

## Partial Purification and Preliminary Characterization of Soluble Leaf Proteins Specific to Virus Infected Tobacco Plants

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### SUMMARY

The 'b' protein components specific to virus infected tobacco leaves (Gianinazzi, Martin & Vallée, 1970) can be partially purified by preferential extraction at pH 2·8. Evidence is presented that they are rich in aromatic amino acids. Results of treatment of the proteins with SDS and subsequent separation by gel electrophoresis in the presence of SDS suggest that b<sub>1</sub>, b<sub>2</sub> and b<sub>3</sub> are composed of the same monomer of mol. wt. about 16000 whilst b<sub>4</sub> is composed of a monomer of mol. wt. about 29000. By purifying and concentrating the soluble protein extracts of water inoculated leaves, further evidence is provided that the 'b' proteins are not normal constituents of healthy tobacco leaves.

### INTRODUCTION

Studying the changes in soluble leaf proteins during the hypersensitive reaction of *Nicotiana tabacum* cv. Xanthi-nc to tobacco mosaic virus (TMV) infection, Gianinazzi, Vallée & Martin (1969), Gianinazzi (1970) and Gianinazzi *et al.* (1970) demonstrated the appearance of four new leaf protein components (designated b<sub>1</sub> to b<sub>4</sub>) in the living tissue surrounding the local lesions; that is, in that tissue showing resistance to secondary infection (Ross, 1961). Furthermore, when resistance to TMV infection was induced in Xanthi-nc plants after systemic infection with a number of viruses (potato virus X, potato virus Y, cucumber mosaic virus, potato aucuba mosaic virus, alfalfa mosaic virus), or in virus-free tissue by the injection of either polyacrylic acid or extract of *Nocardia asteroides*, one to four of these new 'b' protein components appeared (Gianinazzi & Kassanis, 1974; Kassanis, Gianinazzi & White, 1974; Gianinazzi & Martin, 1975). Van Loon & van Kammen (1970) and van Loon (1975) have in addition demonstrated the induction of similar new leaf protein components in *N. tabacum* cv. Samsun NN after infection with TMV, tobacco necrosis virus, tobacco rattle virus and potato virus Y. Recently Barker (1975) challenged these results, claiming that the reported new protein components 'may be normal constituents of tobacco leaves'.

This paper reports a method for the partial purification of the 'b' proteins together with initial steps towards their characterization, and provides further evidence that they are not normal constituents of healthy tobacco leaves.

## METHODS

*Extraction and quantitative analysis of soluble leaf proteins.* *Nicotiana tabacum* cv. Xanthi-nc was grown under greenhouse conditions. 10- to 11-week-old plants were selected for uniformity, trimmed to four fully expanded leaves and transferred to a constant environment room (20 °C, 16000 lux, photoperiod 16 h and 70 % humidity). Half the plants were infected with TMV and the other half inoculated with water (control). After 7 days leaf samples from either the control or TMV-infected (71 local lesions/g tissue for each experiment) plants were ground in a mortar at 4 °C using 1 ml/g leaf of either 0.1 M-tris buffer, pH 8.0, or MacIlvaine buffer (phosphate-citrate buffer), pH 2.8; both buffers contained 0.3% mercaptoethanol (v/v). Extracts were centrifuged at 45000 g for 30 min.

The soluble proteins of the supernatant fluid were precipitated with 20% (v/v) trichloroacetic acid (TCA) during 2 h at 4 °C. The precipitates were collected by centrifuging at 10000 g for 5 min, and washed 3 times in 5% TCA and 3 times in acetone. The final protein pellet was dried under vacuum and dissolved in a known vol. of 1 N-NaOH. The protein concentration was estimated by the method of Lowry as described by Layne (1957) using a standard curve of bovine serum albumin (Sigma no. A-4378).

One ml of the soluble protein extract at pH 2.8 was passed through a G10 Sephadex column (40 × 2.5 cm) and eluted with the pH 2.8 buffer described above, without mercaptoethanol. The purity of the protein solutions obtained was confirmed by analysing the spectral extinction. The concentration of the protein was determined from the extinction at 280 nm and 210 nm as compared with known concentrations of bovine serum albumin. Equivalent measurements on similarly prepared extracts at pH 8.0 were not possible, probably due to the high level of oxidation.

*Electrophoretic studies.* Control and 7-day TMV-infected, centrifuged, leaf extracts made at pH 2.8 were analysed by disc-electrophoresis in 10% (w/v) polyacrylamide gels at 4 °C using the system of Davis (1964) with a running buffer of pH 8.3: (a) directly, (b) after dialysis against 0.1 M-tris buffer, pH 8.0 for 24 h, and (c) after passing through a G25 Sephadex column (50 × 5 cm), using 0.1 M-tris buffer, pH 8.0, as eluant, followed by lyophilization. After electrophoresis the positions of the protein bands were recorded by densitometry (Acta CIII Beckman spectrophotometer) either immediately at 280 nm or at 560 nm after staining with Coomassie blue (Gianinazzi & Kassanis, 1974). The position of the protein band in the gel was expressed as the  $R_f$  value, taking the distance travelled by the bromophenol blue tracking dye as 1.00.

*Molecular weight determination.* The soluble protein extract, prepared from the 7-day TMV-infected leaf extract, passed through a G25 Sephadex column and lyophilized, was dissolved in water. The proteins were separated by disc-electrophoresis in 10% polyacrylamide gels as described above. After separation the 'b' proteins were extracted by cutting discs of the gels in the regions corresponding to  $b_1$ ,  $b_2$ ,  $b_3$  and  $b_4$ , macerating each in 0.125 M-tris-borate buffer, pH 8.9, containing 1% sodium dodecyl sulphate (SDS) and 0.5% mercaptoethanol, and heating at 80 °C for 2 min. The sample suspensions were left to stand for 2 h and the liquid phase, containing the extracted 'b' protein, was analysed by electrophoresis on a polyacrylamide vertical slab containing 0.1% SDS at pH 8.9, using a separating gel of 12.5% acrylamide, 0.13% *N,N'*-methylenebisacrylamide, 0.05% *N,N,N',N'*-tetramethylethylenediamine (modification of the method described by Koenig *et al.* 1970) and a stacking gel containing 3% acrylamide (Davis, 1964). The marker proteins (Sigma) were horse heart cytochrome *c* (mol. wt. 12400), ovalbumin (mol. wt. 43000) and *Bacillus subtilis*  $\alpha$ -amylase (mol. wt. 97000).

Table 1. Amounts of soluble protein extracted at pH 2.8 and pH 8.0 from water-inoculated control and TMV-infected leaves of *N. tabacum* cv. *Xanthi-nc*

pH	Source leaves*	
	Water-inoculated control	7-day TMV-infected
2.8	0.64 ( $\pm 0.080$ )	1.51 ( $\pm 0.074$ )
8.0	6.23 ( $\pm 0.201$ )	7.18 ( $\pm 0.055$ )

\* The figures represent mg soluble protein per ml extract (mean and standard error) measured using the Lowry method.

After electrophoresis the protein bands were fixed in a 1:4:5 (w/v/v) trichloroacetic acid-methanol-water mixture and stained with Coomassie blue as described above. The sizes of the subunits of the 'b' proteins were calculated on the basis of their relative mobilities (R<sub>m</sub>) in comparison with the marker proteins (Weber & Osborn, 1969).

## RESULTS

### *Quantitative analysis of soluble leaf proteins*

The average amounts of soluble protein, measured by the method of Lowry, extracted at pH 2.8 from 7-day TMV-infected and water-inoculated leaves of *Xanthi-nc* tobacco were 79.0 and 89.7% lower, respectively, than those extracted at pH 8.0 (Table 1). The quantity of soluble protein extracted from the control and TMV-infected leaves at pH 8.0 did not differ greatly, whereas at pH 2.8 significantly larger amounts (136%) of soluble protein were extracted from TMV-infected than control leaves ( $P = 0.005$ ). The results for pH 2.8 were confirmed in the spectrophotometric determinations of the soluble proteins extracted and passed through a G10 Sephadex column. Analysis of the spectral extinction of the protein solutions obtained gave a single well-defined peak with a maximum extinction at 279 to 280 nm, which is a valuable index of the 'cleanness' of the soluble protein preparations. The amount of protein determined at either 280 nm or 210 nm in the TMV-infected leaf extracts as compared with the control (I/C) was always significantly greater than 1.0 (280 nm,  $1.80 \pm 0.11$ ,  $P = 0.005$ ; 210 nm,  $1.39 \pm 0.08$ ,  $P = 0.02$ ). The ratio of  $E_{280}:E_{210}$  nm of control leaf extracts was  $0.038 \pm 0.001$ , comparable to that of bovine serum albumin (0.036), whilst in the extracts of the infected leaves it was significantly different from both the latter ( $0.054 \pm 0.003$ ,  $P = 0.01$ ) and the control ( $P = 0.02$ ).

These results suggest that at pH 2.8 more soluble protein is extracted from TMV-infected than from non-infected control leaves and that the soluble protein extracted from the infected tissue may be richer in aromatic amino acids than that from either the control tissue or bovine serum albumin.

### *Qualitative analysis of soluble leaf proteins*

The soluble protein patterns of the centrifuged extracts at pH 2.8 from water-inoculated and 7-day TMV-infected *Xanthi-nc* tobacco leaves are presented in Fig. 1. Fig. 1(a) and (b) represent the protein bands of control and TMV-infected leaves respectively in gels stained with Coomassie blue (560 nm), and Fig. 1(c) and (d) are scans at 280 nm of unstained replicate gels. After staining, four new protein components appeared with R<sub>f</sub> of 0.87, 0.70,

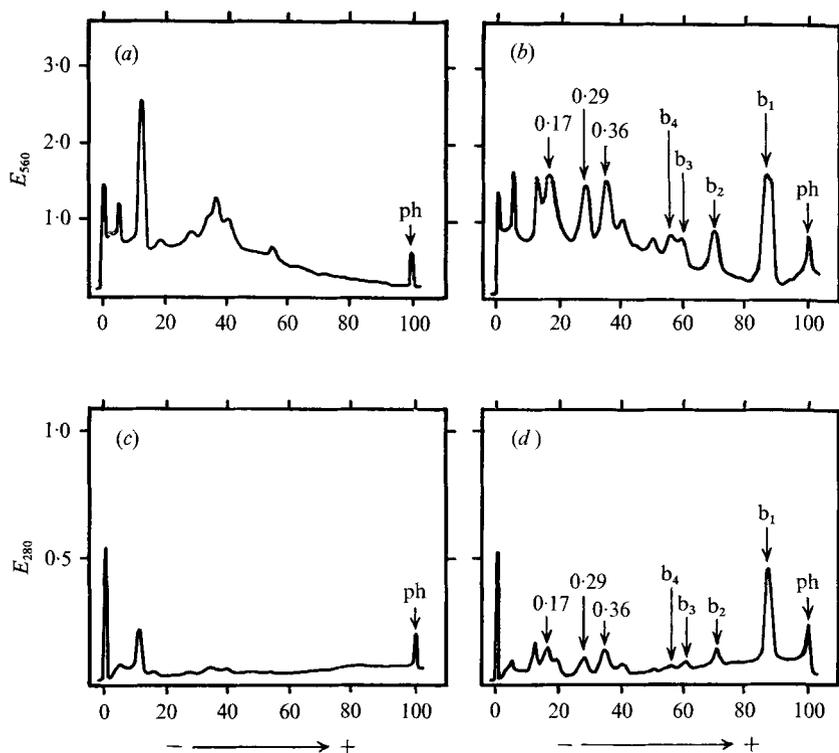


Fig. 1. Densitometer tracings of electrophoretic patterns of soluble proteins of water-inoculated (a and c) and 7-day TMV-infected (b and d) leaves of *N. tabacum* cv. Xanthi-nc extracted at pH 2.8; (a, b) extinction at 560 nm of gels stained with Coomassie blue after electrophoresis of 50  $\mu$ l of centrifuged extract; (c, d) extinction at 280 nm of unstained gels after electrophoresis of 100  $\mu$ l of centrifuged extract.  $b_1$ ,  $b_2$ ,  $b_3$  and  $b_4$ , new protein components; ph, bromophenol blue; additional band changes are indicated by their  $R_f$  values.

0.60 and 0.56 respectively (Fig. 1 b). These were the same as the new protein components previously obtained after extraction of TMV-infected leaves using 0.1 M-tris buffer, pH 8.0, and designated by Gianinazzi *et al.* (1970) as  $b_1$ ,  $b_2$ ,  $b_3$  and  $b_4$ . By comparing the peak areas on the electrophoretic patterns of the 'b' proteins extracted at pH 2.8 with those extracted from the same amount of leaf tissue at pH 8.0, no significant differences were found, although they were always a little larger at pH 2.8. Extraction at pH 2.8 also revealed further differences in the soluble protein composition between the control and TMV-infected tissue at the  $R_f$  positions 0.36, 0.29 and 0.17. In the densitometer tracings obtained at 280 nm only a few peaks were visible in the control (Fig. 1 c) whilst many peaks were present in the TMV-infected extracts and in particular in the same positions as the  $b_1$ ,  $b_2$  and  $b_3$  proteins and at the  $R_f$  0.36, 0.29 and 0.17 (Fig. 1 d).

Identical results were obtained when extracts were dialysed against 0.1 M-tris buffer, pH 8.0 before electrophoretic analysis.

Lyophilization of the extracts which had been passed through G25 Sephadex in no way altered the pattern of the 'b' proteins in the TMV-infected extracts (Fig. 2 b), their  $R_f$  being identical to those in Fig. 1. The only difference was a decrease in the intensity of the band  $R_f$  0.12 in the TMV-infected plant pattern. Furthermore, by lyophilizing the soluble proteins they could be concentrated up to four times as compared with the original extracts. Even

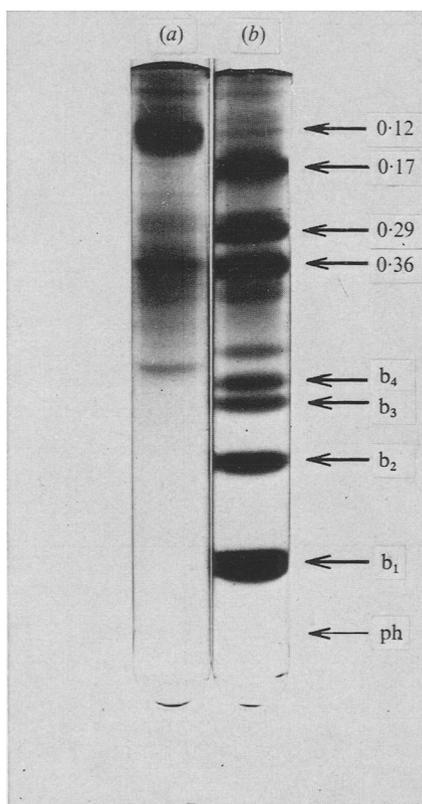


Fig. 2. Electrophoresis of soluble leaf protein extracts (equivalent to 100  $\mu$ l of original extract) of *N. tabacum* cv. Xanthi-nc after having been passed through G25 Sephadex and lyophilized. (a) Water-inoculated control and (b) 7-day TMV-infected leaves:  $b_1$ ,  $b_2$ ,  $b_3$  and  $b_4$ , new protein components; ph, bromophenol blue; additional band changes are indicated by their  $R_f$  values.

at this concentration no protein bands were detected in the electrophoretic pattern of the uninfected control in the positions corresponding to the 'b' proteins. Further concentration of the soluble proteins resulted in overloaded gels.

#### *Molecular weight of the 'b' proteins*

SDS treatments of  $b_1$ ,  $b_2$  and  $b_3$  yielded a single band of protein with a mol. wt. of 16000, showing that they were composed of the same monomer. The single band of protein derived from  $b_4$ , however, had a mol. wt. of 29500 indicating that it was composed of a different monomer (Fig. 3).

#### DISCUSSION

The amounts of soluble protein which can be extracted at pH 8.0 from TMV-infected and water-inoculated leaves of *N. tabacum* cv. Xanthi-nc do not differ greatly. At pH 2.8 considerably less soluble protein is extracted from both, and significantly less from the control than the TMV-infected tissue. However there is no reduction in the amounts of the 'b' proteins extracted at pH 2.8 as compared with pH 8.0 from the TMV-infected leaves. Thus, by using pH 2.8 the 'b' proteins can be partially purified from the mass of soluble protein present in a TMV-infected leaf. Comparison of Fig. 2 in this paper with Fig. 3 of Gianinazzi

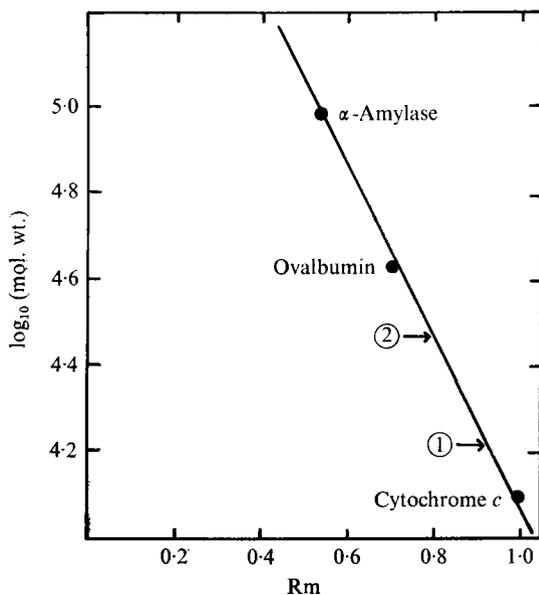


Fig. 3. Relative mobilities (Rm) in 12.5% polyacrylamide+0.1% SDS of different proteins of known mol. wt. (markers) and of the monomers of the 'b' proteins. 1, Rm of monomer of b<sub>1</sub>, b<sub>2</sub> and b<sub>3</sub>; 2, Rm of monomer of b<sub>4</sub>.

*et al.* (1970), Fig. 6 of Gianinazzi & Kassanis (1974) and Fig. 1 of Kassanis *et al.* (1974) clearly illustrates this point.

Because at pH 2.8 the level of oxidation of the extracted proteins is considerably less than at pH 8.0, we have been able to study the protein solutions obtained after passing through a G10 Sephadex column. The  $E_{280}:E_{210}$  ratio of this soluble protein is significantly greater in the TMV-infected leaf extracts than in the control, suggesting that the former may be richer in aromatic amino acids. This is supported by the densitometer tracings at 280 nm of the unstained polyacrylamide gel containing TMV-infected and control soluble protein extracts. There is virtually no absorption at this wavelength by the soluble proteins from control leaves whilst those from TMV-infected leaves produce obvious absorption peaks, especially in the positions corresponding to the 'b' proteins. This suggests that the 'b' proteins are particularly rich in aromatic amino acids.

Further characterization of the 'b' proteins after electrophoresis in SDS gel has shown that the b<sub>1</sub>, b<sub>2</sub> and b<sub>3</sub> protein components can each be broken down to the same monomer, the mol. wt. of which is about 16000, whilst the monomer of b<sub>4</sub> appears to be different, having a mol. wt. of 29500. We suggest that the b<sub>2</sub> and b<sub>3</sub> proteins are polymers of the b<sub>1</sub> protein. The fact that this is a property of the interferon in animal cells (Colby & Morgan, 1971) strengthens the hypothesis previously put forward by Gianinazzi *et al.* (1970) that the 'b' proteins are interferon-like. This possible role of the 'b' proteins is further underlined by the fact that their synthesis is stimulated in healthy tobacco leaves, as interferon is in animal cells, when virus resistance is induced by the injection of polyacrylic acid or bacterial extracts (De Clercq, Eckstein & Merigan, 1970; Galabov & Galabov, 1973; Gianinazzi & Kassanis, 1974; Gianinazzi & Martin, 1975). If this analogy with the interferon of animal cells is correct, it is important that experiments are carried out under well-defined, controlled conditions since small amounts of interferon can be induced by a variety of stimuli.

By lyophilization we were able to concentrate the soluble protein solutions up to four times compared with the original extract. However, even at this high concentration, no new soluble protein components appear in the control uninfected leaf extracts. This is in contradiction to Barker (1975) who claimed that by over-exposing photographs of electrophoretic gels, protein bands could be detected in extracts of uninfected tissues. If Barker is right then a concentration of four times the protein extract should have revealed any possible weak protein bands in the control leaf extracts which are not revealed at the normal concentration. We re-affirm that the 'b' proteins are not normal constituents of tobacco plants and support van Loon's (1976) opinion that 'the conclusion of Barker (1975) that the four new proteins previously thought to occur only in virus-infected tobacco leaves are probably normal constituents is based on an erroneous interpretation of the banding pattern obtained by polyacrylamide gel electrophoresis'.

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