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AN ANTIVIRAL PROTEIN FROM *BOUGAINVILLEA SPECTABILIS* ROOTS; PURIFICATION AND CHARACTERISATION

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Key Word Index—*Bougainvillea spectabilis*; Nyctagynaceae; protein purification; anti-viral protein.

Abstract—An antiviral protein active against mechanical transmission of tomato spotted wilt virus was identified in the root tissues of *Bougainvillea spectabilis* Willd. Bougainvillea Antiviral Protein I (BAP I) was purified to apparent homogeneity from the roots of Bougainvillea by ammonium sulphate precipitation, CM- and DEAE-Sepharose chromatography and reverse phase HPLC. BAP I is a highly basic protein (pI value > 8.6) with an Mr of 28 000. The N-terminal sequence of BAP I showed homology with other plant antiviral proteins. Preliminary tests suggest that purified BAP I is capable of interfering with *in vitro* protein synthesis.

INTRODUCTION

A large number of plants have been reported to contain anti-viral proteins [1–5] and a few have been purified and characterised. These anti-viral proteins are basic proteins with Mr values ranging from 24 000 to 32 000, and act against a range of plant viruses [1, 3, 6, 7]. Tomato spotted wilt virus (TSWV) is an important plant virus whose host range includes more than 400 plant species in both the monocots and dicots. TSWV replicates both in plant hosts and in thrips, which act as transmission vectors [8, 9]. The wide host range of this virus and its vector, as well as the lack of effective management strategies, leads to major worldwide economic losses [10]. Through screening plant extracts against TSWV we found that an extract of *Bougainvillea spectabilis* Willd contained inhibitory activity against this virus. This paper describes the purification and characterisation of Bougainvillea antiviral protein (BAP I) obtained from the root tissues of *B. spectabilis*.

RESULTS AND DISCUSSION

Purification of BAP I

Preliminary studies had shown that antiviral activity was considerably higher in roots than in leaves of Bougainvillea. Roots of Bouginvillea were therefore extracted as described in the experimental section. The majority of the antiviral activity was recovered from a 40–90% ammonium sulphate fraction. The pellet was redissolved in buffer A, dialysed against the same buffer and the dialysate lyophilised. The lyophilized powder was dissolved in buffer B and was fractionated by CM-Sepharose cation exchange chromatography (Fig. 1A) where fractions 51–54 eluting at about 150 mM NaCl contained antiviral activity. The pooled fractions were lyophilized, dissolved in buffer C and further fractionated by DEAE-Sepharose anion exchange chromatography, where antiviral activity came through in the unbound fraction. The fraction was pooled, adjusted to pH 6.0 and was resubmitted to CM-Sepharose cation exchange chromatography (Fig. 1B) where antiviral activity was detected in fractions 44–48 which also coincided with the point where bound protein eluted. RP-HPLC of active fractions from the Fig. 1A pool (Fig. 1C; Trace A) and from the Fig. 1B pool (Fig. 1C; Trace B) separated into two protein peaks, respectively. However, enzyme assay of fractions from the two HPLC separations showed that only the first elution peaks had the same retention times and had BAP I antiviral activity. This purified material was used to characterise the active protein.

Characterisation of BAP I

The purified protein showed a single band on SDS-PAGE (Fig. 2a). Whereas some plant derived antiviral

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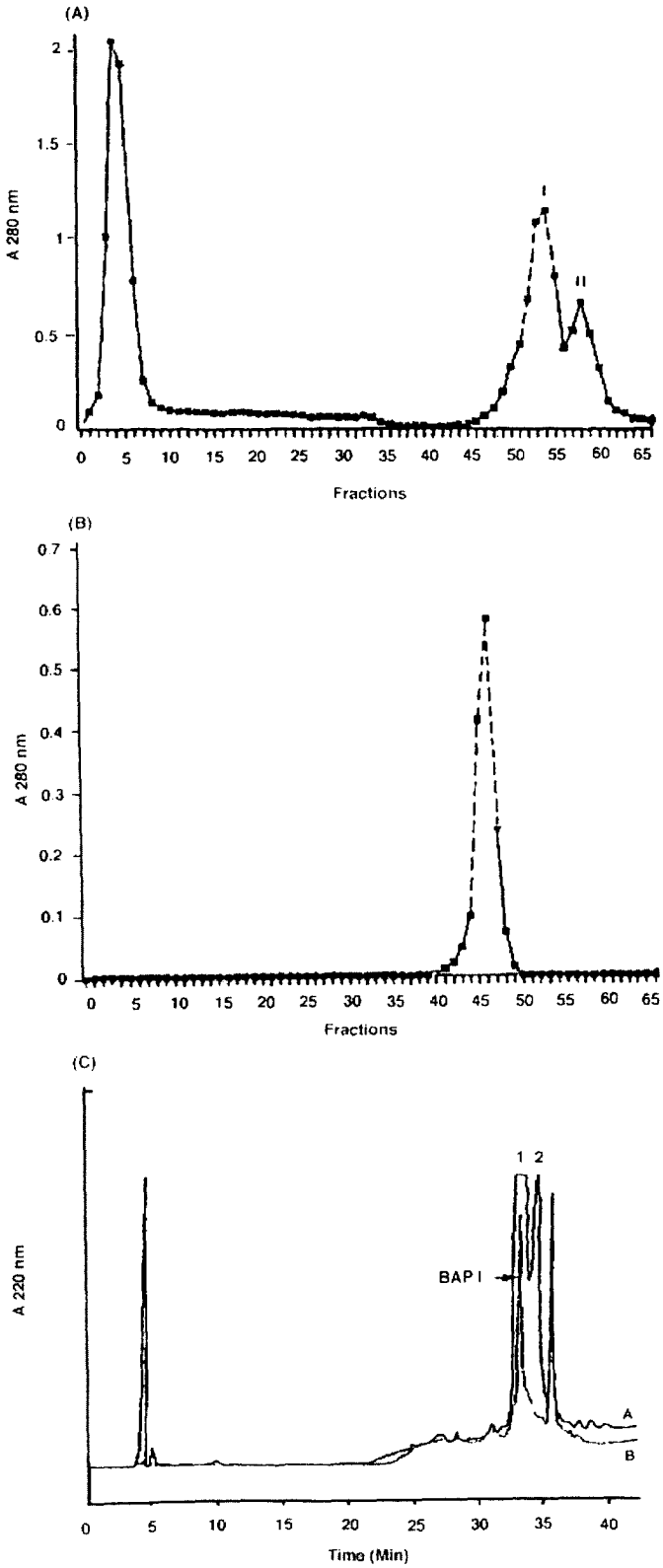

Fig. 1. Chromatographic purification of BAP I. (A) CM-sepharose cation exchange chromatography elution profile of proteins absorbing at 280 nm. I and II indicate fractions containing BAP I enzyme activity. (B) Peak I was pooled (fractions 51–54) and was resubmitted to CM-Sepharose chromatography after the DEAE-Sepharose step (described in experimental). (C) RP-HPLC of peak I (Trace A) from Fig. 1A and of the purified protein (fractions 42–48) in Fig. 1B from the second CM-Sepharose chromatography (Trace B).

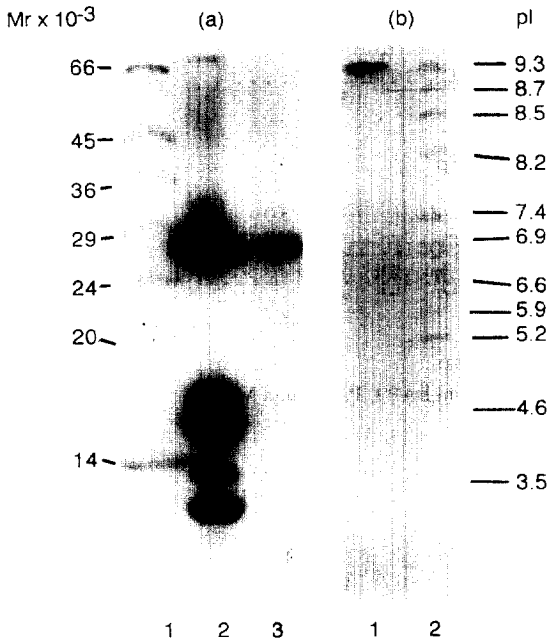

Fig. 2. Electrophoretic analysis of purified BAP I. (a) SDS PAGE. Track 1: molecular weight markers; Track 2: initial crude extract; Track 3: purified BAP I. (b) Isolectric focusing. Track 1: purified BAP I; Track 2: pI markers.

proteins, such as PAP-S, AVP from *Punica granatum*, *Boerhaavia diffusa* and gelonin have been shown to be glycoproteins (13, 15–17), no carbohydrate could be detected in BAP I.

Isoelectric focusing showed the protein to migrate as a single band, to the cathodic end of the focused gel, at a point close to that of trypsinogen (pI 9.3) (Fig. 2B). Although an exact pI could not be determined at this extreme of the gel, BAP I is clearly a very basic protein. Many other plant antiviral proteins have been shown to be basic proteins. For example, the pI of PAP-S is 9.95 [13], 9.8 for MAP [11] and 10.3 for *Spinacea oleracea* antiviral protein [18]. The Mr of BAP I as determined by MALDI mass spectrometry was approximately 28 000. The molecular masses of other antiviral proteins (Mirabilis antiviral protein (MAP), *Phytolacca* antiviral proteins (PAP, PAP II and PAP-S), Dianthin 30 and Dianthin 32) ranges from 24 000 to 32 000 [6, 11, 13].

Figure 3 shows the sequence of residues from the N-terminus of BAPI and a comparison with those of PAP-S [13] and MAP [14]. Homology analysis within this region showed 52% identity between BAP I and PAP-S and 30% identity between BAP I and MAP. BAP I therefore appears to have considerable similarities with previously identified plant antiviral proteins. Preliminary tests suggest that purified BAP I is capable of interfering with *in vitro* protein synthesis, as is the case with many other plant antiviral proteins [19–21]. Given the wide host-range of the tomato spotted wilt virus and its vector (thrips), BAP I clearly has potential for contributing to effective management strategies.

EXPERIMENTAL

Viral inoculum

The TSWV strain was originally obtained from tomato leaves (from the Orchard of Tamil Nadu Agricultural University) showing typical symptoms and was maintained on the primary leaves of its local lesion host, cowpea (*Vigna unguiculata* cv. C.152). The TSWV inoculum was prepared by macerating the leaf tissues from cowpea having ten local lesions with one ml of pre-chilled sodium phosphate buffer, pH 7.0, containing 0.1% 2-mercaptoethanol, using the ice-tray technique [22].

Bioassay

The test material was sprayed or painted on the primary leaves of 5 to 6-days-old cowpea plants (see Fig. 4). After 24 h the leaves were dusted with carborundum powder which served as an abrasive to allow inoculation of the TSWV strain. After 5 minutes the excess inoculum was washed off the leaves. Local lesions developed after 3–5 days were counted, and the percentage inhibition calculated. Water, instead of test material, was applied to control plants.

Materials

From a portion of a mature root cut from *B. spectabilis* tree, the bark was removed and the remaining portion washed, chopped into five pieces and lyophilised.

Chemicals

All chemicals and the dialysis bag were purchased from Sigma Chemical Company, U.S.A., except Na_2HPO_4, NaH_2PO_4 and $(NH_4)_2SO_4$ which were purchased from E. Merck and Glaxo, India.

Extraction

Extraction of antiviral protein was performed at 4°C according to the method of Takanami *et al.* [11] with some modifications. Lyophilised root tissues (100 g) were homogenized with 20 vols. of buffer A (10 mM sodium phosphate buffer, pH 7.2, 0.1% 2-mercaptoethanol) in a Waring blender for 2 × 1 min and the extract filtered through muslin cloth. After centrifugation of the filtrate at 5000 g for 15 min, the supernatant was collected. The residue was re-extracted with 10 vols of buffer A, and the two supernatants pooled. The extracted proteins were fractionated by adding $(NH_4)_2SO_4$ to 40% saturation. After centrifugation at 5000 g for 20 min, the supernatant obtained was adjusted to 90% saturation with

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          1        10        20        30
BAPI      YNTVSFDLGEAE--EEMSTVIQSLRNELANGAP....
PAPS      INTITFDAGNATINKMATFMESLRNEAKDPSL....
          1        10        20        30

52.0% Identity in 25 aa overlap

          1        10        20        30
BAPI      YNTVSFDLGEAEEEMSTVIQSLRNELANGAP....
MAP       APTLETIASLDLNNPTTYLSFLTNIRTKVADKTE....
          1        10        20        30

30.4% Identity in 23 aa overlap
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Fig. 3. Comparison of amino acid sequence data from BAP I, PAP-S and MAP.

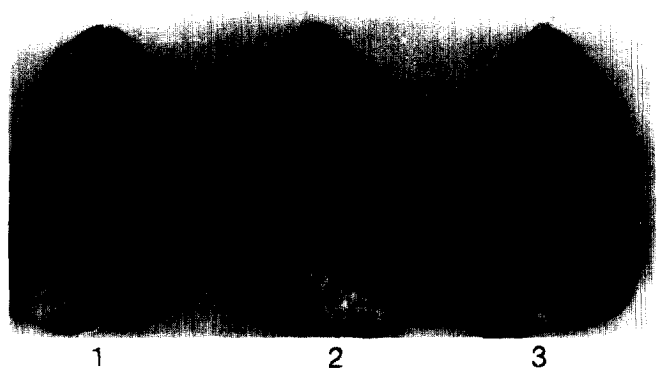

Fig. 4. Bioassay on cowpea, cv. C 152 leaves. (1) Experimental leaf treated with pure BAP I; (2) control leaf treated with water; (3) experimental leaf treated with crude root extract of Bougainvillea.

$(NH_4)_2SO_4$, and the pellet collected by centrifugation. This pellet was dissolved in 10 mM sodium phosphate buffer, pH 7.2, and dialysed against the same buffer for 24 h. The clear dialysate was then lyophilised.

Chromatography

All steps were carried out at 4 °C. The lyophilised powder (750 mg) was dissolved in 20 ml of buffer B (10 mM sodium phosphate buffer, pH 6, 0.1% 2-mercaptoethanol, 25 mM NaCl) and loaded onto a column (25 × 300 mm) of CM-Sepharose equilibrated with the same buffer. After washing with buffer B, bound proteins were eluted from the column with a linear gradient (300 ml total volume) of 25–300 mM NaCl in the same buffer, and fractions (8.6 ml) collected. Protein elution was monitored at 280 nm and ionic strength measured using a conductivity meter. Each fraction was dialysed against distilled water and tested for antiviral activity using the bioassay described above. The active fractions of the first peak were lyophilised. The lyophilised powder was dissolved in buffer C (10 mM sodium phosphate buffer, pH 7.6) and fractionated on a DEAE-Sepharose column (9 × 250 mm) equilibrated with buffer C. The unbound protein fraction eluted by buffer C was adjusted to pH 6 with 0.1 M KH_2PO_4 and NaCl added to 25 mM. This was subsequently loaded onto a CM-Sepharose column (9 × 100 mm) equilibrated with buffer B. After washing with buffer B, bound BAP I was eluted with a linear gradient (70 ml total volume) of NaCl (25–400 mM) in buffer B. The elution profile was monitored at 280 nm, and the ionic strength of the collected fractions (1.5 ml each) was also measured. The fractions were dialysed against deionized water, assayed for activity, and active fractions pooled and lyophilised. The material was then dissolved in 0.1% TFA, and a 100 μl sample was injected onto an Aquapore butyl (C4) column equilibrated with 0.1% TFA. After washing with 0.1% TFA for 10 min, the absorbed proteins were eluted with a linear gradient (0–90%) of acetonitrile: 0.1% TFA over 25 min. The flow rate was 0.7 ml/min. Eluted protein was monitored at 220 nm.

SDS-PAGE

SDS-PAGE was performed using a discontinuous buffer system on 14% acrylamide gels using the

method of Laemmli [23]. Protein bands were stained with Coomassie Brilliant Blue R-250.

Protein concentration

Protein concentration was determined by the Bradford method [24] using bovine serum albumin as standard.

Isoelectric focusing

Isoelectric focusing (IEF) was carried out using the PhastSystem (Pharmacia). The gels were fixed in 20% TCA and stained with Coomassie Brilliant Blue R-250.

N-terminal sequence

Purified BAP I was resuspended in 2% TFA. An aliquot of this solution was loaded onto a hydrophobic sequencing support column. Sequence analysis of the material retained on the column after a wash with 2% TFA was carried out by placing the column in the Hewlett-Packard G 1005A Protein Sequencer, and using routine 3.0 sequencing chemistry.

Molecular weight

BAP I was dissolved in 2% TFA, and 0.5 μl of the solution was spotted onto a stainless steel target precoated with alpha-cyano-4-hydroxy cinnamic acid and analysed in a VGToF Spec Mass spectrometer fitted with a 337 nm nitrogen laser.

Test for sugars

BAP I was tested for the presence of sugars by the method of Dubois *et al.* [26].

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