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Molecular characterization of antiviral proteins, isolated from host plants, pretreated with antiviral glycoprotein, isolated from roots of *boerhaavia diffusa* plants

Abstract

Antiviral proteins were isolated, from host plants, following treatment with an antiviral glycoprotein, extracted from the roots of *Boerhaavia diffusa* plants. It was found to be active against isometric as well as an isometric viruses in their respective hypersensitive and systemic hosts. The biologically active principal was isolated and purified. Estimation of proteins, from the leaf samples of *N.glutinosa* plants, indicated higher concentration of new proteins in plants whose two basal leaves were treated 24 hours earlier with an aqueous solution of the glycoprotein. SDS-PAGE analysis studies on partially purified antiviral principal of protein nature revealed that the size of band in L3 (after 24 hours) was 30 Kda along with very dark and prominent bands. The size of band recorded in rest treatments was of 36 Kda and 50 Kda.

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Introduction

A large number of wild and cultivated plants are known to contain endogenous proteins that act as virus inhibitors.[1-9] These antiviral substances ,mostly of protein nature, are referred as ribosome-inactivating proteins.[6,10] These proteins have been studied in *Phytolacca* Americana,[4,11,12] *Mirabilis Jalapa*[13,14] and *Trichosantheskirilowii*.[15] These proteins show antiviral activity when mixed with virus inoculum and are found to be localized extracellularly in plants.[16,17] On the other hand, some virus inhibitors of plant origin have been reported to induce systemic resistance in non-treated parts of plants also and thereby preventing infection of viruses.[6,7,9,18-22] One such glycoprotein isolated from *B. diffusa* roots has prevented virus infection and multiplication in plants.[18,19,21] It has shown very high antiviral activity when mixed with viruses *in vitro* and provoked the plant system to produce new protein(s) in the treated plants which is the actual virus inhibitory agent (VIA).[19] This glycoprotein thus induced antiviral state in the plants through formation of *de novo* synthesized protein and perhaps is active in signaling the activation of defense mechanism in susceptible hosts.[23,24]

High antiviral activity was observed in the sap extracted from the leaves of host plants treated with *Boerhaavia diffusa* glycoprotein.[19,23] No such activity was detected in the sap from leaves of non-treated (control) plants. Obviously sap from treated leaves contained some antiviral agent (AVA)/protein which was absent from normal (untreated) plants. Induced antiviral agent was found to be serologically unrelated with different viruses. Possible mechanism of action of this antiviral protein was studied *in-vitro* and *in-vivo* against a few plant viruses and it was established that the antiviral glycoprotein induced systemic resistance in treated plants and thus further infection is prevented.[24] This communication reports molecular characteristics of induced antiviral agent(s) (AVA)/proteins from host plants.

Materials and methods

The maintenance of virus cultures, preparation of virus inocula, preparation and isolation of *Boerhaavia diffusa* antiviral glycoprotein, extraction of AVA from host plants and methods of treatments were the same as described earlier.[19,23] Seeds of *Nicotiana. glutinosa*L., a hypersensitive host for tobacco mosaic virus were sown in sterilized compost soil in earthen pots. Plants of the same height, age and vigor, having four fully expanded and identical leaves, were selected for experimental work. All the experiments were conducted in an insect free glass house/wire net house at about 22 + 60 C. The data was analyzed statistically by the test of comparison between the control and the individual treatment (check versus treatment) to test for the significance of the activity of AVA.[25]

Maintenance and purification of Tobacco mosaic virus (TMV)

Maintenance of the pure culture of tobacco mosaic was done by its successive inoculations on *N. tabaccum* var. NP 31 plants. Purification and preparation of virus inocula were the same as described earlier.[19]

Extraction of AVA from leaves of *N.glutinosa* plants, pre-treated with purified *B. diffusa* inhibitor,at different time intervals

About 200 gram fresh, non treated upper leaves of *N. glutinosa* plants, two basal leaves on the same plant had been sprayed 24 hours earlier with *Boerhaavia diffusa* glycoprotein,[21] were harvested, washed thrice with distilled water, blotted dry and stored in Frigidaire for 12 hours. Frozen leaves were thawed and crushed in a mixer grinder with 200 ml of 0.1M phosphate buffer (pH 7). Pulp obtained was squeezed through two folds of muslin cloths. Sap was then centrifuged at 3000g for 15 minutes. Supernatant thus, obtained was centrifuged at 1,20,000g for 90 minutes. Then the more or less opalescent brown supernatant fluid was immediately filtered to remove some lipid materials. The filtrate was dialyzed against 100 times volume of phosphate buffer for 48 hours at 40C. The non-dialyzable fractions were centrifuged at 5000g for 15minutes. Biologically active non-dialyzable material was mixed with petroleum ether in equal amounts. Mixture was then shaken vigorously in a separating funnel for 15 minutes and allowed to settle. The two layers (upper solvent and lower aqueous) were collected separately. Aqueous fraction was kept in an oven to evaporate the solvent, if any. An equal volume of a saturated solution of ammonium sulphate was added to this fraction and kept overnight in a Frigidaire. After 12 hours the mixture was

centrifuged at 8000g for 15 minutes. The precipitate obtained was suspended in 10 ml of phosphate buffer. The solution was centrifuged at 8000g for 30 minutes and the supernatant obtained was dialyzed at 40C against the same buffer by continuous stirring for 48 hours. After dialysis the soluble fraction was first centrifuged at 3000g for 20 minutes so as to remove any precipitated material. The clear supernatant was later on centrifuged at 1, 20,000g for 90 minutes. The supernatant showing antiviral activity was lyophilized. The lyophilized material was designated as antiviral agent (AVA).

Estimation of the concentration of new proteins induced in N.glutinosa plants by the application of *B. diffusa* inhibitor

The concentration of new proteins, induced in upper non treated leaves of *N.glutinosa* plants, whose lower two leaves were treated 6 ,12 , 24 and 36 hours earlier with antiviral glycoprotein isolated from the roots of *B.diffusa* plants ,were estimated by colorimetric method.[21,23,26]

SDS-PAGE analysis of purified induced AVA, extracted from the leaves of *N.glutionsa* plants

Protein samples were analyzed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by staining with comassie brilliant blue.[27]

Results

Data presented in Table 1 have clearly indicated maximum protein contents (156.11 mg/g) in leaf samples taken from *N.glutinosa* plants, pretreated 24 hours earlier, on basal two leaves, with an aqueous solution of glycoprotein isolated from the root of *B.diffusa* plants, followed by leaf samples taken 12 hours(145.58 mg/g) ,36hours (137.06 mg/g) and 6 hours later (125.15 mg/g). It was revealed from Figure 1 that none of local lesions appeared on the leaves of *N. glutinosa* plants, two lower leaves of which were treated 24 hours before tobacco mosaic virus challenge, with an aqueous solution of antiviral substance from the roots of *B.diffusa* plants. This clearly indicated that the antiviral substance in *B.diffusa* was translocated in whole of the plant, if applied either on lower or upper leaves before virus inoculation. However a few local lesions appeared on the leaves of *N. glutinosa* plants, in which the antiviral substance was, applied 6 or 12 hours before virus challenge. On the other hand, a large number of local lesions appeared on the leaves of untreated (control) plants, pretreated with sterile water instead of anti viral substance.

Table 1 Estimation of Protein contents (induced AVA) in *Nicotiana glutinosa* leaves following the application of partially purified *Boerhaavia diffusa* inhibitor at different time intervals (Optical density recorded at 750 nm)

Treatment	6 hrs mg/g	12 hrs mg/g	24 hrs mg/g	36 hrs mg/g
*B. diffusa inhibitor*treated	125.15	145.58	156.11	137.06
Control	77.73	82.7	84.4	79.31
Sem±	1.346	0.473	0.653	0.471
CD at 5%	4.659	1.636	2.258	1.629

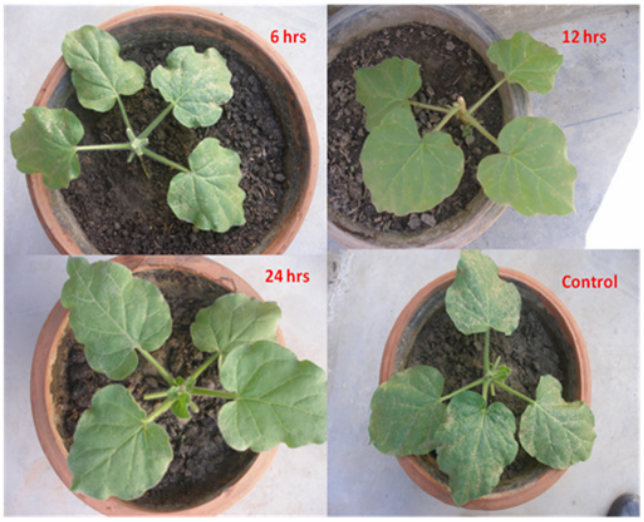

Figure 1 Induction of systemic resistance in upper untreated leaves of *N. glutinosa* plants(lower leaves in each plant were treated with aqueous root extract/ antiviral substance from *B. diffusa* plants 6,12 and 24 hrs before tobacco mosaic virus challenge) control (untreated plants).

SDS-PAGE analysis of partially purified antiviral principal of proteinaceous nature isolated from leaf extract of two upper non-treated leaves of *N. glutinosa* plants whose two lower leaves were treated 24 hours earlier with an antiviral glycoprotein isolated from root extract from *B. diffusa*plants, revealed very dark and prominent bands (L3).The size of band was of 30 Kda .However, SDS-PAGE analysis studies of induced AVA in L1 (juice extracted after 6 hours) ,L2 (juice extracted after 12 hours) and L4 (juice extracted after 36 hours) exhibited very faint and inconspicuous bands . On the other hand, no such protein band was observed in juice extracted from 2 upper leaves from control (untreated) plants. The size of band recorded was 36 Kda and 50 Kda in all the treatments (Figure2,3).

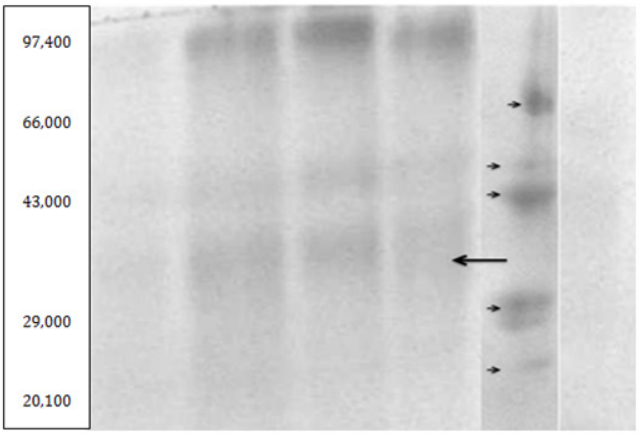

Figure 2 SDS-PAGE analysis of induced AVA extracted from leaves of *N. glutionsa* plants, pretreated with *Boerhaavia diffusa* inhibitor : L1= 6 hrs, L2= 12 hrs, L3= 24 hrs, L4= 36 hrs, M= marker (mw- 14.3- 97.4 kDa) and control (untreated/ distilled water treated) leaves after L5= 6 hrs, L6= 12 hrs, L7= 24 hrs, L8= 36 hrs and L9 (control). (L=Lane).

Discussion

Experimental finding revealed that partially purified *B. diffusa* inhibitor induced systemic resistance in treated and non-treated parts of plants several hours after treatment. When the inhibitor was applied by spraying, prior to virus inoculation, on to the basal leaves

of the test plants, the local and systemic interference developed from 6 hours onwards reducing local lesion number by 100% at 24 hours. Maximum protein contents were also recorded in samples, collected from upper two leaves of plants 24 hours after the application of partially purified *B. diffusa*inhibitor (156.11 mg/g) on basal two leaves of *N. glutinosa* plants. SDS-PAGE analysis of partially purified *B. diffusa*inhibitor revealed that size of band was 30 Kda , SDS-PAGE analysis of induced AVA showed very dark and prominent bands in L3 (after 24 hours) and size of band recorded was 36 Kda and 50 Kda in all the treatments. AVA was effective up to a dilution of 1:500 and 1:1000 respectively. It was non dialyzable but inactivated at 95 0C for 10 minute with longevity *in vitro* up to 3 to 4 month , when stored at freezing temperature (4-10 0C) but lost its antiviral activity after 4 month. The extracted AVA was active at pH 10 but inactivated at pH 4.

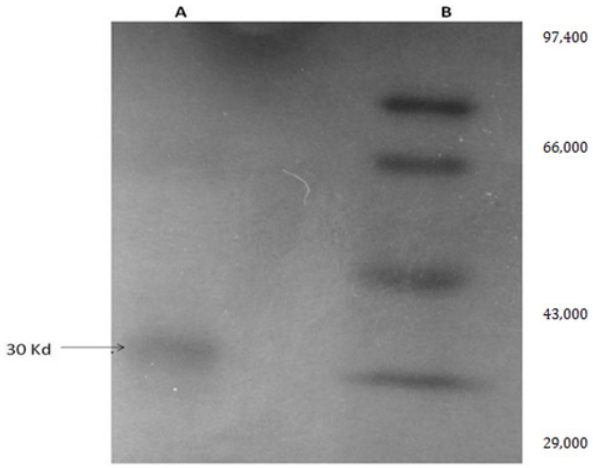

Figure 3 SDS-PAGE characterization of Partially Purified *Boerhaavia diffusa* Inhibitor, Lane A-BD inhibitor and Lane B-Molecular Marker (mw-14.3-97.4 kda).

The glycoprotein stimulated natural viral defense mechanism existing in susceptible plants and provided systemic protection of a very high degree.[18] The systemic resistance inducers (SRIs) present in roots of *B.diffusa* induced strong systemic resistance in several susceptible hosts against viruses, reacting hypersensitively or systemically, when applied/sprayed 24 hours before virus inoculation or when regularly (weekly) sprayed, by ordinary sprayers, soon after emergence of seedlings.[28] By using certain modifiers/spreaders and micro-propagating the plants of *B. diffusa*on suitable modified MS medium, the antiviral activity has been markedly improved and prolonged.[29]

The SRIs were purified using modern protein purification techniques and were identified as basic glycoprotein with molecular weight in the range of 16-20 kDa. The systemic resistance inducing proteins (SRIPs) were extremely thermos table and retain biological activity upon incubation with pronase, trypsin, and pepsin. The SRIPs are non-phytotoxic, promote plant growth, improve crop yield and quality and show a broad spectrum of protection. Following treatment with BD, the treated hosts accumulated a new virus inhibitory agent (VIA) in treated and non treated parts of plants. The induced virus inhibitory agent (VIA) shows characteristics of protein and reduces infectivity of viruses both *in vitro* and *in vivo.* The SRIPs and VIAs are immunologically two distinct proteins. The VIA is neither host nor virus specific and is not accumulated.[23]

Verma and Awasthi[19] also reported that the AVA was synthesized when test plants were pre-treated with BD inhibitor. It was neither virus specific nor host specific as the antiviral activity was found in different virus/host combinations[30-32] Verma *et al.,* 1979. The inhibitor containing AVA when incubated with viruses reduced the infectivity of viruses. AVAs from the leaves of a few host plants were characterized and found to be as low molecular weight proteins.[23] The AVA production in test hosts was maximum after 24 hours treatment with BD inhibitor followed by 12,6 and 0 hours. Induced Proteins in host plants function as a signal molecule and is of great interest, as it has a role in stimulating the defense system of plants against infection of viruses. The inhibitor was a basic glycoprotein (70-80% protein + 8-13% carbohydrate) with a molecular weight of 16-20 kDa as determined by gel-filtration chromatography.[21,33] The protein has a PI greater than 9 and gives a molecular weight of 30 kDa on SDS-PAGE. Glycoprotein, a systemic resistance inducer, from *B. diffusa* was studies earlier[6-9,18,20,34,35] which has molecular weight 16-20 kDa. Physico-chemical characteristics of systemic resistance inducers from many other plants were studied.[18,19,22]

The work has demonstrated for the first time that the inducible plant defense system against viruses can be switched on after treatment with certain highly specific basic phytoproteins[24,28] and has opened a new field of 'Plant Immunology'. This more recently developed novel strategy of immunizing plants using the phytoproteins showed great promise as it is versatile, nonspecific and entirely risk free. A meaningful virus disease control, through host plant resistance, using non pathogen product has been achieved for the first time. The SRIPs indirectly prevent virus infection and perhaps are active in signaling the activation of defense mechanisms in susceptible hosts. Upon treatment these systemic resistance inducing proteins (SRIPs) provoke the plant to produce a new defensive protein in the treated plants, which is the actual virus-inhibitory protein (VIA). These SRIPs, like interferon, are the only natural substances with the proven ability to inhibit *in vivo* virus infection and replication and will be very useful for immunization of susceptible plants against commonly occurring viruses. This strategy of defense in plants, in outcome, but not mechanism is comparable to the inducible defense system in animals. Although specific immunoglobulins have not been found in plants, but many new defensive proteins are formed in plants following treatment with a large number of different agents. These defensive proteins are effective against diseases caused by fungi, bacteria and viruses. These defensive proteins effective against cellular pathogens are more common and induced in greater quantities and number, whereas, induced proteins effective against viruses are formed in smaller quantities and hence their detection and purification was difficult. Cellular pathogens during attachment have the ability to elicit defense responses in plants, whereas, viruses since are directly delivered into the cell, the attachment process is by passed and hence they are not able to elicit strong defense response to produce detectable amounts of defensive proteins. Some phytoproteins, which have some chemical similarity to viruses when applied externally in proper concentrations, have the capacity to elicit defense response by producing greater quantities of defensive proteins effective against viruses.[31]

Plant virus interactions are therefore mechanistically distinct from those involving other biotic agents and thus, the basis of resistance is likely to differ as well. The resistance inducing proteins (SRIPs) are extremely thermostable and retain biological activity upon incubation with pronase, trypsin, and pepsin. The induced VIP was further purified and characterized. It shows characteristics of protein and reduces infectivity of viruses both *in vitro* and *in vivo*. The VIA is neither host nor virus specific and is not accumulated in the presence of actinomycin-D.[23] Immunization by plant products

that stimulate the plant's natural disease resistance mechanism may provide a new strategy for crop protection against viruses. By using certain modifiers/spreaders and micropropogating the plants of *B. diffusa*on suitable modified MS medium, the antiviral activity has been markedly improved and prolonged.[36] Thus, a modest beginning towards successful virus control has been made.

Acknowledgments

None.

Conflicts of interest

None.

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