Indian Journal of Agricultural Sciences **81** (1): 74–8, January 2011

Purification and evaluation of antiviral proteins from *Bougainvillea* x *buttiana* against *Helicoverpa armigera*

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Received: 16 December 2009; Revised accepted: 24 September 2010

ABSTRACT

An open reading frame (ORF) of cDNA encoding 35.49 kDa ribosome-inactivating/antiviral protein (RIP/AVP) from the leaves of *Bougainvillea* x *buttiana* cv Mahara, was cloned into an expression vector and expressed in *Escherichia coli* as a simple protein. The recombinant protein, designated as BBAP1, was tested for its insecticidal activity against *Helicoverpa armigera* (Hübner)(Lepidoptera: Noctuiidae) – the most important insect-pest in agriculture. Initially, to appreciate the insecticidal activity, water extract of *B.* ×*buttiana* leaves (1: 10; w/v) was assayed against 5-day old larvae of the test-insect with intoxicated natural diet (Frenchbean pods). Larval mortality never exceeded 50% in the test-concentrations (maximum, 1: 1 ratio of extract and water). For testing BBAP1, wheat-germ-based artificial diet was taken in the diet-surface incorporation assay. Neonates of the target insect were reared on the intoxicated diet at three dosages, viz 0.127, 1.27 and 12.7 ng/g diet, till eclosion and oviposition by the emerged and mated adult moths. Growth and development as affected by BBAP1 is discussed in this pursuit. Growth and developmental indices for highest dosage were 2.23, 2.52 as against 5.6, 3.3 and 4.21, 3.16 in the control and buffer blank populations, respectively. Toxicity of the said AVP even at nanogram levels like those in *Bacillus thuringiensis* (Bt) insecticidal crystal proteins (ICP), implies that BBAP1 has the potential to be used in frontier plant-protection technology in particular the insect-resistant transgenics.

Key words: Antiviral protein (AVP), BBAP1, *Bougainvillea* x *buttiana*, Growth and development, *Helicoverpa armigera*, Insecticidal, Transgenics

Presence of antiviral principles has been reported from large number of plants, however, only a few of these inhibitory substances have been purified and characterized. *Bougainvillea spectabilis* and *B. glabra* have been reported to contain antiviral principles (Baranwal and Verma, 1993; Bolognesi *et al*. 1997). Narwal *et al*. (2001a,b) described two antiviral proteins (AVP) from the leaves of another species of *Bougainvillea* (*B.* x *buttiana* cv Enid Lancester) inducing resistance against TMV and SRV. Similarly, AVP consisting of two polypeptide bands (33 and 28 kDa) was purified from the leaves of *B.* x *buttiana* cv Mahara (Bhatia *et al*. 2004).

Many of the AVPs have ribosome-inhibiting *N*-glycosidase activity and designated as ribosome-inactivating proteins (RIPs). RIPs have been linked to defense by antiviral, antifungal, and insecticidal properties demonstrated *in vitro* and in transgenic plants (Nielsen and Boston 2001). Two RIPs from *Mirabilis expansa* have shown antifungal as well as antibacterial activity (Vivanco *et al*. 1999). One such RIP gene, a full-length cDNA encoding RIP/AVP from *B.* x*buttiana* leaves was isolated. The cDNA consisted of 1364–nucleotides (NCBI GenBank Acc. No. DQ 013264) with an open reading frame (ORF) of 960 nucleotides encoding to 35.49 kDa protein of 319 aminoacids. The deduced protein has been designated as BBAP1 (*B.* x *buttiana* AVP1). The ORF was cloned into an expression vector, pMAL-c2X and expressed in *E. coli* as a fusion protein of ~78 kDa (Choudhary *et al*. 2008a). The cleaved and purified recombinant BBAP1 exhibited ribosome-inhibiting rRNA *N*-glycosidase activity, and imparted a high level of resistance against the tobacco mosaic virus (Choudhary *et al.* 2008a) as well as sunnhemp rosette virus (Choudhary *et al.* 2008b). Thus, such RIPs have a potential to be used as biopesticides. However, for testing the activity of AVP, the fusion protein as such cannot be used. Therefore, in the present study the

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ORF was cloned into another expression vector and expressed as a simple protein. Investigations carried out by Choudhary *et al.* (2008a,b) also suggested, BBAP1 to be a multifunctional protein. In furthering the research, herewith we verified the insecticidal property of this BBAP1, taking the most important insect pest *Helicoverpa armigera* as the target insect. This has developed resistance to conventional pesticides, and therefore quite reasonably need searching for new and novel molecules inclusive of botanicals. Keeping this in view, insecticidal activity of native antiviral protein isolated from *Bougainvillea* leaves was tested with expectations, if found insecticidal, this fact will add substance and credibility to this BBAP1.

MATERIALS AND METHODS

As the expression vector, pET–19b (Novagen) has got multiple cloning sites (*Nde*I – *Bam*HI), 960 bp ORF was PCR amplified by designing gene-specific primers having cloning sites *Nde*I in the forward primer and *Bam*HI in the reverse primer.

Forward primer: 5' ACTGACCATATGATGAAGCCAC TAGGGGGGCT 3'

Reverse primer: 5' GTTGAGGATCCT TAGCCAATGATTGTTTCTAGTTTTCAAGT 3'

The PCR reaction mixture (100 µl) contained: 100 ng cDNA(ORF), 10 µl 10x PCR buffer, 1µl dNTPs (10 mM) mix, 100 pmol each of forward and reverse primers, and 3.0 U *Taq* polymerase. The thermocycler programme used was 5 min at 94°C; 32 cycles each of 1 min at 94°C, 1 min at 54°C and 1 min at 72°C, and finally 7 min at 72°C.

The PCR products and vector were digested with *Nde*I and *Bam*HI restriction enzymes and subsequently the cDNA cloned into the vector pET–19b at *Nde*I and *Bam*HI sites. The positive clones were confirmed by digesting with restriction enzymes and colony PCR using Bangalore-Genei PCR kit. *E. coli* strain BL21 (DE3) competent cells (Novagen) were transformed with the clone pET-19b. A single colony from the BL21 (DE3) transformed plate was inoculated and checked for the expression of the desired protein (~37 kDa) after inducing with 1 mM IPTG.

For protein purification, the BL21 (DE3) transformants were grown in LB medium up to O.D. ~0.6 and then induced with 1 mM IPTG for protein expression. The cells were harvested and sonicated in 20 mM Na-phosphate buffer, *p*H 6.9; containing 500 mM NaCl and 20% sucrose and then centrifuged at 10 000 rpm, for 30 min. at 4°C. On SDS-PAGE analysis, 80% of induced protein was found in the supernatant and rest 20% in inclusion bodies. As the expressed protein has got histidine tag, it was purified using Novagen His-bind resin column according to manufacturer's protocol. The eluted protein with histidine tag (36.5 kDa) was further purified using DEAE-Cellulose (Sigma-Aldrich) and CM-Sepharose (Sigma-Aldrich) columns. The fractions containing protein were pooled, dialyzed against water and then lyophilized. Before use, the lyophilized BBAP1 was dissolved in 50 mM Na-phosphate buffer, *p*H 7.5.

The restriction enzymes used were of Bangalore-Genei and all the biochemicals/chemicals were of molecular biology grade or of high purity.

The well-developed freshly harvested leaves of *B.* x*buttiana* cv Mahara were washed with distilled water, water absorbed using tissue paper and thoroughly dried at ~40°C in an air-oven for 2–3 days. Fine powder of the dried leaves was prepared using a grinder. For preparing its extract, water (1: 10; w/v) was used. The leaf powder was mixed with half of the water, stirred well and left for ~72 hr at room temperature (~25 °C). After that 20 mg PVPP/g leaf powder and rest half of the water were added, mixed well and homogenized in a waring blender. The pulp was squeezed through 2 layers of muslin cloth, and the filtrate was clarified by centrifugation at 12 000×g for 20 min twice. The clear supernatant (water extract) was collected and stored in a refrigerator at least for six months.

Laboratory culture of test-insect (*H. armigera*) was maintained in the environmental simulation chamber (temp. 27±1°C, 65±5% RH, 16: 8 h scoto/photophase regime) on wheat-germ-based artificial diet (Patent Application No. 1618/DEL/2008, dated 7/07/08) in the Division of Entomology, New Delhi; during 2008–09. *Bougainvillea* aqueous extract was used in different dilutions in distilled water (1: 1, 1: 3, 1: 5, 1: 7 and 1: 10; v/v). The laboratory reared larvae were pre-conditioned at ambient temperature. Five-day old larvae of uniform size were sorted out and used in the insect bioassay.

Water extract of *B.* x*buttiana* leaves was evaluated against second instar (5–d-old) larvae by 'dip-method' using Frenchbean pods. The pods were cut into pieces of approx 4 cm length, dipped into the *Bougainvillea* extract and then air-dried. Treated pod pieces were then transferred to clean crystal vials (6 cm dia) with moistened filter paper and one larva was released in each vial. Each treatment comprised 24 larvae (N=24). Untreated control Frenchbean pods were dipped in distilled water only. Mortality was recorded in these treatments 24, 48, 72 and 96 hr after exposure. Moribund insects were counted as dead. Probit analyses could not be carried out due to lack of requisite range (ie 10–90%) of larval mortality.

Effect of the BBAP1 on the growth and development of test insect was studied. Two gram artificial diet was added per assay vial (20 ml capacity). Different dosages of BBAP1 (0.127; 1.275 and 12.75 nanogram/gram diet) were dissolved in buffer and added to the vials @ 75 µl/vial. Individual neonates were released onto each assay vial (N=30, replication=3). Biological parameters, viz larval weights (5–d, 7–d and 9–d-old), larval period, larval mortality, pupal weights, pupal period, and pupation percentage, adult emergence, survival – all were recorded. Oviposition by the emerged moths after mating was recorded. Hatching

percentage and total developmental period was calculated. Growth indices (GI) and Developmental indices (DI) were calculated for each treatment.

GI = [% pupation/larval period] (Saxena and Khan 1974)

DI = [% adult emergence/total developmental period] (Sharma and Agarwal 1982). Statistical analysis was done using INDOSAT (version 8.1).

RESULTS AND DISCUSSION

Cloning and expression of the gene in E. coli

The positive pET-19b clones were digested with the restriction enzymes (*Nde*I, *Bam*HI) and colony PCR was done. Fig 1 shows the PCR amplification of 960bp ORF.

E. coli BL21(DE3) cells containing cDNA encoding for AVP from the leaves of *B.* x*buttiana,* cloned in an expression vector (pET-19b), were grown in LB medium containing IPTG to induce BBAPI expression. The expressed protein was purified by following the column chromatographic techniques. On SDS-PAGE analysis, a clear band (36.5 kDa) of purified recombinant antiviral protein BBAP1 (with histidine tag), and two prominent bands (33 and 28 kDa) of native antiviral proteins in the water extract (WE) of the leaf powder were seen (Fig 2).

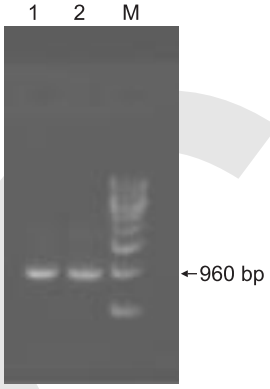

Fig 1 Agarose (1%) gel showing PCR amplification of cDNA (AVP gene). Lanes - M: 500 bp marker; 1 and 2: PCR products (960 bp)

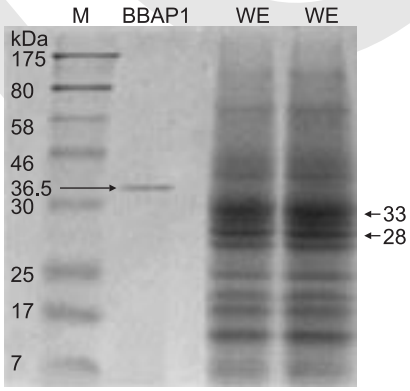

Fig 2 SDS–PAGE (12%) analysis of recombinant antiviral protein BBAP1 with histidine tag, and of water extract (WE) of leaf powder of *Bougainvillea* x *buttiana* cv Mahara

Preliminary trial with aqueous extract of bougainvillea

No larval mortality on 24 hr exposure period was observed in any treatment. Larval mortality never exceeded 50% in the test-concentrations even after 72 hr of exposure (maximum, 1: 1 ratio of distilled water and crude extract) (Fig 3). Of course, detailed investigation was not carried out on growth and development as affected by crude extract.

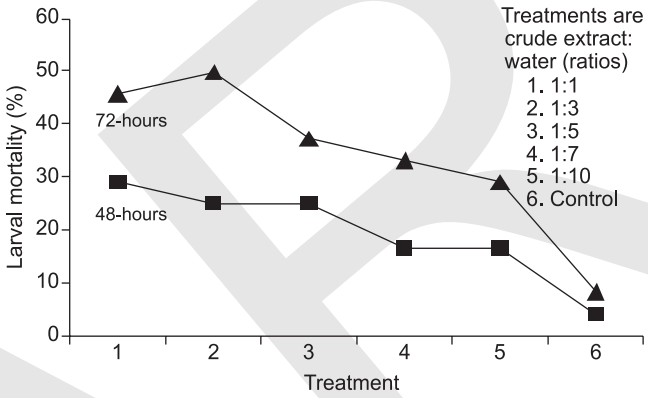

Fig 3 Effect of *Bougainvillea* water extract on *Helicoverpa armigera* larval mortality

Testing recombinant AVP-BBAP1

Various biological attributes of *H. armigera* as affected by BBAP1, are summarized in Table 1. Maximum larval mortality (62.5%) was observed in the highest dosage tested (12.7 ng/g diet). Adult emergence of 75% was recorded in this treatment as against 88 – 91% in blank treatments (control and buffer-control). The growth and developmental Indices (GI and DI) as calculated were presented in the bar-diagram (Fig 4). Calculated GIs and DIs were 4.03, 3.0 and 2.23; and 2.91, 2.64 and 2.52 corresponding to three BBAP1 test-dosages (0.127, 1.27 and 12.7 ng/g diet) as against 5.60, 3.3, and 4.21, 3.16 in the untreated populations (control and buffer

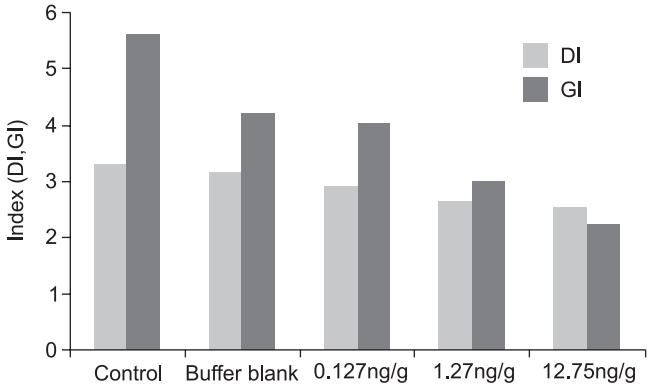

Fig 4 Growth (GI) and developmental indices (DI) of *Helicoverpa armigera* as affected by BBAP1

Table 1 Effect of antiviral protein (BBAP1) from *Bougainvillea* on growth and development of *Helicoverpa armigera*

Biological attributes at various life-stages	Control (sterile water)	Buffer blank	BBAP1 treatments at nanogram level (ng/g of diet)		
			0.127	1.27	12.7
Larva					
Weights (5-d old) mg	16.3 [a]	14.3 [a]	15.6 [a]	13.4 [ab]	12.3 [b]
(7-d old) mg	75.9 [a]	74.3 [a]	71.9 [a]	57.3 [b]	51.0 [b]
(9-d old) mg	267.5 [a]	263.0 [a]	263.9 [a]	241.4 [ab]	239.0 [b]
Mortality (%)	8.33 [a]	19.2 [b]	33.3[c]	50.0 [c]	62.5 [d]
Period (days)	16.4 [a]	16.8 [a]	16.6 [a]	16.7 [a]	16.8 [a]
Pupa					
Weight (mg)	287.7 [a]	291.4 [a]	278.6 [a]	291.1 [a]	285.5 [a]
Period (days)	11.1 [a]	11.1 [a]	12.2 [a]	12.8 [a]	12.9 [a]
Pupation (%)	91.7 [a]	70.8 [b]	66.7 [b]	50.0 [c]	37.5 [d]
Adult					
Moth emergence (%)	90.9 [a]	88.2 [a]	83.8 [a]	77.8 [a]	75.0 [b]
Survival (%)	83.3 [a]	62.5 [b]	62.5 [b]	37.5 [c]	29.2 [c]
Oviposition (eggs/gravid female)	901 [a]	875 [a]	254 [b]	193 [c]	174 [c]
Hatching (%)	85.4 [a]	80.2 [a]	69.3 [b]	61.1 [b]	49.4 [c]
Total developmental period (days)	27.5 [a]	27.9 [a]	28.8 [a]	29.5 [a]	29.8 [a]

Figures are mean of three replicates, N (insects) = 30
Rows following common alphabets are statistically at par (P=0.05)

blank). Few insecticidal proteins were reported to be toxic and detrimental to growth and development of target insect (like *H. armigera*). In the same laboratory, following the same diet-surface incorporation assay, under similar rearing conditions. Gupta *et al*. (2000) reported the LC_{50} values for *Bacillus thuringiensis* (Bt) insecticidal crystal proteins (ICP), Cry 1Ac against *H. armigera* to be <10 ng/cm^2. Though, probit analyses could not be done in this assay due to lack of requisite mortality range, nevertheless the resultant reduced GI and DI obtained clearly depicts the detrimental effects of BBAP1 on the target insect. Toxicity of the said AVP even at nanogram levels like those in Bt ICP, implies that BBAP1 has the potential to be used in plant protection technology, in particular the insect-resistant transgenics. Several plant RIPs were toxic to insects and could inhibit *in vitro* protein synthesis of housefly (Gatehouse *et al*. 1990; Ferrari *et al*. 1991). Zhou *et al.* (2000) reported the toxicity of cinnamomin – a type II RIP to bollworm (*H. armigera*) and mosquito (*Culex pipines pallens*). The LC_{50} value of this RIP was calculated to be 1 839 ppm against *H. armigera*. Our study implies the toxicity of BBAP1 at nanogram levels, demonstrating its superiority and suitability for applied aspects in transgenic development endeavours.

ACKNOWLEDGEMENTS

Authors are thankful to the ICAR and University Grants Commission for grant of Emeritus Schemes.

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