

Isolation and Characterization of Antiviral Protein From *Salsola Longifolia* Leaves Expressing Polynucleotide Adenosine Glycoside Activity

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Abstract: Disease control of economically important crops using non-costly and environmentally non-harmful methods is a necessity. Due to their wide antimicrobial activities in general and their antiviral activities in particular, ribosome-inactivating proteins (RIPs) have a very strong potential in plant defense. A new type -1 single chain RIP named SLP was purified from the leaves of *Salsola longifolia* by ammonium sulphate fractionation, anion exchange on DE-cellulose chromatography and cation exchange chromatography on CM-cellulose. This new RIP's molecular mass was 32 KDa with homology to single-chain ribosome inactivating protein. Reverse transcriptase polymerase chain reaction detected the ribonuclease activity of SLP from the examined species showing SLP to be a broad spectrum RIP that depurinates not only its own ribosomes but also other heterologous plant ribosomes (*Phaseolus vulgaris*). SLP also showed deoxyribonuclease activity against pBlue Script SK⁺ plasmid DNA at moderate SLP concentration which led to nick super coiled DNA and then to nicked circular form. By increasing RIP concentration, it transformed the nicked DNA into a linear form. SLP also showed to possess a powerful antiviral activity.

Keywords: Isolation, Antiviral protection, *Salsola longifolia* leave

Introduction

RIPs are plant proteins capable of inactivating ribosomes. Due to their biological activities toward animal and human cells, RIPs are very important in biological and biomedical research. Some RIPs also have a big role in plant defense (Peumans et al., 2001; Kim et al., 2003; and Vepachedu et al., 2005), and hence can be used in plant protection against different pathogens. Those RIPs are known as antiviral proteins (Stirpe et al., 1992; Van Damme et al., 2001; Girbes et al., 2004; and Corrado et al., 2005).

Most RIP-catalytic ribonuclease activity, via the single depurination of the large rRNA upon treatment with acidic aniline, releases the RIP diagnostic fragment (Barbieri et al., 1993). Although a few studies in the literature state that some RIPs show both DNase and RNase enzymatic activity, many studies show that RIPs' enzymatic activities are mostly DNase activities (Hudak et al., 2000 and Choudhary et al., 2008) and depurination of capped mRNAs (Hudak et al., 2002). Recent studies on the other hand, show that RIPs may also induce cell death by apoptosis (Sikriwal et al., 2008).

Although, It is a broad belief that RIPs have characteristic N-glycosidase activity and that this activity inactivates the ribosomes, and inhibits protein synthesis irreversibly (Van Damme et al., 2001), it is still kind of vague how these antiviral proteins operate to inhibit virus infection (Gu et al., 2000; Park et al., 2004; and Stripe, 2004). Those antiviral / RIPs genes are therefore resistant genes, which can be isolated and transferred to crops that have economic value to develop virus resistant plants (Moon, et al., 1997; Zoubenko et al., 2000; and Vandebussche et al., 2004).

Searching the available literature, it is clear that many type-1 RIPs have been isolated and characterized in details and the search for novel type-1 RIPs with interesting properties is still a very active research topic. Since, the majority of type-1 RIPs at the current time are isolated from dicotyledons plants of families like *Cucurbitaceae*, *Chenopodiaceae*, *Caryophyllaceae*, *Euphorbiaceae*, *Nectaginaceae* and *Phytolaccaceae*, the isolation and characterization of novel type-1 RIPs from other dicots is again a very hot research topic to stress and confirm the biological activities of type-1 RIPs. It is well known that RIPs represent a family of isoenzymes and consequently a family of multigenes that can be expressed in different plant species. Highest activity was found in the mature seeds, 1-fold more than that in the root, and 5-fold more than that in the leaves of *Saponaria officinalis* (Stirpe et al., 1983). Developmental and environmental statuses as well as the plant tissue are the various factors that each RIP gene can express its own pattern accordingly. This study has been performed to determine the structure analysis of RIP gene(s), on the DNA level, for *S.longifolia*. The direct PCR amplification has been undertaken in order to achieve that. In this study, a type-1 RIP from *Salsola longifolia* Forssk (*Chenopodiaceae*) is isolated and its enzymatic and molecular characterization and antiviral activity are studied.

Materials and Methods

Virus inoculum

Two viruses were used; TNV and BYMV for testing the virus inhibitory activities of leaf extract and purified preparation of *S.longifolia*. The viruses were maintained on the local lesion host (*Phaseolus vulgaris*) and systemic host (*Vicia faba*), respectively. The plants were grown in small plastic pots containing soil, and sand mixture. The pots were kept in an insect free glass house. Virus inocula were prepared by homogenizing the infected leaves with 20mM sodium-potassium phosphate buffer, pH 7.0, with a sterilized pestle and mortar. The contents were squeezed through two layers of muslin cloth and the filtrate was centrifuged at 12,000g for 10mins. The clear supernatant was used as virus inoculum after suitable dilution with distilled water.

Plant viral bioassay

Seeds of *P.vulgaris* were grown in 15cm plastic pots; each pot containing 8 seeds, and kept in the green house under natural conditions. Those plants were used as local lesion host to quantitatively measure the activity of TNV-D. Ten primary leaves of 10-13 days old french bean plants were inoculated with TNV. *Vicia faba* plants were used as the systemic host for BYMV, and the inoculation was done on the fourteenth day stage. Inoculation was done under green house conditions at 25 ± 5 °C, by dusting virus inoculum with Carborandum (600 mesh). Ten replicates were made for each virus inoculation.

The antiviral bioassay of the purified SLP-32 was done on the test plants with same height, and age. For each treatment, ten replicates of equal size were used. The purified 32kD protein from *S.longifolia* leaves was applied on the test plant leaves. For controls, test plant leaves were treated with only buffer and virus inoculum. After one hour, the protein treated leaves were washed with distilled water and gently blotted dry. The leaves were then sprinkled very lightly with 600mesh carborundum powder and inoculated gently and uniformly with virus inoculum. After inoculation, leaves were washed with distilled water. In case of TNV infection, plants were observed for the development of lesions for 3-5 days. The inhibitory activity of the proteins was calculated in terms of percentage inhibition using the following formula: %inhibition = (Cont-Treat)/Cont x 100% , where, Cont is average number of lesions in control plants, and Treat is the average number of lesions in treated plants.

As for the BYMV infection, plants were observed for the development of systemic symptoms for 14 days. The inhibitory activity of the proteins was calculated according to the ratio between obviously infected plants (showing systemic symptoms) to the total inoculated plants.

Protein extraction and purification from *S.Longifolia* leaves

Plant materials from fresh and dry leaves (100g) were ground in liquid nitrogen, homogenized in three volumes of extraction buffer (25mM NaPO₄, pH 7.0, with 250mM NaCl, 10mM EDTA, 5mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride, and 1.5% [w/v] polyvinylpolypyrrolidone), and then centrifuged for 30mins at 10,000g. The supernatant was brought to 20% (w/v) ammonium sulfate by continues stirring. The mixture was chilled for one hour and then centrifuged again for 30mins at 10,000g. The supernatant was precipitated with 90% (w/v) ammonium sulfate and centrifuged at 14,000g for 30mins. The pellet was suspended in 10mM sodium phosphate buffer, pH 7.8, and dialyzed against the same buffer. After dialysis, the clear supernatant was applied to DE-52 cellulose column (2.2 cm x 10cm) pre-equilibrated with the buffer. The flow-through fractions were collected and applied to a CM-52 cellulose column (2.2 cm x 10cm) pre-equilibrated with 10mM sodium acetate buffer, pH 5. The column was eluted with a linear gradient of 0-0.3 M NaCl in the same buffer. Fractions with inhibitory activity towards virus infection were pooled, dialyzed extensively against water and freeze-dried.

Estimation of soluble proteins

Protein content of leaf extract at each step of purification was estimated by the method described by Lowry et al., (1951).

SDS-polyacrylamide gel electrophoresis

Discontinuous SDS-PAGE was carried out in 12% separating gel with a 5% stacking gel according to Laemmli (1970). The proteins were visualized by staining with 0.1% Coomassie brilliant blue R-250.

Effect of actinomycin D on antiviral activity

Actinomycin D (ActD 20 μ g ml⁻¹) was applied at different time intervals (0, 6, and 10h, respectively) following treatment with purified antiviral protein on the same leaves of *P.vulgaris*. An equal number of leaves in control sets were treated with ActD alone, antiviral protein alone and buffer alone. After 24h, all the leaves were inoculated with TNV and observed for lesion development.

Assay for N-glycosidase activity

Total RNA was isolated from the leaves of *Phaseolus* and *Salsola* using the RNeasy Plant Mini Kit (Qiagen USA) according to manufacturer's instructions. RT-PCR was performed to detect diagnostic depurinated rRNA fragment which is specific for the enzymatic action of RIPs. RT-PCR was carried out using the Titan One Tube RT-PCR system (Roche, cat. No. 1888382) PCR product (about 250 bp) represents the depurinated RNA using two specific primers (Sense Primer I: 5' AACGTAGTACGAGAGGAAC 3', AntiSense Primer II: 5' AAGTCGTCTGCAAAGGATT 3') as described by Kataoka *et al.*, (1992).

DNase activity

The alkaline hydrolysis mini-preparation method of Ahn et al., (2000) was used to isolate pBlueScript SK⁺ plasmid DNA. To gain information like the size, quantity and quality of the isolated plasmid DNA, the agarose (1%), gel electrophoresis was conducted. To conduct the gel electrophoresis, 1 µg of plasmid DNA was incubated with increasing amount of purified SLP-32 in a final volume of 25 µl. The reaction mixture was then incubated at 37°C for 4 h. After those 4 hours on incubation, 5µl loading solution (30% Ficoll, 200mM EDTA, 0.25% bromophenol blue, and 0.25% xylene caynole FF) was added. Electrophoresis was conducted under non-denaturing condition in a 1% agarose gel. Ethidium bromide was used to stain the gel in order to visualize the DNA bands.

Results and Discussion

Fresh and dry *S.longifolia* leaves were both used in preparing the extracts that inhibited the formation of local lesions by Tobacco Necrosis Virus (TNV) on *P.vulgaris* plants. The antiviral protein was purified from the leaves of *S.longifolia*. Purification of antiviral protein from *S.longifolia* leaves

After collecting the leaves of *S.longifolia*, they were washed with tap water and dried at room temperature. Those leaves were then used to extract the antiviral activity. Around, 2600mg of soluble proteins was obtained from 100g of leaves. Bioassay of crude extracts against TNV, on *p.vulgaris*, showed a very high activity (97.3% inhibition). *S.longifolia* protein was then purified to homogeneity in two steps. The first step of purification resulted in one major peak of DE-52 cellulose chromatography showing the antiviral inhibitory activity against TNV (figure 1A). In this fraction a combination of a major protein of 32KD and minor proteins were present, as revealed by SDS/PAGE analysis. The second purification step removed the minor proteins by CM-52 cellulose chromatography (figure 1B), resulting in a homogeneity and a protein fraction (the first peak) with a molecular mass of 32KD as revealed by SDS/PAGE analysis (figure 2). That protein was then characterized and designated as SLP-32. The inhibitory activity and relative outputs in the various steps of purification are summarized in table 1.

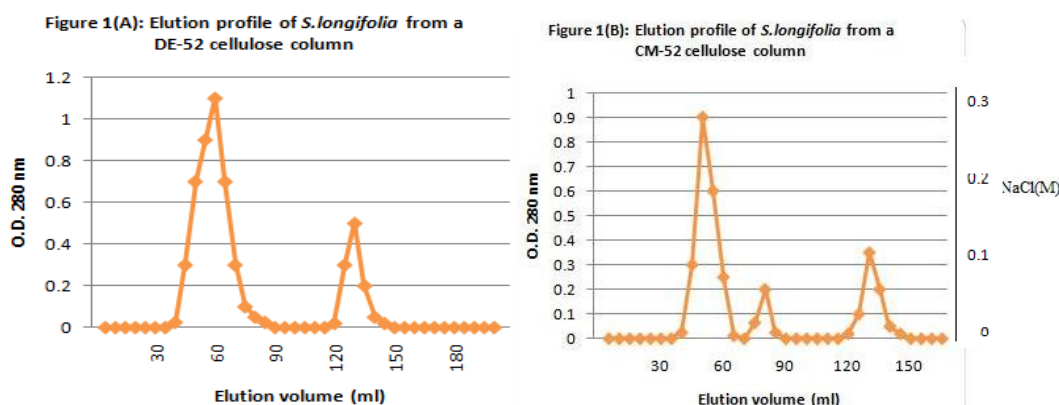


Table 1: Purification of antiviral protein (SLP-32) from the leaf extract of *Salsola longifolia*

Protein fraction	Total protein (mg) from 100g of fresh and dried leaves	Percent inhibition
Crude extract	61.34	97.3
Ammonium sulphate fraction 70-90%	50.29	95.8
DE-cellulose chromatography		
Unadsorbed fraction peak1	25.74	96.2
Unadsorbed fraction peak2	8.23	15
CM-cellulose chromatography		
Peak1	7.82	95.3
Peak2	3.06	9.4
Peak3	4.9	7.53

Molecular weight of SLP-32

SDS-PAGE [12.5% (w/v) gel] was used to determine the molecular weight (Mr) of the purified *S.longifolia* as described by Laemmli(1970). It came out to be 32 KDa (figure 2). This purified salsola antiviral protein has been designated as SLP-32.

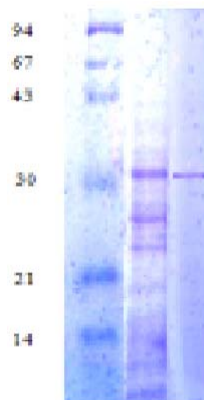


Figure 2: SDS-PAGE of purified SLP-32 isolated from *Salsola longifolia* leaves. Lane 1, from the left, is the molecular mass markers; Lane 2, is the crude extract; Lane 3, is the purified fraction of SLP-32. The gel was stained with Coomassie Brilliant Blue R-250. The positions of molecular mass standards (in KDa) are shown at the left.

Effect of actinomycin-D on antiviral activity

SLP-32 made *P.vulgaris* resistant to TNV. This resistance was inhibited when actinomycin-D (Act-D, 20 µg/ml) was applied immediately after treating the plant with SLP-32 treatment. actinomycin-D however, failed to inhibit the resistance response to a greater extent when applied 6 and 10 hours respectively, after SLP-32 treatment (table 2). Act-D inhibits DNA-directed rRNA synthesis because it binds to double helical DNA by intercalating between the bases. Since, *P.vulgaris* resistance to TNV was inhibited as a result of immediate application of Act-D, following the SLP-32 treatment, then this protein might have induced the synthesis of new substances interfering with the virus or enhance the production of already existent ones, thereby altering the susceptibility of the host plant. The antiviral property of SLP-32 is not reversed however, if Act-D is applied from 6 to 10 hours after protein treatment due to the fact that the substances involved in antiviral effect are already present and the late application of Act-D doesn't interfere in their synthesis. This property is similar to that shown by *C.inerne*, *C.aculeatum* and *C.cristata* antiviral proteins (Verma et al., 1996) and by *A.tricolor* leaves antiviral protein (Sribash et al., 2006).

Table 2: Effect of Actinomycin D (ActD) on the antiviral activity of SLP-32 against TNV infection

Treatment	Average lesion number	% Inhibition
Buffer control	89.3	
ActD (20µg/ml)	84.6	
SLP-32 (+ve control)	4.6	94.8
(SLP-32)+ActD after 0h	77.8	12.8
(SLP-32)+ActD after 6h	4.19	95.3
(SLP-32)+ActD after 10h	5.3	94.1

Control sets were treated with buffer alone or with ActD solution alone (20µg/ml), purified SLP-32 was applied on leaves of *P.vulgaris*. In one set, actinomycin (ActD 20µg/ml) was applied immediately, and in other sets ActD treatment was given after 6 and 12 h of SLP-32 application.

Assay for N-glycosidase activity

Reverse transcriptase polymerase chain reaction (RT-PCR) and specific primers designed from the universally conserved region of 12 nucleotides in rRNA of both eukaryotes and prokaryotes (Kataoka et al., 1992) were then used for the assay of rRNA depurinating activity for SLP-32 on *S.longifolia* and *P.vulgaris* leaf ribosomes through detection of RIP diagnostic fragment. Amplifications of cDNA from 25S rRNA, isolated from *S.longifolia* and *P.vulgaris* by RT-PCR, revealed amplified product about 250 bases (figure 3), which corresponds to RIP diagnostic fragment of target ribosomes. Since RIPs inhibit protein synthesis by deglycosylating rRNA through cleaving the sugar-phosphate back bone at depurination sites (Tumer et al., 1997), these results indicate that SLP-32 is a broad spectrum RIP that depurinates its own ribosomes. These results resemble those reported by Kurinov (1999) on the pokeweed antiviral protein (PAP), and the heterologous ribosomes from other plant species (*P.vulgaris* ribosomes) as well. This is also in accordance with the results of Taylor and Irvin (1990) on *Phytolacca americana*, Park et al., (2004) on *Chenopodium album*. These ribosomes inactivation and consequently cell death is the major cause of viral inhibition of RIP- associated activity (Taylor et al., 1994; Hao et al., 2001; Desmyter, 2002; Sawasaki et al., 2008; and Sikriwal et al., 2008).

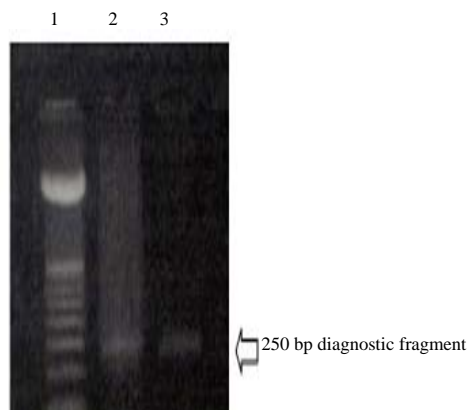


Figure 3: 1.2% Agarose gel electrophoresis of RT-PCR amplified product of RIP diagnostic fragment isolated from the leaves of *Phaseolus* (lane 2) and *Salsola* (lane 3) treated with SLP-32. Lane 1 represents the DNA molecular weight marker (100, 200, 300, 400) bp.

DNase activity

pBlueScript SK⁺ DNA has three forms, namely supercoiled, nicked, and linear. Upon incubation of pBlueScript SK⁺ DNA with different amounts of SLP-32, it was clear that the degree of supercoiling was altered. Upon incubation of the supercoiled form of pBlueScript SK⁺ DNA with 10 and 20 μ g of SLP-32, it first nicked giving a nicked circular form. This nicked circular form moved slower than the supercoiled DNA form through the agarose gel. The SLP-32 concentration was then increased to 30 μ g causing the emergence of the third form of DNA namely, the linear form. This linear form of DNA migrated faster than nicked circular form and slower than the supercoiled form. Since RIPs cleave only the supercoiled DNA by nicking before linearizing the strand cleavage, the above results implies that the DNase activity of the SLP-32 was conformation specific (Lau et al., 1998, Barbieri et al., 2000 and Choudhary et al., 2008). To confirm that the band shift was not caused by the binding of SLP-32 onto pBlueScript SK⁺ DNA, the two species were separated before electrophoresis by digestion with proteinase K, after the DNA was incubated with SLP-32. Afterwards, the DNA was extracted with an equal ration of phenol and chloroform and precipitated with ethanol. Same changes in the bands pattern were confirmed by the electrophoretic analysis (figure 4).

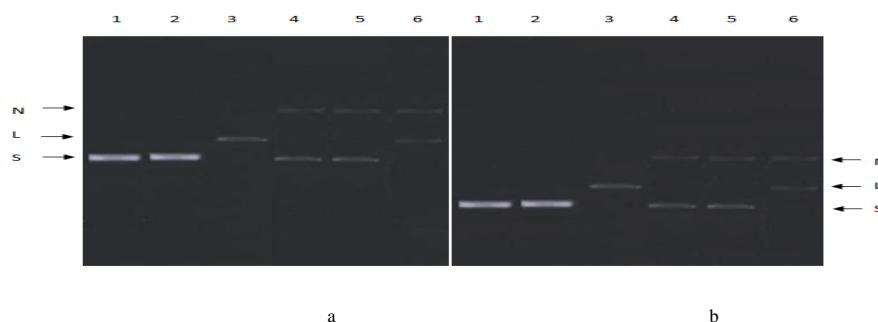


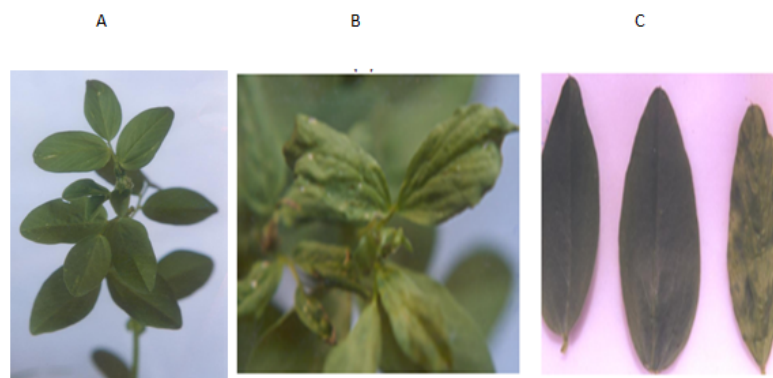
Figure 4: a) DNase activity test. Incubating with 10 or 20 μ g of SLP-32, pBlueScript SK⁺ DNA supercoiled form (S) shows nicked form (N) (lanes 4 and 5) and with 30 μ g of SLP-32 shows linear form (L) (lane 6). Lanes: 1) plasmid; 2) control; 3) plasmid restricted with *Eco* RI. b) DNase activity test after proteinase K treatment. Same changes in banding patterns indicating that band shifting was not because of the binding of the protein with the plasmid DNA.

Antiviral bioassay for SLP-32

Antiviral activity of plant extracts was always used to determine the potential of identifying new RIPs. Isolation of RIPs helps studying their biological activities as well as determining the mechanism of action of these RIPs on the infection process produced by several plant viruses. The results indicated that SLP-32 significantly inhibited local lesion formation by TNV on *P. vulgaris* causing 95-97% inhibition, as in table (1). Effects of different RIP concentrations, from *S. longifolia* against bean yellow mosaic virus (BYMV) systemically infected *V. faba* plants, was also studied to determine the concentration causing the highest percent inhibition. Results in table (2) show that the higher the concentration of RIP the higher the percent of inhibition showing 94.1% inhibition at 100 μ g/ml of RIP concentration. These results were consistent with those reported by Watanabe et al. (1997), Zoubenko et al., (2000), Nielsen and Boston (2001), and Iglesias et al., (2005). There are many possibilities to explain the antiviral activity of RIPs against plant viruses. RIPs can act on the viral nucleic acids through their PAG activity, or can act on the host by selectively killing the infected cells, thus preventing the virus from replicating and propagating to neighbor cells. RIPs can also act indirectly by activating the plant defense system (Peumans et al., 2001; Gholizadeh et al., 2004; and Sikriwal et al., 2008). Figure (5) below show the healthy *vicia faba* plants, the plant inoculated with SLP-32 and BYMV, and the plant inoculated with the virus.

Table 2: Effects of different RIP concentrations extracted from *S.longifolia* on *Vicia faba* plants inoculated with BYMV

Concentration of <i>S.longifolia</i> RIP µg/ml	% Inhibition of
5	29.0
10	34.1
30	38.0
40	42.3
50	66.7
60	69.5
70	76.3
80	89.3
90	93.75
100	94.1

Figure (5): (A) Healthy *Vicia faba* plants (control); (B) *Vicia faba* plants systemically infected with Bean Yellow Mosaic Virus (BYMV); (C) Left is *Vicia faba* plants inoculated with SLP-32 and the virus; middle is the healthy *Vicia faba* plants (control); right is the systemically infected *Vicia faba* plants with BYMV.

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