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Further Studies on the Antiviral Agent Isolated from Host Plants, Pre-treated with *Boerhaavia diffusa* Glycoprotein

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Abstract

Induced antiviral agent(s), following treatment with *Boerhaavia diffusa* glycoprotein in several host plants, was purified by fractionation with organic solvents followed by precipitation with ammonium sulphate, dialysis, ultracentrifugation and filtration through Amberlite, Polyamide, Silica gel, DEAE cellulose and Sephadex gel columns. Biologically active material was eluted in two different fractions. Purified Antiviral Agent(s) (AVA) was active selectively against anisometric and isometric viruses. It was hydrolyzed by Trypsin and Pronase but not affected by RNAs or DNAs. It showed protein nature. Molecular weight of AVA fractions was 8000-10,000 and 32,000-35,000 daltons.

Keywords: *Boerhaavia diffusa*; Antiviral agent; Systemic induced resistance

Introduction

Many higher plants are known to contain endogenous proteins that act as virus inhibitors [1-3]. All these belong to a class of proteins called ribosome-inactivating proteins (RIPs). These proteins have been studied in *Phytolacca americana [4], Mirabilis jalapa [5]* and *Trichosanthes kirilowii* [6]. These proteins show antiviral activity when mixed with virus inoculum and are found to be localized extracellularly in the plants [7,8]. 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 [3,9-13]. One such glycoproteinaceous substance isolated from *B. diffusa* roots has prevented virus infection and multiplication in plants [14,15]. 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) [16]. This glycoprotein thus induces 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.

Singh and Awasthi [17,18] reported that the aqueous root extract of *B. diffusa* effectively reduced mungbean yellow mosaic and bean common mosaic virus disease in mungbean and urdbean along with increased grain yield in field conditions. Later Awasthi and Kumar [19-22] found that weekly sprays significantly prevented infection, multiplication and spread of Cucumber mosaic virus, Bottle gourd mosaic virus, Cucumber green mottle mosaic virus and Pumpkin mosaic virus in cucurbitaceous crops. Kumar and Awasthi [23] reported that infection and spread of cucumber mosaic disease was prevented. Singh and Awasthi [24] evaluated various medicinal plants for the management of yellow mosaic disease of mungbean (Vigna radiata). Yadav et al. [25], Awasthi and Yadav [26] management of viral diseases of tomato by seed treatment and foliar sprays of *Boerhaavia diffusa* root extract.

High antiviral activity was found in the sap extracted from the leaves of host plants sprayed with *Boerhaavia diffusa* glycoprotein [16,26-29]. No such activity was detected in the sap from leaves of non-treated (control) plants. Obviously sap from treated leaves contains some antiviral agent (AVA)/ protein which is absent in normal plants. Induced antiviral agent had inactivated serologically unrelated and morphologically different viruses, when mixed *in vitro* [30]. This communication reports isolation, biophysical and chemical characteristics of induced antiviral agent(s) (AVA) /proteins from several host plants.

Materials and Methods

Details of raising of test hosts, maintenance of virus cultures, preparation of virus inocula, preparation and isolation of *B. diffusa* glycoprotein, extraction of AVA and methods of treatments were the same as described earlier [30]. All the experiments were conducted in an insect free glass house/ wire net house at about 22 + 60°C. The data were 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 [31].

Purification of antiviral agent

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 [32], 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 0.1 M phosphate buffer (pH 7). Pulp obtained was squeezed through two folds of muslin cloths. Sap was then centrifuged at 3000 g for 15 minutes. Supernatant thus, obtained was centrifuged at 1,20,000 g 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 40°C. The non-dialyzable fractions (bag contents) were centrifuged at 3000 g for 15 minutes. 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

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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 3000 g for 15 minutes. The precipitate obtained was suspended in 10 ml of phosphate buffer. The solution was centrifuged at 3000 g for 25 minutes and the supernatant was dialyzed at 40°C against the same buffer by continuous stirring for 48 hours. After dialysis the soluble fraction was first centrifuged at 3000 g for 25 minutes so as to remove any precipitated material. The clear supernatant was later on centrifuged at 1,20,000 g for 90 minutes. The supernatant showing antiviral activity was lyophilized. The lyophilized material was designated as antiviral agent (AVA). Further fractions were separated by eluting through columns of Amberlite, Polyamide, Silica gel, DEAE cellulose and Sephadex G 75. For comparison, upper leaves from *N. glutinosa* plants sprayed with distilled water were also harvested and processed by exactly the same procedure. Partially purified material from non-treated plants (controls) was termed as "mock AVA". This was further purified by the same procedure as described for AVA.

Packing of the column and elution

A-Amberlite: A glass column of 2.5x35 cm was packed upto 10 cm with activated red Amberlite crystals (IR 120, B.D.H. Chemicals Ltd., England). 25 mg of active material was dissolved in 1ml distilled water and centrifuged. The clear supernatant was applied on to the top of the Amberlite, and 10 fractions of 2 ml each were eluted with distilled water. Excessive washing with a 5% Ammonia solution in water again activated the column. Antiviral activity of all the fractions was tested against TMV on *N. glutinosa* plants.

B-Polyamide: Polyamide has a property to adsorb the phenolic substances by hydrogen bonding and the solvents of increasing pH can elute the adsorbed substances. Polyamide (B.D.H. Chemicals Ltd. England) activated with a 5% solution of ammonia in water, washed thoroughly with 0.01 M phosphate buffer till pH becomes neutral was loaded in a glass column of 0.5 cm x 20 cm up to 10 cm. A flow rate of 0.2 ml/hr was maintained and fractions of 2 ml each were collected. 25 mg lyophilized material was dissolved in 1 ml of 0.01 M phosphate buffer and applied by a sample applicator on to the top of the buffer. After the material had been adsorbed, it was eluted with the same phosphate buffer and 5 fractions of 2 ml each were collected. Further elution was counted out with methanol and another 5 fractions, of 2 ml each were collected as earlier. Exhaustive washings with 5% ammonia solution in water regenerated the polyamide. Five more fractions, of 2 ml each, with 5% ammonia solution were also collected. Polyamide was finally washed with distilled water till the washings were neutral.

C- Silica gel: Silica gel (B.D.H. Chemicals Ltd. England) for column chromatography, swelled in distilled water for 48 hours, was packed up to 20 cm in a 2 x 50 cm glass column. The column was equilibrated with phosphate buffer. 25 mg lyophilized material dissolved in 1 ml phosphate buffer was applied on to the top of the gel and 20 fractions, of 2 ml each, were collected. The elution was done further by 5% methanol and 10 factions, of 2 ml each, were again collected. Antiviral activity of all the fractions was tested against TMV in *N. glutinosa* plants.

D–DEAE cellulose: Diethylaminoethyl cellulose (Standard, Carl Schleicher and Schwell Co., Capacity 0.9 meg./g) was used as anion exchanger. Several gradients of Nacl concentrations were applied as elluents.

DEAE-cellulose was swelled in distilled water for 6 hours and then washed with 0.1 M NaOH for 30 min. The pH of DEAE cellulose was brought to neutral by repeatedly washing with distilled water. Thus neutralized DEAE cellulose was again washed with 0.1 N HCl for 30 minutes and neutralized by washing with distilled water. A glass column of 80 cm in length and 0.8 cm in diameter was packed up to 16 cm with DEAE cellulose. The column was equilibrated with 0.01 M phosphate buffer, pH 7.0. 25 mg of lyophilized sample was dissolved in 1 ml of 0.01 M buffer and loaded on to the top of DEAE-cellulose. It was then eluted with the same buffer and 5 fractions, of 4 ml each, were collected. In the second step 0.5 M NaCl in phosphate buffer was used for elution and 6fractions, of 4 ml each were collected. Fractions eluted with buffer and NaCl were dialyzed against 0.01 M buffer. Antiviral activity of fractions was tested against TMV in *N. glutinosa* and SRV in *C. tetragonoloba* plants.

E-Sephadex gel: Sephadex G75 (Pharmacia, Fine Chemicals, Uppsala, Sweden), was prepared and packed up to 60 cm into a 2.5 x 100 cm column which allowed a flow rate of 0.2 ml/min. The column was equilibrated with phosphate buffer. All operations were performed at 40°C. The partially purified and freeze dried AVA was dissolved in phosphate buffer (50 mg/ml) and applied on to the top of Sephadex gel. It was then eluted with the same buffer and sixty fractions, of 5 ml each, were collected. The absorbance of each fraction was recorded at 280 nm. The elution volume (Ve) of the fraction showing antiviral activity (against isometric and anisometric viruses) was calculated and the ultraviolet absorption was recorded at 220-340 nm.

Molecular weight of the induced antiviral agent (AVA)/ proteins

The molecular weight of induced antiviral agent(s)/ protein(s) in the purified preparation was determined by Sephadex gel filtration method [30,33]. Partially purified material (25 mg in 1 ml phosphate buffer containing 2 mg of blue Dextran 2000) was layered under the buffer on to the top of the column of Sephadex G75. As described earlier, sixty fractions of 5 ml each were collected. Four proteins of known molecular weight (Cytochrome C., 12, 500 - Vallabhi Bhai Patel Chest Institute, New Delhi; Lysozyme, 14, 000 - Serva Fein Biochemicals, Germany; Chymotrypsin, 24, 500 - British Drug house, India and Bovine Serum Albumin, 68,000 - Central Drug Research Institute, Lucknow) were also passed through the same column and sixty fractions, of 5 ml each, for each reference protein, were also collected. The void volume (Vo) of the column was determined by measuring the elution volume (Ve) of the Blue Dextron 2000 band front. The elution volume (Ve) and the absorbance at 280 nm of all the fractions of each reference protein and the antiviral agent(s) AVA was determined. A standard graph was prepared by the plots of Ve/V0 ratios of these proteins against their logarithmic molecular weight and the molecular weight of AVA was derived from it [16,33,34].

Qualitative tests for proteins, carbohydrates, phosphorus, nucleic acid, phenolics, alkoloids and lipids

Chemical tests were performed for the presence of these groups as described earlier [35-41].

Thin layer chromatography

The purified samples were hydrolyzed with 6 N HCl in a sealed vessel for 36 hours and then evaporated to dryness under reduced pressure to remove excess HCl. The residue obtained was dissolved in 2 ml distilled water and centrifuged at 3000 g for 15 minutes. The supernatant solution (20-40 µl) was spotted on glass plates coated with thin layer of silica gel and two-dimensional chromatography was performed. The chromatograms were developed in n-butanol: acetic acid: water (4:1:5) and amino acids were located with 0.2% ninhydrin

in acetone [42,43]. For comparison two-dimensional chromatography of the 'Mock AVA' was also performed in the same way.

Quantitative analysis of proteins

The proteins in the AVA and 'mock AVA' were estimated separately by Folin phenol reagent [44].

Biophysical characteristics of AVA: Biophysical characteristics of AVA like dilution, thermal inactivation, longevity, effect of pH and Hclo4 were studied in the same way as described for crude AVA [16]. Check experiments with 'mock AVA' were also performed in same way.

Enzymatic digestion

Effect of trypsin: The enzyme solution consisted of 4 µg/ml of Trypsin (BDH Chemicals Ltd., England) in 0.1 M phosphate buffer, pH 7.6. Substrate solution was prepared by dissolving 2.5 mg/ml of lyophilized AVA. Equal volumes of enzyme and substrate solutions were mixed and incubated at 370°C for 0, 10, 30 and 60 minutes. The solutions were then diluted to 1:10 and dialyzed against the same buffer for 6 hours with constant stirring at 40°C.

The antiviral activity of non-dialyzable AVA or purified protein digested with trypsin, pronase ribonuclease or deoxyribonuclease was tested. Leaves of the plants served as control were inoculated with TMV diluted with the 'mock AVA' digested with enzymes in the same way or diluted with respective enzyme solutions to the final concentration of the virus, so that control and treated solutions had the same enzyme and virus concentration.

In a separate set of control experiments, the antiviral activity of the AVA or 'mock AVA' was tested after being kept at 370°C for same time period. In this case, the AVA preparations were diluted 1:2 with the standard buffer instead of the enzyme solution.

Effect of pronase: Pronase (Sigma Chemicals Company, U.S.A.) was dissolved (5 µg/ml) in 0.1 M phosphate buffer pH 7.3. The substrate solution was prepared as above. Equal volumes of these solutions were mixed and incubated at 370°C for 0, 10, 30 and 60 minutes. The solutions were then dialyzed against the same buffer as above. After centrifugation antiviral activity of supernatant was tested against TMV in *N. glutinosa* plants. Control for both the cases consisted of 'mock AVA' sample, phosphate buffer or bovine serum albumin preparation.

Effect of ribonuclease or deoxyribonuclease: The enzyme solution consisted of 4 µg/ml of either of RNAs or DNAs (Sigma Chemicals Company, U.S.A.) in phosphate buffer. Lyophilized AVA, 'mock AVA', RNA or DNA solution in phosphate buffer alone served as substrate. Equal volumes of either of two solutions were mixed and incubated at 370°C for 0, 10, 30 or 60 minutes. Antiviral activity was then assayed against TMV on *N. glutinosa* plants.

Antiviral spectrum of purified AVA

Earlier tests indicated that crude AVA invariably reduced infectivity of isometric as well as anisometric viruses in their hypersensitive hosts when mixed *in vitro* and then infectivity was assayed.

Hence each of the fraction eluted through Sephadex gel was screened for antiviral activity *in vitro* against Tobacco mosaic virus (in *Chenopodium amaranticular, Datura stramonium* and *Nicotiana glutinosa*). Sunnhemp rosette virus (in *Chenopodium amaranticolor* and *Cyamopsis tetragonoloba*), Cucumber green mottle mosaic virus (in *Chenopodium amaranticolor*) and Gomphrena mosaic virus (in *Chenopodium amaranticolor* and *Vigna sinensis)*.

Results

Partially purified fractionated material with organic solvents when eluted through the columns of several resins, like amberlite and silica gel, did not exhibit any antiviral activity. Inhibitory material may either be adsorbed and could not be eluted later on or inactivated by these substances. Biologically active material could not be fractionated through the ion exchanger like DEAE cellulose column as well. However, it was successfully purified through the column of polyamide. Antiviral activity was detected in fraction number 2 and 3 (eluted with phosphate buffer). The colourless fractions completely inhibited tobacco mosaic virus infectivity. Optical density reading as well as chemical tests indicated the presence of proteins in fractions 2 and 3 only (Figure 1). Remaining fractions obtained after elution with methanol or ammonia was non-proteinaceous and did not show any antiviral activity. Protein containing fractions from AVA and 'mock AVA' were lyophilised and further fractionated on a Sephadex G75 column.

The elution profile of the inhibitor from a Sephadex gel column (Figure 2) showed that the inhibition ranged from fraction numbers 21-23 and 32-34 in which protein concentration was also high. Six protein peaks were observed with AVA but only two of them have shown antiviral activity. However 'mock AVA' had only four peaks (Figure 2). Ultraviolet absorption spectra of fraction 22 and 33 revealed the maximum and minimum absorption at 278 and 240 nm respectively (Figure 3).

Molecular weight of induced antiviral agent(s)/ protein(s)

The void volume (Vo) of the column, was 60ml (determined by Blue Dextron 2000) and the elution volumes (Ve) of Cytochrome C, Lysozyme, Chymotrypsin and Bovine serum albumin were 150 ml, 140 ml, 120 ml and 75 ml respectively. Elution volume of antiviral proteins was 110 ml (fraction 22) and 165 ml (fraction 33). A plot of Ve/Vo ratios of standard proteins (of known molecular weight) against their logarithmic molecular weight suggested that the molecular weight for new proteins is in between 32,000-35,000 daltons (fraction-22) and 8,000-10,000 daltons (fraction-33) (Figure 4).

Qualitative tests (chemical nature)

Chemical tests confirmed the presence of proteins in both the active samples. Tests for carbohydrates, phosphorus, nucleic acids (RNA and DNA sugars) phenolics, alkaloids, sugars, and lipids were negative.

Thin layer chromatography: Two dimensional thin layer chromatography of fraction 22 indicated the presence of 7 amino acids viz., alanine, aspartic acid, glycine, leucine, proline, tyrosine and valine, while fraction 33 contained 9 amino acids viz., alanine, arginine, glutamid acid, histidine, leucine, methionine, lysine, threonine and tyrosine. Amino acid analyzer confirmed the presence of these amino acids. Quantitatively fraction 22 and 33 contained 70-86% and 80-92% protein respectively.

Biophysical characteristics of AVA are summarized in Table 1.

Enzymatic digestion: Proteolytic enzymes trypsin and pronase adversely affectd antiviral activity of induced proteins which resulted in complete loss of activity. However, its antiviral activity remained unaffected by ribonuclease and deoxyribonuclease treatments (Table 2).

Antiviral spectrum of AVA fractions

Antiviral activity of different fractions, of AVA, eluted after Sephadex gel filtration clearly indicated that fraction 22 completely inhibited Tobacco mosaic, Sunnhemp rosette and Cucumber green

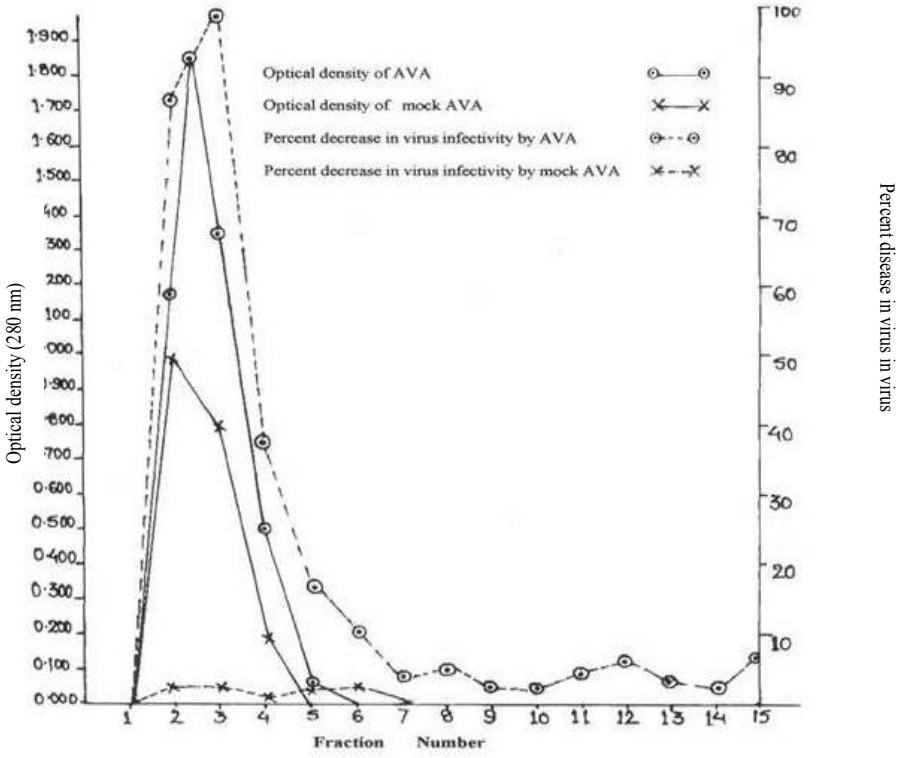

Figure 1: Fractionation of AVA through polyamide column.

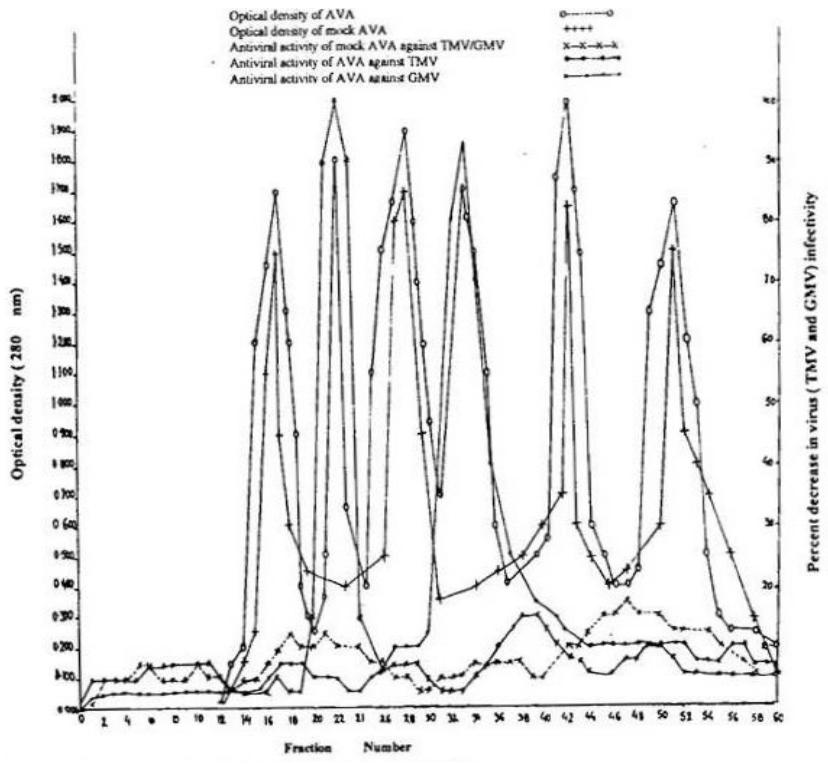

Figure 2: Fractionation of AVA through Sephadex G-75 Column.

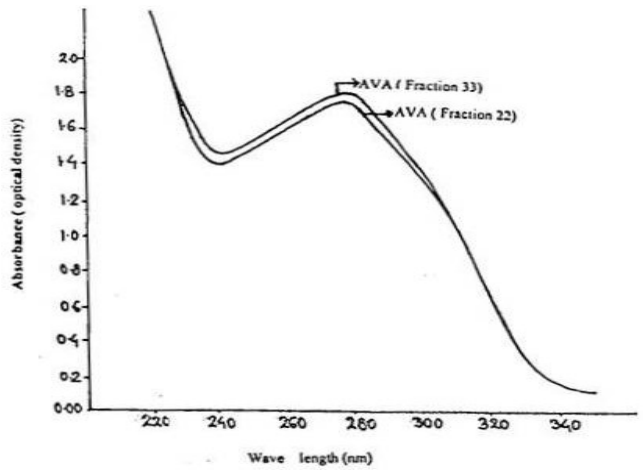

Figure 3: Ultraviolet light absorption spectrum of purified antiviral agent (fraction 22 and 33).

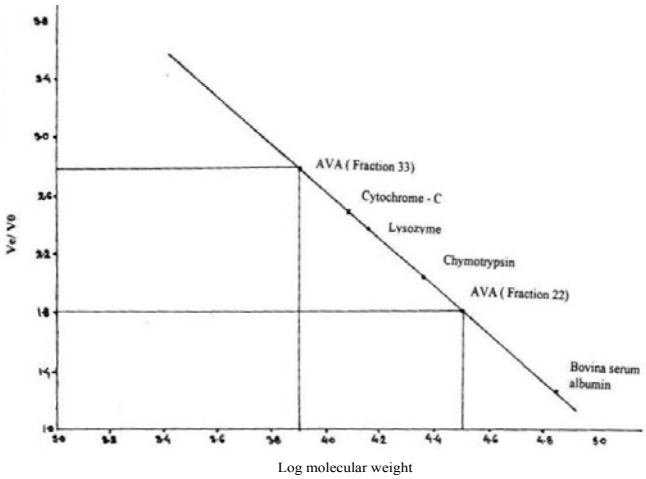

Figure 4: Molecular weight.

mottle mosaic viruses (anisometric viruses) in their hypersensitive hosts. However, it could not affect infectivity of Gomphrena mosaic virus (isometric virus) while fraction 33, which has shown significant inhibition of Gomphrena mosaic virus had no appreciable effect on anisometric viruses. If fraction 22 and 33 were pooled they were as much inhibitory both against isometric as well as anisometric viruses (Table 3).

Discussion

B. diffusa glycoprotein has been found to be effective in inducing strong systemic resistance in several susceptible hosts, by triggering immunity signals. It appears that in susceptible hosts this ability to produce defensive substances is present in a 'cryptic' form and can be evoked by triggering action of some specific inducer; *B. diffusa* glycoprotein. The stimulus provided by such bio molecules trigger signalling events that affect the whole plant. The stimulus leads to increase in steady–state levels of defence gene transcripts throughout the plant, within a few hours of treatment [11]. The 'induced antiviral agent' was isolated from several host plants and purified. This specific substance induced by B.D. glycoprotein in host plants turned out to be

Treatment	Results	
	Fraction –22	**Fraction-33**
Dilution	Active up to 0.002 mg/ml	Active up to 0.002 mg/ml
Heat sensitivity	Inactivated	Inactivated
Longevity	Active up to 6 months	Active up to 6 months
Dialyzability	Non- dialyzable	Non- dialyzable
Absorption on animal Charcoal or celite	Adsorbed	Adsorbed
Effect of trypsin	Inactivated	Inactivated
Effect of pronase	Inactivated	Inactivated
Effect of RNAs	Not affected	Not affected
Effect of DNAs	Not affected	Not affected
Nature	Proteinaceous	Proteinaceous

Table 1: Biophysical characteristics of purified AVA.

Treatment (virus inoculum mixed in a ratio of 1:1 with)	Percent decrease in virus infectivity			
	Incubation period (min)			
	0	**10**	**30**	**60**
Controls				
(i) Phosphate buffer	Nil	Nil	Nil	Nil
(ii) AVA	100a	100a	100 a	100 a
Mock AVA	Nil	Nil	Nil	Nil
Trypsin	29a	23a	37b	32 a
Pronase	16b	24b	26a	35b
RNAs	Nil	Nil	24a	29a
DNAs	Nil	Nil	Nil	Nil
Treated				
Trypsin + AVA	23b	Nil	Nil	Nil
Trypsin + mock AVA	Nil	Nil	Nil	Nil
Trypsin + buffer	Nil	Nil	Nil	Nil
Pronase + AVA	Nil	Nil	Nil	Nil
Pronase + mock AVA	Nil	Nil	Nil	Nil
Pronase + buffer	Nil	Nil	Nil	Nil
RNAs + AVA	100a	100a	100a	100a
RNAs + Mock AVA	Nil	Nil	Nil	Nil
RNA + buffer	Nil	Nil	Nil	Nil
DNAs + AVA	100a	100a	100a	100a
DNAs + Mock AVA	Nil	Nil	Nil	Nil
DNAs + buffer	Nil	Nil	Nil	Nil

Differences due to treatments are significant, a = at 1% level, b = at 5% level

Table 2: Enzymatic digestion of AVA.

Virus	Host	Percent decrease in virus infectivity		
TMV	*C. amaranticolor*	Fraction-22	Fraction-33	Fraction22 and 33 pooled
	D. stramonium	96a	12	95a
	N. glutinosa	98a	29b	98 a
SRV	*C. amaranticolor*	100a	17	100a
	C. tetragonoloba	82a	15	88a
CGMMV	*C. amaranticolor*	100a	23b	100a
GMV	*C. amaranticolor*	95a	-19	98a
	V. sinensis	25b	92a	95a
		14	100a	99a

Differences due to treatments are significant, a = at 1% level, b = at 5% level

Table 3: Antiviral spectrum of AVA.

a basic protein with a molecular weight between 32,000-35000 daltons and 8,000-10,000 daltons. The protein is not very stable and its activity was affected by trypsin and pronase but was unaffected by RNAs and DNAs.

Singh et al. [28,45,46] reported the prevention of yellow mosaic disease of mungbean and urdbean by clarified aqueous root extract of *B. diffusa*. Six sprays of *B. diffusa* root extract (10%) reduced 80-90 per cent disease incidence and increased nodulation, plant height primary and secondary branches, pod formation and grain yield. Awasthi and Singh [47] reported that the most effective treatment was seed treatment with *B. diffusa* root extract + three foliar spray which exhibited 65.36% reduction in disease incidence.

Virus inhibitors of plant origin preventing infection of virus in the untreated parts of test plants were first observed by Mc Keen [48]. He found inhibition of Cucumber mosaic virus infection in the untreated opposite primary leaf of cowpea whose other primary leaf was treated with an extract of pepper (*Capsicum frutescens*). No tests were done on upper leaves. He speculated that the inhibitor had translocated from one primary leaf to other leaves. However, no conclusive evidence was given of the transportability of virus inhibitor.

The first non – multiplying and non-translocating antiviral agent inducing systemic resistance in upper, non-inoculated leaves was a polysaccharide isolated from the culture filtrate of the fungus *Trichothecium roseum* [49]. Induction of such systemic resistance by substances from higher plants was first demonstrated by leaf extract of brinjal [50] and subsequently by root extract of *B. diffusa* [9]. Since then a number of plants have been reported to possess systemic resistance inducing substances, all of which are proteinaceous [3,11,26,28,29,45,51]. Sela and Applebaum [52] reported occurrence of an antiviral factor from virus infected plants which was later on purified and was found to be proteinaceous in nature [53-55].

The precise mechanism involved in systemic inducer mediated resistance involves formation of a virus neutralizing protein (VNP or VIA/AVA) which inactivates virus in-vitro and thus interferes with an event that is early in the infection cycle of the virus [3]. Following immunization with B.D. glycoprotein, plants respond rapidly and one component of the response is the rapid accumulation of virus neutralizing substance(s) mainly protein(s). Its formation is prevented by actinomycin D [16]. The production of antivirally active proteins by B.D. glycoprotein seems to be a defense response of plants to viral infections and could be utilized for practical purposes.

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