter before injection.

Electrophoresis

Electrophoresis was carried out on polycrylamide gel in Tris–glycine buffer (pH 8.3) as described previously[17,18]. Electrophoresis was performed for 1.5 h at 370 V and 4°C, then the gels were stained with 0.03% Coomassie Brillant Blue R 250 in methanol–water–acetic acid (5:5:1) and destained with methanol–water–acetic acid (5:5:1).

RESULTS

On the analytical column, isocratic conditions with various proportions of 2-propanol–5% formic acid in aqueous 5% formic acid were not suitable because all the b proteins were coeluted in trailing peaks. In the same way, with 0.1% aqueous

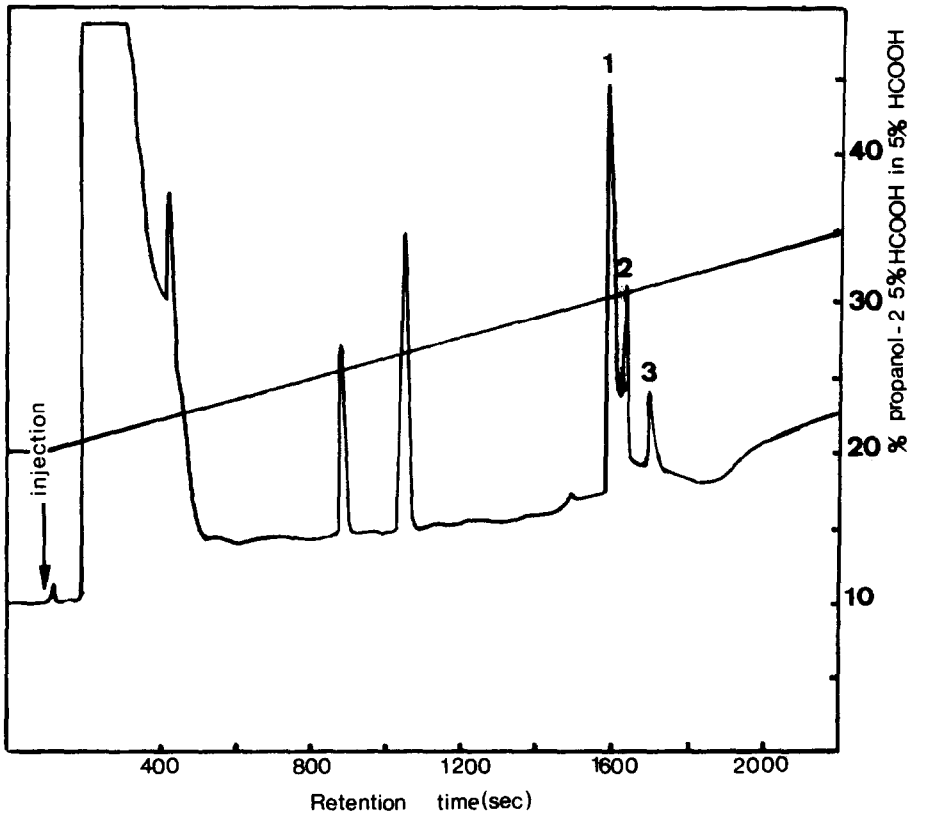

Fig. 2. Chromatogram of roughly purified sample from *N. tabacum* cv. *xanthi* nc seven days after TMV infection (25 μg/ml). Column: LiChrosorb RP-8, 10 μm (25 cm × 4 mm I.D.). Conditions as in Fig. 1. Peaks: 1 = protein b_1; 2 = protein b_2; 3 = protein b_3.

trifluoroacetic acid and 1% aqueous formic acid in 2-propanol, the resolution was not satisfactory and b proteins seemed to be hardly retained on the RP-8 support.

Different gradients ranging from 0 to 40% of 2-propanol–5% formic acid in aqueous 5% formic acid were tested. The best resolution of the three standard proteins was obtained with a linear gradient between 20% and 35% of 2-propanol–5% formic acid for 35 min (Fig. 1).

Under rapid analysis conditions, with a gradient time less than 1 h, separation between b_1 and b_2 did not occur if the gradient started below 10% of 2-propanol.

The roughly purified extract chromatographed under the above conditions showed the same resolution (Fig. 2) and proteins b_1, b_2 and b_3 were separated and quantitated.

The retention times were tested, and the variations did not exceed 2%. The recovery was about 95% for each b protein.

The injection of large volumes (up to 150 µl) of protein mixtures did not affect the resolution. Using RP µBondapak C_{18} (9 µm) and a 20–40% gradient of 2-propanol–5% formic acid in 5% aqueous formic acid we obtained a good peak shape

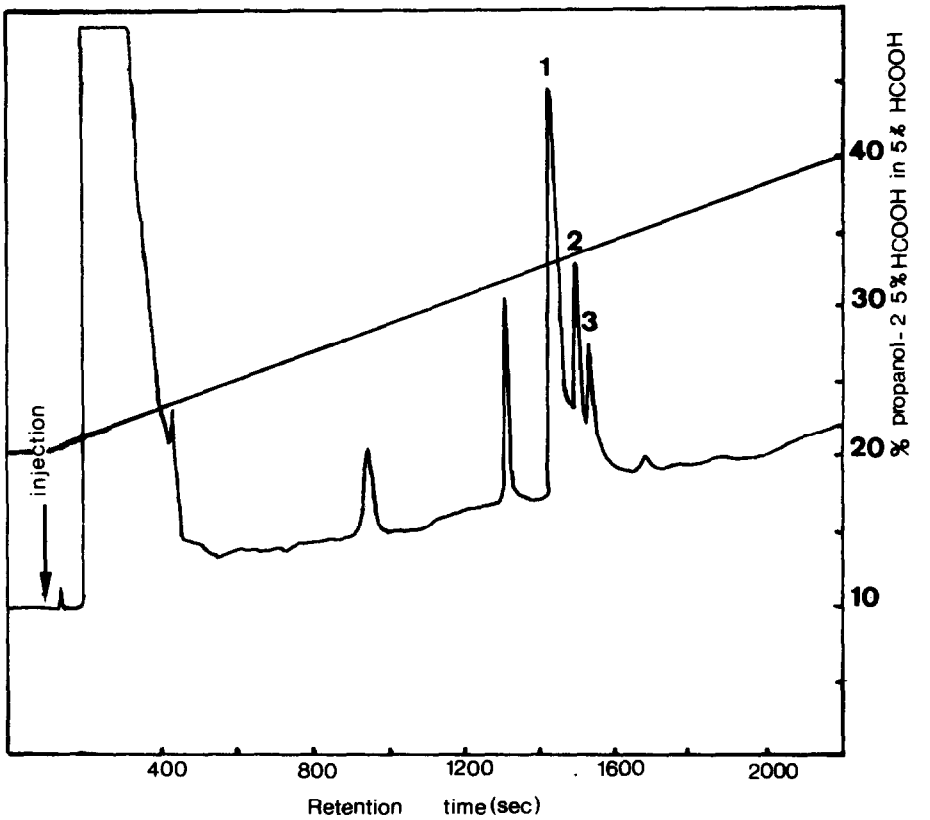

Fig. 3. Chromatogram of 25 µl of roughly purified samples from *N. tabacum* cv. *xanthi* nc seven days after TMV infection (25 µg/ml). Column: µBondapak C_{18}, 9 µm (30 cm × 3.8 mm I.D.). Linear gradient of solvents A–B from 20:80 to 40:60 in 30 min. Flow-rate, 1 ml/min; room temperature; peak detection, 280 nm; pressure, 2500–3500 p.s.i. Peaks: 1 = protein b_1; 2 = protein b_2; 3 = protein b_3.

but the peak of protein b_3 was less separated from the other two on this column (Fig. 3).

Increasing of the support hydrophobicity by lengthening the carbon chain and silanizing of SiOH residues on the silica support was not suitable and gave a poorer resolution of b proteins than the shorter octyl group of LiChrosorb RP-8. In addition, protein b_4 was not eluted under the above chromatographic conditions, probably owing to a retention on the C_8 silica support. This protein probably needed an increase in ionic strength in order to be eluted.

Using a detector sensitivity of 0.04 a.u.f.s., we established that the response was linear with injections of up to 120 ng of protein, but the detection limit was about 50 ng.

Owing to its sensitivity, this technique demonstrated the presence of b_1 proteins in healthy *N. tabacum* cv. *xanthi* (50 ng/g of fresh leaf) (Fig. 4) and a increase in their concentration in the hypersensitive plant after TMV infection with a maximum (80 μg/g of fresh leaf) seven days after virus inoculation (Fig. 5).

On a preparative LiChrosorb RP-8 (7 μm) column, using a detector sensitivity of 0.4 a.u.f.s., we obtained a good resolution for the three b proteins with a linear

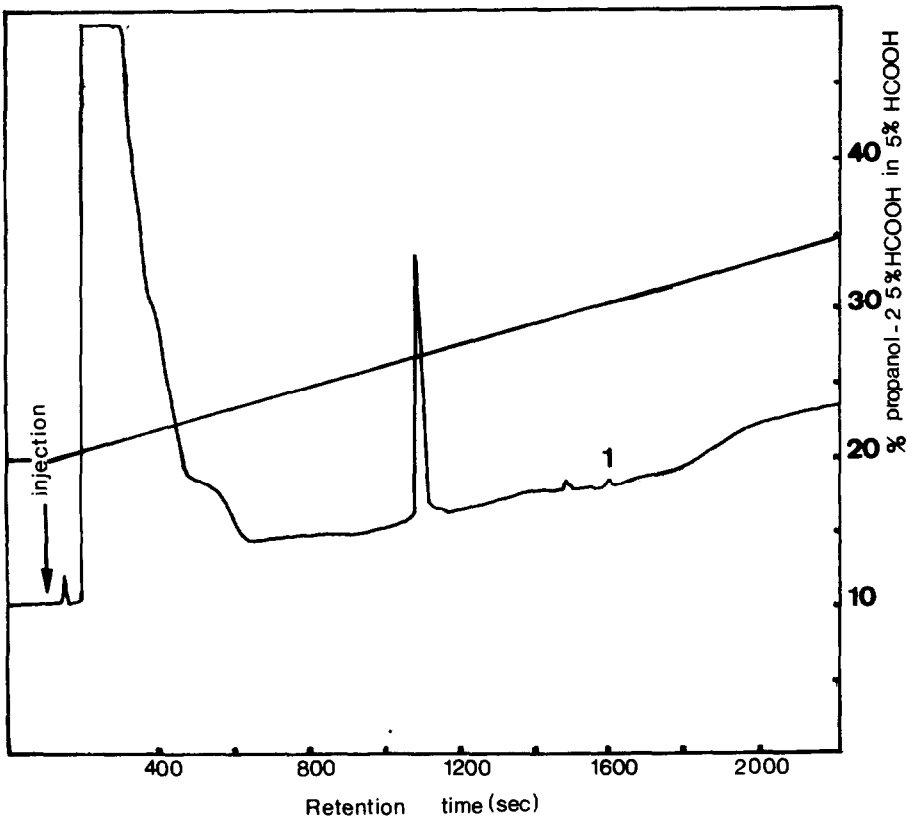

Fig. 4. Chromatogram of roughly purified samples from healthy *N. tabacum* cv. *xanthi*. Column: LiChrosorb RP-8, 10 μm (25 cm × 4 mm I.D.). Conditions as Fig. 1. Peaks: 1 = protein b_1.

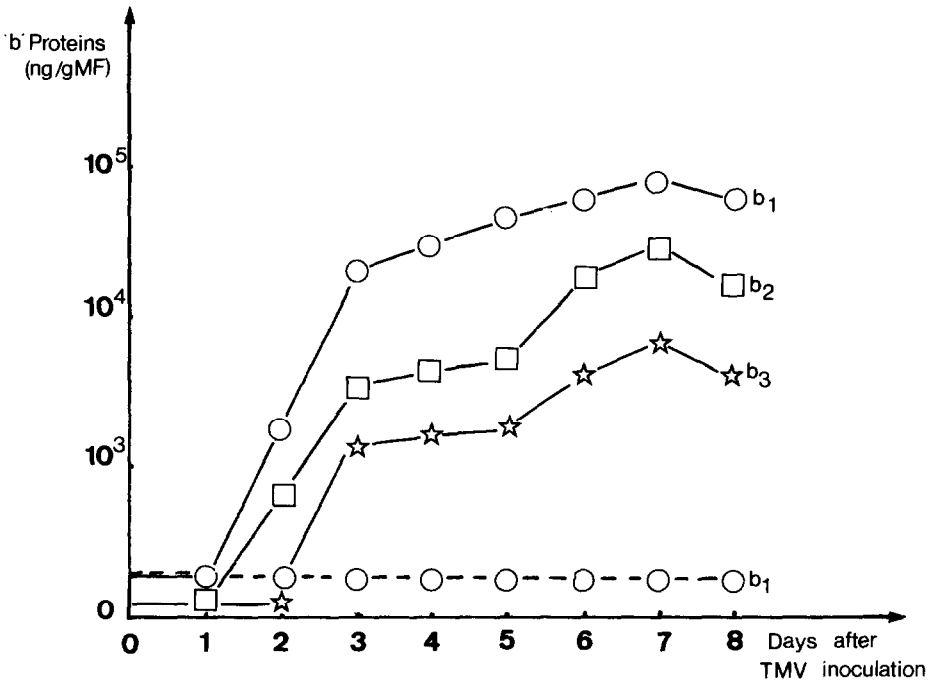

Fig. 5. Kinetic accumulation of b proteins from *N. tabacum* ev. *xanthi* nc 50 days old. Extract from uninoculated leaves (broken line) and TMV (25 μg/ml) infected leaves (solid line). Proteins b_2 and b_3 were undetectable in healthy plants by this technique.

gradient from 20 to 35% of 2-propanol–15% formic acid in aqueous 15% formic acid for 35 min. The chromatogram was identical with those obtained on the analytical LiChrosorb RP-8 (10 μm) column (Fig. 6). Under these chromatographic conditions we collected, from the roughly purified extract, the fractions corresponding to each peak of the b proteins. The collected fractions were analysed by electrophoresis for purity evaluation (Fig. 7), and one typical band occurred for each peak.

CONCLUSION

This technique using a LiChrosorb RP-8 column and 2-propanol–formic acid as the mobile phase, which has been well tested with water-soluble and hydrophobic proteins[19,20], is a very powerful method for the separation and quantification of three b proteins in a complex mixture of proteins with high sensitivity (the detection limit for b proteins is 50 ng) and good recoveries (90%). In addition, the solvents used, with analytical C_8 and C_{18} supports, are fully compatible with the pH range of silica supports (2–8), which was not the case in previously reported methods using high organic acid concentrations for the effective chromatography of proteins[21].

However, an increase in the ionic strength (15% formic acid on preparative LiChrosorb for efficient chromatography of b proteins seemed to show different behaviour between the two silica supports, analyticals RP-8 (10 μm) and preparative RP-8 (7 μm). On the preparative LiChrosorb RP-8 (7 μm), the b proteins were not

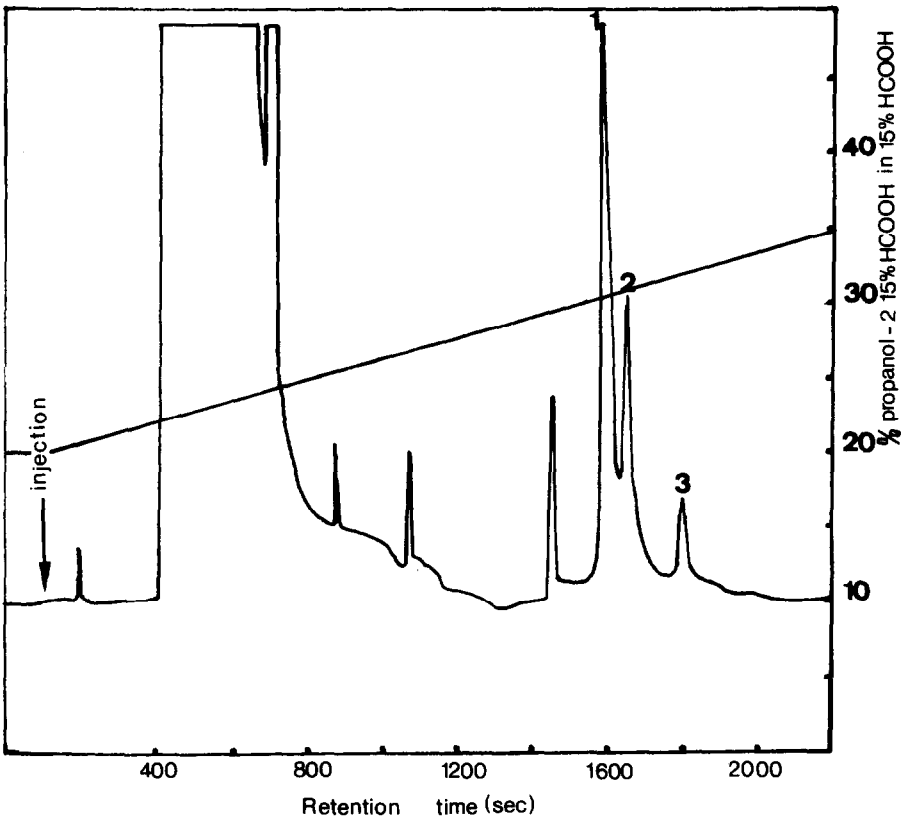

Fig. 6. Chromatogram of roughly purified samples from *N. tabacum* cv. *xanthi* nc. seven days after TMV infection (25 mg/ml). Column: LiChrosorb RP-8, 7 μm (25 cm × 10 mm I.D.). Solvent A, 2-propanol–15% formic acid; solvent B, aqueous 15% formic acid (pH 1.3); linear gradient of A–B from 20:80 to 35:65 in 35 min. Flow-rate, 2 ml/min; room temperature; peak detection, 280 nm; pressure, 1500–2000 p.s.i. Peaks: 1 = protein b_1; 2 = protein b_2; 3 = protein b_3.

eluted with 5% formic acid (RP-8 analytical conditions) and eluted as a training peak with 10% formic acid.

In fact, this method permits the rapid analysis of three b proteins in the same 45-min assay, a result which could not be obtained by indirect enzyme-linked immunosorbent assay (ELISA)[22]. Further, despite a better sensitivity, the immunoassay requires specific antisera against each protein and, as reported [22], purified b_2 and b_3 from *N. tabacum* cv. *xanthi* are serologically closely related to b_1.

We also demonstrated that the b proteins and interferons[23,24] show very similar chromatographic characteristics on reversed-phase supports. The confirms, together with other physico-chemical analogies[5], the partial identity between b proteins in tobacco tissues and interferons in animal cells; hence it could be very useful, in future work, to compare more closely these two classes of molecules (activity, biosynthesis, physiological implications).

We conclude that the reported method should play a valuable role in the study of b proteins, especially when they are involved in intermediate steps in some general

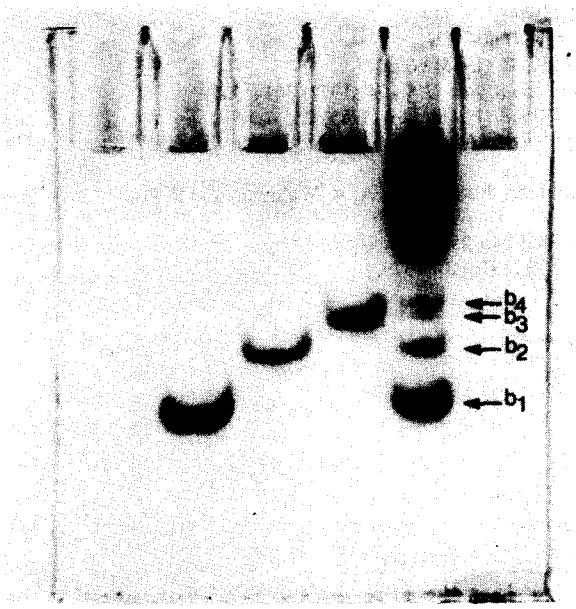

Fig. 7. Electrophoretic pattern of b proteins collected after preparative HPLC on LiChrosorb RP-8, 7 μm. Electrophoresis was conducted at 4°C for 15 min at 2 mA per tube and subsequently for 150 min at 4 mA per tube in 15% acrylamide gels. Gel 1, peak 1 = protein b_1; gel 2, peak 2 = protein b_2; gel 3, peak 3 = protein b_3. Gel 4, injection of roughly purified sample from *N. tabacum* cv. *xanthi* infected by TMV (25 μg/ml).

defence mechanisms in higher plants[25]. In fact, this technique allows the purification of large amounts of b proteins for further biochemical investigations, and this should contribute to a clearer understanding of their biological function in relation to fungal and viral infection.

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