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Ribonuclease, Deoxyribonuclease, and Antiviral Activity of *Escherichia coli*-Expressed *Bougainvillea* x*buttiana* Antiviral Protein 1

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Abstract—A full-length cDNA encoding ribosome-inactivating/antiviral protein from the leaves of *Bougainvillea* x*buttiana* was recently isolated. The coding region of cDNA was cloned and expressed in *Escherichia coli*, and the protein product was designated as BBAP1 (*Bougainvillea* x*buttiana* antiviral protein 1). BBAP1 showed ribonuclease activity against *Torula* yeast RNA. It also exhibited depurination activity against supercoiled pBlueScript SK^+ plasmid DNA in a concentration dependent manner, and was found to convert nicked circular DNA into linear form only at higher concentration. On bioassay, BBAP1 exhibited antiviral activity against sunnhemp rosette virus infecting *Cyamopsis tetragonoloba* leaves in which 95% inhibition of local lesion formation was observed.

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Extracts of many plant tissues are known to possess antiviral activity [1]. In several cases, this activity has been shown to be due to proteins inhibitory to protein synthesis called ribosome-inactivating/antiviral proteins (RIPs/AVPs) [2]. RIPs are rRNA *N*-glycosidases that remove a specific adenine (A4324 in rat liver 28S rRNA) from the universally conserved sarcin/ricin loop of the large rRNA inactivating eukaryotic as well as prokaryotic ribosomes [2]. In addition to having *N*-glycosidase activity [3-7], some AVPs also act as polynucleotide: adenosine glycosidases as they act on RNA species other than ribosomal, including viral RNAs and poly(A) and also depurinate DNA [8-12]. AVPs have been shown to depurinate capped mRNAs [13]. Recently, some AVPs have been reported to exhibit superoxide dismutase activity [14, 15], phospholipase activity [16], and antioxidant activity [17, 18]. Though AVPs appear to have a variety of substrates, the exact mechanism through which these operate to inhibit virus infection is not known [19-21].

Bougainvillea, a popular ornamental plant, is a member of family Nyctaginaceae. Its leaves [12, 22] as well as roots [23] serve as a very good source of RIPs/AVPs. Native AVPs from the leaves of *Bougainvillea* x*buttiana* exhibited rRNA *N*-glycosidase activity [7], RNase activity against viral RNA of tobacco mosaic virus (TMV) and sunnhemp rosette virus (SRV), and DNase activity against plasmid $pBSK^+$ supercoiled DNA [10]. Recombinant AVP bouganin, encoded by a cDNA isolated from the leaves of *B. spectabilis*, was found to be least toxic as native bouganin, illustrated on living human cells (JY B-cell line) [24], thus making bougainvillea AVPs suitable for practical applications in agriculture as well as in pharmaceuticals.

In view of this, a cDNA (GenBank Acc. No. DQ 013264) encoding ~35 kD antiviral protein from the leaves of *B.* x*buttiana* has been isolated in our laboratory. The open reading frame (ORF) was cloned and expressed in *Escherichia coli*. The recombinant protein exhibited ribosome-inhibiting *N*-glycosidase activity as well as antiviral activity against TMV (unpublished results) and has been designated as *Bougainvillea* x*buttiana* antiviral

Abbreviations: AVP) antiviral protein; BBAP1) *Bougainvillea* x*buttiana* antiviral protein 1; ORF) open reading frame; PAP) pokeweed antiviral protein; RIP) ribosome-inactivating protein; SRV) sunnhemp rosette virus; TMV) tobacco mosaic virus.

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protein 1 (BBAP1). The molecular mass of the recombinant BBAP1 is slightly higher than the native bougainvillea antiviral protein (33 kD) [18]. In the present study carried out with the recombinant BBAP1, we further report its RNase activity against *Torula* yeast RNA, DNase activity against supercoiled plasmid DNA, and antiviral activity against SRV.

MATERIALS AND METHODS

Plant materials. Leaves of *B.* x*buttiana* cv. Mahara were collected from the Bougainvillea Garden of our Institute. Seeds of *Cyamopsis tetragonoloba* (Guar, var. M-83) were obtained from the Regional Research Station, Rajasthan Agricultural University, Jaipur. The SRV culture was originally obtained from the Department of Botany, Lucknow University, Lucknow. Virus inoculum was prepared by homogenizing the infected leaves of *Crotolaria juncea* with 20 mM sodium phosphate buffer, pH 7.0. The content was filtered and centrifuged at 12,000*g*. The supernatant was collected and used as virus inoculum on *C. tetragonoloba* plants leaves.

Expression and purification of recombinant BBAP1. *Escherichia coli* TB1 transformed with pMAL-c2X expression vector containing ORF encoding BBAP1 was grown in the presence of the *lac* Z_α inducer isopropyl-thio-β-D-galactoside. The induced fusion protein (78 kD) consisting of maltose binding protein and recombinant protein (35 kD) was purified by affinity column chromatography using resin specific to maltose binding protein as per the manufacturer's instruction (New England BioLabs Cat. No. E 8000 S). The fusion protein was cleaved with factor Xa, which is a site-specific protease supplied along with the kit. A Sephadex G-100 column was used to get purified recombinant BBAP1. Protein homogeneity was checked by 12% SDS-PAGE.

RNase activity. To determine if BBAP1 could degrade RNA, a method for a substrate-based RNase activity gel assay was adopted [25]. BBAP1 sample was prepared in 50 mM Tris-HCl, pH 6.8, containing 0.1% SDS, 0.01% bromophenol blue, but without the addition of reducing agents. As a positive control for RNase activity, 2 μg RNase A was prepared in the same buffer. The sample and control were run through 12% SDS-PAGE containing 2 mg/ml *Torula* yeast RNA in the resolving gel. Following electrophoresis, gel containing lanes for BBAP1 and RNase A was cut longitudinally to check RNase activity. This gel portion was washed with 25% isopropanol in 10 mM Tris-HCl, pH 7.0, to remove SDS. The isopropanol was removed by further washing in 10 mM Tris-HCl, pH 7.0, containing 2 μM $ZnCl_2$. Protein in the gel was renatured by incubation in 100 mM Tris-HCl, pH 7.0, at 50°C for 1 h. The gel was stained with 0.2% Toluidine Blue-O in 10 mM Tris-HCl, pH 7.0, for 10 min and destained in distilled water until transparent bands appeared. Another portion of gel containing lanes for protein markers and BBAP1 was stained with Coomassie brilliant blue to indicate the location of BBAP1.

DNase activity. Plasmid DNA (pBlueScript SK^+) was isolated by alkali hydrolysis mini-prep method [26]. The purity and quantity of plasmid DNA was checked on 1% agarose gel. The activity of DNase was determined as described earlier [9, 10], except that it was assayed in the absence of Mg^{2+}. Plasmid DNA (4 μg) was incubated with serially increasing amount of purified BBAP1 in a final volume of 25 μl. The reaction mixture was incubated at 37°C for 4 h. At the end of the incubation period, 5 μl loading dye (6×; MBI Fermenta, USA) was added. Electrophoresis was carried out under non-denaturing condition in 1× TBE buffer in a 1% agarose gel. DNA bands were visualized by staining with ethidium bromide.

Antiviral activity of BBAP1. For each treatment, three plants of *C. tetragonoloba* with two leaves of equal size were used. The *E. coli*-expressed protein of ~35 kD was applied on the leaves at the rate of 50 μg per leaf. After 1 h, the protein treated leaves were washed with distilled water and gently blotted dry. The leaves were then rubbed very lightly with 600-mesh carborundum powder and inoculated gently and uniformly with SRV virus inoculum. For control, test plant leaves were treated with only buffer and virus inoculum. Ten minutes after inoculation, the leaves were washed with distilled water. The plants were observed for the development of lesions for 3-4 days. The inhibitory activity of the protein was calculated in terms of percentage inhibition using the following formula: inhibition (%) = $(C - T)\cdot 100/C$, where C is average number of lesions in control plants, T is average number of lesions in treated plants.

RESULTS AND DISCUSSION

There is not yet clear understanding of the mechanism(s) whereby RIPs/AVPs help in inducing resistance or in providing protection against viruses [19-21]. The information available so far suggests that these can manifest their effect either by stopping the spread of pathogen [27] or acting indirectly by inducing host resistance [15]. In view of this, the purified recombinant *B.* x*buttiana* leaf antiviral protein, BBAP1, was characterized in terms of RNase, depurination of supercoiled double stranded DNA, and antiviral activity.

RNase activity. RNase activity of BBAP1 was tested using *Torula* yeast RNA as a substrate. To rule out the possibility that the RNA degradation is due to contaminating nucleases, substrate-based RNase activity gel assay was done. On staining for RNA, the appearance of unstained band indicated the position of RNase activity of BBAP1 (Fig. 1, lane *3*). Moreover, the single unstained band was found to co-migrate with protein band of

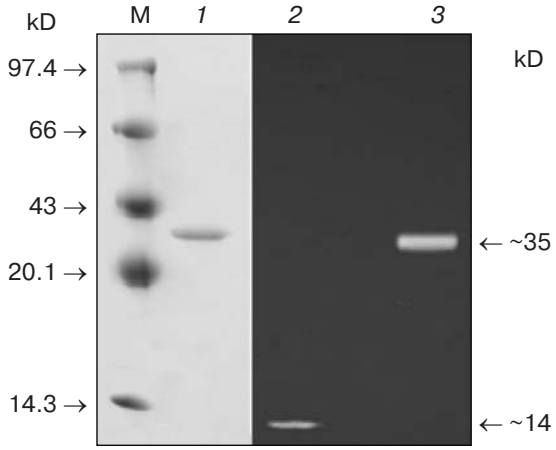

Fig. 1. Substrate-based RNase activity gel assay of recombinant BBAP1. BBAP1 (lanes *1* and *3*) and RNase A as positive control (lane *2*) were separated using 12% SDS-PAGE already containing *Torula* yeast RNA. Following electrophoresis, lanes containing protein markers (lane M) and BBAP1 (lane *1*) were cut off from gel and stained with Coomassie brilliant blue to locate the protein. The rest of the gel was stained with Toluidine blue-O to indicate the presence of RNase activity of BBAP1 (lane *3*) and that of positive control, 2 μg RNase A (lane *2*).

recombinant BBAP1 (Fig. 1, lane *1*). Thus, these results clearly indicated that BBAP1 degraded the RNA and it has RNase activity. Though with the use of purified recombinant BBAP1 the possibility of contaminating RNase activity was reduced, it does not completely rule out its presence until or unless such RNases are removed by dye-chromatography using Red-Sepharose. Lane *2* in Fig. 1 showed the positive control wherein 2 μg of RNase A was used. The clear band observed at ~14 kD indicated that RNase A was renatured and exhibited activity when analyzed in the same manner.

The RNase activity results from depurination at adenine residue, and the depurinated RNAs are susceptible to degradation *in vivo* by cellular pathways that eliminate aberrant RNAs as suggested by Hudak et al. [8]. They proposed a novel mechanism suggesting that for depurinating activity of pokeweed antiviral protein (PAP), rRNA rather than viral mRNA may be the preferential substrate under normal conditions, since 80% of RNA of the cell is rRNA. However, during viral infection, the level of viral RNA increases significantly, beyond the normal level of rRNA. In this situation, AVPs possibly inhibit translation by binding to the cap structure and depurinating the mRNA, and the depurination of capped viral RNA may be the primary mechanism for the antiviral activity. According to them, PAP degraded capped luciferase transcripts and behaved as an RNase at high concentration. Parikh et al. [27] reported that AVPs do not target the entire capped message, but display specificity for viral RNAs or other cellular message involved in virus replication. Masayuki et al. [28] observed 50% inhibition of local lesion formation in cowpea leaves when treated with an RNase-like glycoprotein (figaren) from *Cucumis figarei* 24 h before and 1 h after incubation with cucumber mosaic virus. Not only this, double stranded RNA extracted from tobacco tissues infected with cucumber mosaic virus was also degraded by figaren. These observations suggest that there may be a positive correlation between RNase activity and antiviral activity.

Similar results have been reported earlier with antiviral proteins like MAP from *Mirabilis jalapa* [29], CIP-29 from *Clerodendrum inerme* [11], CCP-27 from *Celosia cristata* [9], and AAP-27 from *Amaranthus tricolor* [30].

DNase activity. When pBSK$^+$ supercoiled dsDNA was incubated with increasing concentrations of recombinant BBAP1 (1, 3, and 5 μg), it was observed that the extent of supercoiling was obviously altered and the plasmid DNA converted to the relaxed and linear forms depending upon protein concentration (Fig. 2), as has been reported earlier for native BBAP [10]. The pBSK$^+$ DNA when incubated with 1 and 3 μg of BBAP1, first gave a nicked circular (relaxed) form (Fig. 2, lanes *3* and *4*), which moved significantly slower than the supercoiled DNA in agarose gel. When the BBAP1 concentration was further increased to 5 μg, the linear form of DNA also emerged, which migrated faster than the nicked circular form but slower than the supercoiled DNA (lane *5*). Initially, in untreated plasmid DNA, two bands of relaxed and supercoiled forms of DNA were prominent (lane *1*), and just to show the linear form of DNA, pBSK$^+$ DNA was incubated with *Eco*RI (lane *2*). The results obtained in the present study are in agreement with earlier reports [9, 29, 31, 32]. The present study and earlier reports suggest that AVP/RIP-associated depurination of supercoiled dsDNA was conformation specific, i.e. AVPs/RIPs cleave only supercoiled DNA in a two-step process, first nicking a single strand before linearizing the DNA by second strand cleavage.

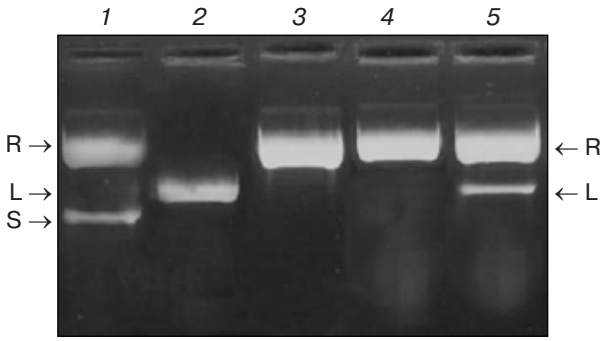

Fig. 2. DNase activity of recombinant BBAP1. Agarose gel (1%) showing cleavage of supercoiled (S) plasmid DNA (pBSK$^+$) into relaxed (R) and linear (L) forms. Lanes: *1*) pBSK$^+$ DNA (control); *2*) pBSK$^+$ DNA linearized with *Eco*RI (control); *3-5*) plasmid DNA treated with 1, 3, and 5 μg recombinant BBAP1, respectively.

Fig. 3. Bioassay of recombinant BBAP1 for its antiviral activity. Antiviral bioassay of purified recombinant protein by a local lesion test using *C. tetragonoloba* as test plant and SRV for infection. a) Control: infected plant treated with buffer only. b) Test: plant treated with recombinant protein BBAP1 and subsequently infected with the virus.

The mechanism of DNase activity of RIPs/AVPs is still controversial [33]. Some reports suggested that the DNase activity of RIPs is due to contamination, whereas others suggested that RIPs might have an additional domain that is responsible for DNase activity. In the present study, the possibility of endogenous or contaminating DNase activity has been ruled out, firstly by taking *E. coli*-expressed recombinant BBAP1 and secondly by assaying of the activity in the absence of Mg^{2+}. This is because many nucleases require divalent metal ions for their activity [34].

Antiviral activity of recombinant BBAP1. Bougainvillea leaves and roots are known for the presence of RIPs/AVPs [7, 10, 22-24]. In the present study, application of recombinant BBAP1 at the rate of 50 μg per leaf, exhibited 95% inhibition of viral infection (data not presented) on *C. tetragonoloba* leaves infected with SRV (Fig. 3). These results are in conformity with our earlier results obtained with native BBAP consisting of two polypeptides, wherein >95% inhibition of viral infection in *C. tetragonoloba*/SRV and tobacco/TMV system was observed [10, 22].

In conclusion, the present study in which purified *E. coli*-expressed antiviral protein BBAP1 was used proves that bougainvillea AVP has RNase and DNase activity. It also suggests that BBAP1 is a multifunctional protein like many other RIPs/AVPs, and its virus inhibitory activity may be directly correlated to its enzymatic activities, such as ribosome-inhibiting *N*-glycosidase, RNase, and DNase activity.

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