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The Purification and Properties of One of the 'b' Proteins from Virus-infected Tobacco Plants

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SUMMARY

The b_1 protein, produced in leaves of *Nicotiana tabacum* cv. Xanthi-nc following infection with tobacco mosaic virus, has been purified to homogeneity by a procedure which involves gel chromatography and absorption on to DEAE-cellulose. One gel chromatography step was sufficient when the procedure was applied to leaf extracts made in an acid buffer, whereas two were necessary with extracts made at pH 8. The final product migrates as a single protein band on electrophoresis in both acrylamide and SDS–acrylamide gels. Its mol. wt. is estimated to be 15000 by electrophoresis and 14200 by ultracentrifugation. Amino acid analysis suggests that it contains about 136 residues of which 39 are potentially acidic, 13 basic and 16 aromatic. The absorbance coefficient $A_{280\,nm}^{1\%}$ is estimated to be 18·9. No evidence was found for the presence of a nucleotide component.

INTRODUCTION

When *Nicotiana tabacum* varieties react hypersensitively to infection with tobacco mosaic virus (TMV), local necrotic lesions appear on the leaves at the sites of infection and the virus is restricted to the cells of the lesion and the immediately adjacent tissue (Martin & Gallet, 1966; Israel & Ross, 1967). The leaves become resistant to further infection with TMV and this induced resistance extends to uninoculated leaves on the plant (Ross, 1961*a*, *b*). The resistance is not specific for the inducing virus but holds for others which produce necrotic lesions on these plants.

Analysis of the leaves of both Xanthi-nc and Samsun NN tobacco by acrylamide gel electrophoresis demonstrates that resistant leaves contain at least four proteins that are not present in uninfected plants (Gianinazzi *et al.* 1969; Van Loon & Van Kammen, 1970). These proteins, designated b_1 to b_4, seem to be involved in the induced resistance; they appear in inoculated leaves at the same time that resistance develops and they slowly disappear at temperatures where the resistance disappears (Gianinazzi & Kassanis, 1974; Van Loon, 1975). Moreover, at least three of these proteins appear when plants are made resistant to virus infection by injection of polyacrylic acid. Attempts to demonstrate that the proteins directly prevent the spread or multiplication of virus have not so far been successful (Kassanis *et al.* 1974). However these attempts are continuing and require a supply of native purified b proteins. Recently Gianinazzi *et al.* (1977) partially purified these proteins and suggested that b_2 and b_3 are dimer and trimer forms of b_1, whereas b_4 is not so closely related. However the extraction conditions used involved treatment in conditions acid enough to affect the tertiary structure of proteins and to remove any

enzymic or biological activity that they possessed. We therefore describe a convenient method of purifying b_1 to homogeneity which does not involve harsh conditions and gives a characterized product which is suitable for testing in biological systems.

METHODS

Buffers. The following three buffers were used: buffer A containing 84 mM-citric acid, 32 mM-Na_2HPO_4, 14 mM-2-mercaptoethanol and 6 mM-L-ascorbic acid at a final pH of 2·8; buffer B containing 100 mM-tris-HCl, 1 mM-EDTA, 5 mM-sodium diethyl dithiocarbamate and 14 mM-2-mercaptoethanol, final pH 8·0 at 25 °C; buffer C containing 50 mM-tris-HCl, 1 mM-EDTA and 3 mM-2-mercaptoethanol, final pH 8·0 at 25 °C. Buffers A and B were used in the extraction of the proteins from the tobacco leaves at low and neutral pH respectively; buffer C was used throughout the purification of the b_1 protein. Further additions to buffer C are specified when they were used.

Protein determination. Protein was determined by the methods of Lowry *et al.* (1951), after precipitation with 25 % trichloroacetic acid, and of Bradford (1976). Bovine serum albumin was used as the standard and its concentration determined spectrophotometrically, assuming that a 1 % (w/v) solution has an absorbance of 6·0 at 280 nm.

Protein hydrolysis and analysis. The b_1 protein was dialysed against distilled water and then lyophilized. Duplicate samples were hydrolysed under nitrogen in 6 N-HCl containing 10 mM-phenol for 24, 48 and 72 h at 110 °C. The samples were analysed with a Technicon amino acid analyser. Tryptophan in the b_1 protein was determined spectrophotometrically by the method of Bencze & Schmid (1957).

Acrylamide gel electrophoresis. This was performed on 10 % (w/v) gels crosslinked with 0·27 % *N,N'*-methylenebisacrylamide at pH 8·3 as described by Ornstein (1964) and Davis (1964) or on the same gels containing sodium dodecyl sulphate (SDS) buffered with phosphate at pH 7·2 as described by Weber & Osborn (1969) and modified by Cohen (1973). Samples for SDS-polyacrylamide gel electrophoresis were denatured by heating at 100 °C for 5 min in 1 % SDS containing 100 mM-2-mercaptoethanol and 10 mM-sodium phosphate, pH 7·2. The following marker proteins were used for the estimation of the subunit mol. wt. of the b_1 protein: cytochrome *c* (mol. wt. 12400), TMV coat protein (17700), α-chymotrypsinogen (25900), carbonic anhydrase (29500), lactate dehydrogenase (36000), ovalbumin (43000) and bovine serum albumin (68000). The polyacrylamide gels were stained for protein using Coomassie brilliant blue G250 (Reisner *et al.* 1975; Holbrook & Leaver, 1976). The SDS-polyacrylamide gels were also stained for protein using Coomassie Brilliant Blue (Weber & Osborn, 1969) and destained by immersion in a solution containing 14 % acetic acid and 10 % methanol.

Gel filtration. Sephadex G-50 (fine) was mixed with excess buffer C for 24 h, degassed and poured into the preparative (84 × 5 cm) and analytical (140 × 1 cm) columns. The columns were calibrated using blue dextran, carbonic anhydrase (mol. wt. 29500), myoglobin (17800) and cytochrome *c* (12400).

Ion exchange chromatography. The DEAE-cellulose (DE-32; Whatman) was treated with 0·5 N-HCl followed by 0·5 N-NaOH as recommended by the manufacturer, washed with distilled water, suspended in several changes of buffer C, degassed and poured into a column (12 × 2·5 cm).

Ultracentrifugation. This was performed on a Spinco model E analytical ultracentrifuge. The mol. wt. of the b_1 protein was determined by the high speed sedimentation equilibrium method in buffer C at 15 °C and 48000 rev/min. The b_1 protein had previously been

Table 1. *Purification of b_1 protein*

Step	Extract made at pH 2·8		Extract made at pH 8·0		
	Volume (ml)	Total protein (mg)	Volume (ml)	Total protein (mg)	Presence of b_1
Leaf extract	217	78·3	180	643	+
1. Chromatography on Sephadex G-50					
Pooled fractions of void volume	227	31·1	265	544	−
Pooled fractions containing b_1	326	40·4	324	80·7	+
2. Chromatography on DEAE-cellulose					
Unabsorbed material	318	19·8	304	19·0	−
Material eluted with buffer containing 140 mM-NaCl	232	11·8	121	22·4	−
Material eluted with buffer containing 200 mM-NaCl	63	4·8	34	5·9	+
3. Chromatography on Sephadex G-50					
Pooled fractions containing b_1	—	—	17·6	3·4	+

dialysed extensively against buffer C and was used at 0·4 mg/ml. The density of the buffer was determined pycnometrically. The partial specific volume of the protein was calculated from the amino acid composition as described by Cohn & Edsall (1943).

Absorption coefficient. The absorbance and protein concentration of the b_1 protein were measured in buffer C. The protein concentration was measured from a synthetic boundary experiment carried out at 15 °C in the ultracentrifuge using the method of Babul & Stellwagen (1969).

Plant material, inoculation and extraction of protein. Batches of 12 *Nicotiana tabacum* cv. Xanthi-nc plants were grown in 12 cm pots in a glasshouse. When the plants were about 8 weeks old the leaves were inoculated with TMV, the lower leaves with 1 μg TMV/ml and the upper leaves with 5 μg TMV/ml. After 7 days, leaves showing symptoms were removed, deribbed and homogenized with 1 vol. of either buffer A or buffer B in a Kenwood blender at high speed for 2 min. The homogenate was filtered through 4 layers of muslin and centrifuged at 20000 *g* for 15 min. The supernatant was taken as the starting material for the remainder of the preparation and is referred to as the extract.

RESULTS AND DISCUSSION

Purification of b_1 protein

The b_1 protein could be purified from extracts made at pH 2·8 by a rapid two-step process (Table 1). In the first step, up to 200 ml of the extract was chromatographed on Sephadex G-50 equilibrated with buffer C. The b_1 protein, as judged by acrylamide gel electrophoresis of the fractions, was slightly retarded on the column, and well separated from a large proportion of the soluble leaf protein which was eluted in the void volume (Fig. 1). This step also removed material of low molecular weight and exchanged the buffering solution for one more suitable for the next step. Fractions containing b_1 protein were then loaded on to a DEAE-cellulose column also equilibrated in buffer C. The b_1 protein was tightly absorbed and could not be removed by washing with buffer C containing 140 mM-NaCl; other proteins present were either not absorbed or, like the b_2, b_3 and b_4 proteins, were absorbed and eluted with the salt wash (Table 1). Essentially homogeneous

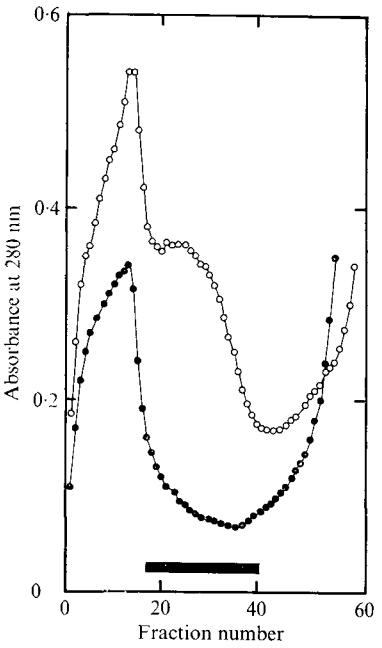

Fig. 1.

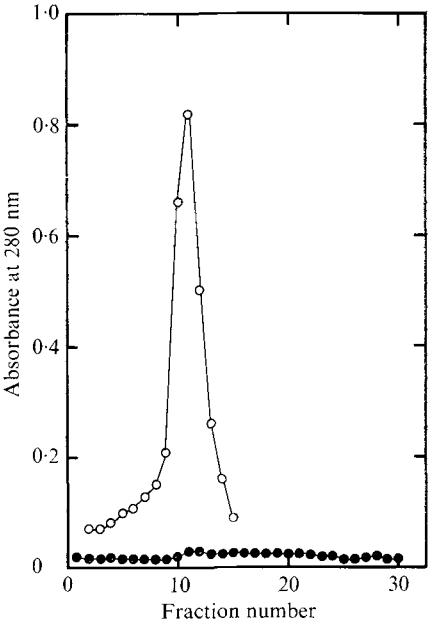

Fig. 2.

Fig. 1. Chromatography of leaf extracts on Sephadex G-50. Extracts were made in the pH 2·8 buffer from uninoculated leaves (●) and TMV-infected leaves (○). Samples (200 ml) were chromatographed on the Sephadex G-50 column (85 × 5 cm) and fractions of the eluate (about 13 ml) were collected and their absorbance at 280 nm was measured. The bar marks fractions, derived from the TMV-infected plants, which contain the b_1 protein.

Fig. 2. Elution of b_1 protein from DEAE-cellulose. The appropriate eluate fractions derived from the chromatography of an extract of TMV-infected leaves on Sephadex G-50, were pooled (300 ml) and absorbed on to a column of DEAE-cellulose (12 × 2·5 cm) equilibrated with buffer C. The column was washed with buffer C containing 140 mM-NaCl, and b_1 (○) was then eluted with buffer C containing 200 mM-NaCl. Little or no protein was recovered when the procedure was applied to extracts made from uninoculated leaves (●).

b_1 protein was recovered from the DEAE-cellulose with buffer C containing 200 mM-NaCl (Fig. 2). It was pooled, dialysed and concentrated by freeze-drying and then redissolved in 1 to 2 ml buffer C, redialysed against this buffer and stored at 0 to 4 °C.

The b_1 protein could also be purified from leaf extracts made in non-acid conditions from infected plants by essentially the same procedure. Such extracts made at pH 8·0 contained much more protein than those made at pH 2·8, including a protein(s) of high molecular weight which had low mobility on acrylamide gels. A small proportion of this protein(s) contaminated the b_1 protein eluted from DEAE-cellulose. However, it was readily removed, after concentration by freeze-drying, by a second gel filtration on a column (140 × 1 cm) of Sephadex G-50 leaving essentially pure b_1 protein.

About 5 mg of purified b_1 protein were obtained from the 600 mg of soluble protein extracted from the leaves of 12 plants, suggesting that it represents about 1 % of soluble protein 7 days after infection. This figure, which is likely to be an underestimate, assumes that the protein is spread throughout the leaf, whereas it is likely to be more concentrated in regions adjacent to necrotic lesions (Rohloff & Lerch, 1977). No b_1 protein could be detected in extracts made at pH 2·8 from uninfected plants or in the appropriate fractions derived from applying the purification procedure to these extracts (Fig. 1 and 2).

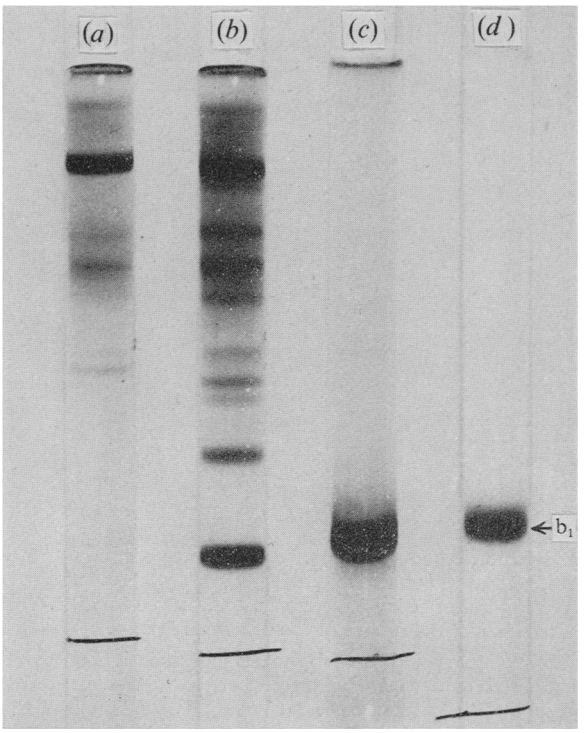

Fig. 3. Gel electrophoresis of leaf extracts and of purified b_1 protein. Extracts made at pH 2·8 from uninoculated leaves (*a*) and TMV-infected leaves (*b*) were analysed by electrophoresis in acrylamide gels at pH 8·3. Purified b_1 protein was analysed similarly (*c*) and also at pH 7·2 in the presence of SDS (*d*).

The elution of the b_2, b_3 and b_4 proteins from the DEAE-cellulose column by a lower salt concentration than was necessary to elute the b_1 protein, suggests that these proteins may not be polymers of b_1 (Gianinazzi *et al.* 1977); recently Rohloff & Lerch (1977) have suggested that the four proteins are two pairs of charge isomers.

Purity of b_1 preparations

Purified b_1 preparations gave a single Coomassie blue-staining band on electrophoresis in acrylamide gels at pH 8·3, and also in gels containing SDS at pH 7·2 (Fig. 3). Examination of their behaviour during ultracentrifugation by interference optics (Fig. 4) also suggested that the preparation had one component. The u.v. spectra of the preparations were typical of proteins uncontaminated with nucleic acid: the ratio A_{280}/A_{260} for the spectrum shown in Fig. 5 was 1·84.

Molecular weight of the b_1 preparations

The mol. wt. of the single component in b_1 preparations was 15000 as determined from its mobility in SDS–acrylamide gels (Fig. 6), and 14200 as determined from its sedimentation in the ultracentrifuge (Fig. 4). Its mol. wt. determined from gel filtration on Sephadex G-50 was apparently 18500, higher than the other values; however, few marker proteins were used in this determination and the behaviour of proteins on gel filtration in the absence of SDS is influenced by molecular shape as well as size. The average of the values deter-

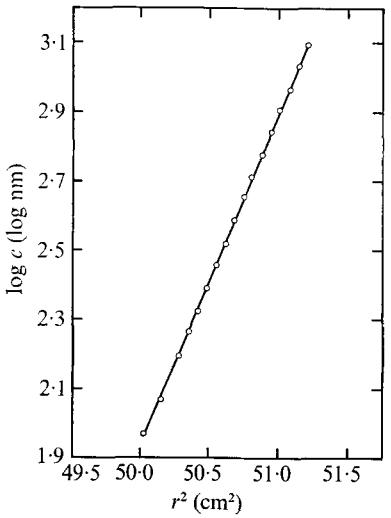

Fig. 4.

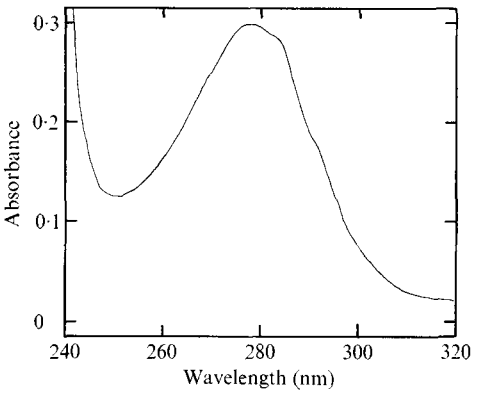

Fig. 5.

Fig. 4. High speed sedimentation equilibrium of b_1 protein. A solution of b_1 protein (0·4 mg/ml) in buffer C was centrifuged at 48000 rev/min at 15 °C. After 8 h, when the protein had reached equilibrium, it was photographed using interference optics. The log interference fringe displacement (log c) was plotted against the square of the distance from the centre of rotation (r^2).

Fig. 5. Ultraviolet absorption spectrum of b_1 protein. A solution of b_1 protein was dialysed extensively against buffer C at 0 to 4 °C and centrifuged to remove denatured material. The solution was diluted in the dialysis buffer to 0·16 mg b_1/ml and its u.v. absorbance compared with that of the dialysis buffer was measured using a Beckman model 25 spectrophotometer.

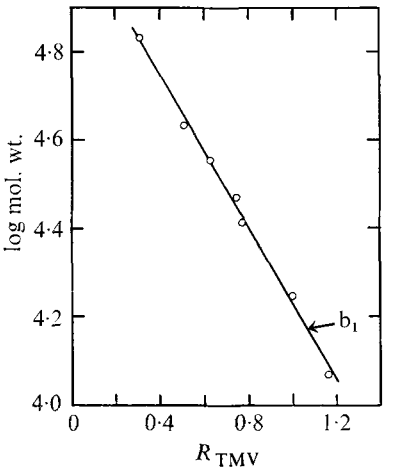

Fig. 6. Molecular weight of b_1 protein by electrophoresis in SDS–acrylamide gels. The mobility of b_1 and the reference proteins was determined relative to the mobility of TMV-protein in SDS-acrylamide gels, and the resulting R_{TMV} plotted against log mol. wt.

mined by the first two methods, 14600, is a little lower than the 16000 determined using SDS-electrophoresis by Gianinazzi *et al.* (1977). The b_1 protein has a lower mol. wt. than that found for the novel protein (E_1; mol. wt. 22000) which appears in extracts of leaves of cucumber which are infected with tomato spotted wilt virus (Tas & Peters, 1977).

Table 2. *Amino acid composition of* b_1

	mol/100 mol	Amino acid residues/15 000 g protein
Aspartic acid	15·5	20·9 (21)
Threonine	4·1	5·5 (6)
Serine	6·6	8·9 (9)
Glutamic acid	12·4	18·1 (18)
Proline	3·5	4·7 (5)
Glycine	9·0	12·1 (12)
Alanine	11·2	15·1 (15)
Valine	9·4	12·6 (13)
Methionine	0·9	1·2 (1)
Isoleucine	0·6	0·8 (1)
Leucine	4·2	5·7 (6)
Tyrosine	8·1	10·9 (11)
Phenylalanine	0·9	1·2 (1)
Histidine	4·0	5·4 (5)
Lysine	2·1	2·8 (3)
Arginine	3·6	4·9 (5)
Tryptophan	3·0	4·0 (4)
Cysteine	ND*	ND
		Total 136

ND* = Not determined.

Composition of b_1

Amino acid analysis of hydrolysates of b_1 frequently contained small amounts of unidentified components that are probably unhydrolysed peptides. These are less evident after longer periods of hydrolysis and are neglected in the amino acid composition shown in Table 2.

A high proportion of residues are potentially acidic (29 %) and comparatively few (9 %) are basic. The low partial specific volume of b_1 ($\bar{v}$ = 0·706), calculated from the amino acid composition reflects the high proportion of acidic residues and glycine. It is not known what proportion of residues are amidated, but the behaviour of the protein on DEAE-cellulose suggests that the proportion is not high. The content of tyrosine and tryptophan is, as suggested by Gianinazzi *et al.* (1977), relatively high; this is consistent with a high absorption coefficient which was estimated to be 18·9 ($A^{1\%}_{280\,nm}$).

Purified preparations of b_1 are currently being tested for proprties that may be relevant to its possible role in localizing virus infection.

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