Isolation and Characterization of Two Protein Isoforms with Antiviral Activity from *Chenopodium album* L Leaves†

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Two antiviral proteins (AVPs) named CAP-I and CAP-II isolated and purified from the leaves of *Chenopodium album cv Pusa Bathua 1* were found to inhibit tobacco mosaic virus (TMV) and sunnhemp rosette virus (SRV) infection on their respective host plants. The molecular weight of both the AVPs was found to be 24 kD. They were devoid of carbohydrate moiety and were highly basic with pI ~10.2. However, they differed with respect to amino acid composition and N-terminal sequence. They also differed with respect to IC₅₀ values, and CAP-I was found to be 2.5 fold more effective than CAP-II in inhibiting viral infection.

**Key words**: *Chenopodium album*, antiviral proteins, purification, characterization.

A number of plant species belonging to different taxonomic families viz., Amaranthaceae, Chenopodiaceae, Euphorbiaceae, Gramineae, Nyctaginaceae and Pinaceae have been reported to possess inhibitors of plant virus infection(1-3). Most of these substances have been found to be of proteinaceous nature and are known as antiviral proteins (AVPs). The distribution of inhibitors in the different tissues of plants may vary from species to species(4-6). The occurrence of antiviral protein isoforms in the same or different organs of the same plant is a common feature. The isoforms may differ from each other in one to many respects like, molecular mass, amino acid sequence, amino acid composition and the mechanism of their action (7,8).

*Chenopodium*, a plant which grows wild as well as cultivated and used as green vegetable has been exploited for its antiviral properties. Several species of *Chenopodium* are known to possess viral inhibitory activity(9-12). Two antiviral proteins from the leaves of wild growing *Chenopodium album* have already been partially purified in our laboratory (13). The antiviral proteins from edible plants like *C. album* give an added advantage of avoiding any risk of inducing allergic response by consumption of transgenic plants expressing the genes encoding such proteins. In this paper, we report the purification to homogeneity and characterization of two antiviral protein isoforms from *C. album cv Pusa Bathua 1*.

**Materials and Methods**

**Source plant and test plants** — *Chenopodium album L cv Pusa Bathua 1* was used as source plant from which the antiviral proteins were isolated and purified. Two hypersensitive hosts, *Nicotiana glutinosa* for TMV and *Cyamopsis tetragonoloba* for SRV were used as test plants in order to test the antiviral activity during the isolation and purification of antiviral proteins. These plants were grown in insect-free glass house. Raising of the test plants, maintenance of viruses and preparation of virus inocula were carried out as described earlier (14). At different steps of AVP purification, only *C. tetragonoloba* / SRV system was used for bioassay purpose. However, the antiviral activity of purified AVPs was tested against both TMV and SRV on their respective local lesion hosts.

**Assay of antiviral activity** — For bioassay the method as described by Narwal et al (14) was used and per cent inhibition of lesion formation due to viral infection calculated by using the following formula :

\[
\% \text{ inhibition} = \frac{(C - T)}{C} \times 100
\]

where, \(C\) is the average number of lesions in control leaves and \(T\) is the average number of lesions in treated leaves.

**Purification of antiviral proteins** — Antiviral proteins (AVPs) present in leaves of *C. album* at peak vegetative
growth stage were purified by following various steps viz., ammonium sulphate fractionation, ion-exchange chromatography and size exclusion chromatography as per the method already described (13). 60 g dried leaves were used as starting material to have sufficient yield of AVPs.

Estimation of soluble proteins — Protein content of leaf extract at each step of purification was estimated by the method of Lowry et al (15) using bovine serum albumin (BSA) as standard.

Molecular weight determination — Molecular weight of purified proteins was determined by electrophoresis on 12% SDS-gel along with molecular weight markers (Dalton Mark VII-L™, Sigma) using the method of Laemmli (16).

Carbohydrate detection — Presence of carbohydrates in the purified protein fractions was detected by Molisch’s test and Periodic acid-Schiff’s (PAS) reagent test. In Molisch’s test, ovalbumin and BSA were used as positive and negative controls, respectively. In case of PAS test, purified protein fractions were run along with a positive (ovalbumin and fetuin) and a negative (BSA) control on 12 % SDS-PAGE, and then PAS staining was performed (17).

Determination of isoelectric point — Isoelectric point of the purified proteins was determined by isoelectric focussing using slab gel method (18). Ampholytes of narrow pH range (pH 8.0-10.5) were used.

Amino acid analysis of purified proteins — Amino acid analysis of the two antiviral proteins was done as per the method of Heinrikson and Meredith (19) by using reverse phase-high performance liquid chromatography (RP-HPLC). From the known amounts of standard amino acids and their peak areas, the weight percentages of different amino acids were calculated for the two proteins. To estimate the tryptophan content of each protein sample, method of Spies and Chambers (20) was followed.

N-terminal sequencing — The purified protein samples were run on the 12% SDS-polyacrylamide gel and blotted onto PVDF membrane. Blotting protocol as specified by IIT, Bombay was followed. N-terminal sequencing was got done from National Facility for Protein Sequencing (Indian Institute of Technology, Bombay). The method followed for the sequencing was based on Edman chemistry.

Determination of IC_{50} values — For this study, two different hypersensitive host / virus systems, N. glutinosa / TMV and C. tetragonoloba / SRV were used. Different quantities of the purified AVPs were applied on to the test host plants and subsequently challenge inoculated with the viruses. Per cent inhibition of the lesion formation for different quantities of the two AVPs was calculated. The quantity of the purified AVP (µg leaf^{-1}) which exhibited approximately 50 % inhibition of virus infection (here taken in the range of 45 % to 55 %) was designated as IC_{50} value of that AVP.

Results

Bioassay of leaf extract for its antiviral activity against SRV and TMV on their respective local lesion hosts, C. tetragonoloba and N. glutinosa, showed very high activity (92.0 % and 98.2 %, respectively). The extracted soluble proteins from the leaves were used for ammonium sulphate (AS) fractionation. The proteins were fractionated in five different fractions (0-20, 20-40, 40-60, 60-80, and 80-100 per cent saturations, respectively) and bioassay of each of the fractions was carried out which showed the presence of bulk of antiviral activity in 40-60 % (95.4 % inhibition) and 60-80 % (93.3 % inhibition) AS saturated fractions (Table 1). These fractions have been designated as 40-60 % ASSF and 60-80 % ASSF, respectively, and were subjected to ion-exchange chromatography for further purification.

The two ASSFs were initially subjected to DEAE-Cellulose chromatography. Elution of 40-60 % ASSF, resulted in two fractions viz. unadsorbed (^{40}40-60 % ASSF) and adsorbed (^{40}40-60 % ASSF). Similarly, elution of 60-80 % ASSF gave two fractions viz. ^{60}60-80 % ASSF and ^{60}60-80 % ASSF. Testing of these fractions showed that in both the cases, high antiviral activity was present in unadsorbed fractions (95.3 % and 97.2 % inhibition, respectively) (Table 1).

^{40}40-60 % ASSF and ^{60}60-80 % ASSF were then chromatographed on CM-Sepharose. When ^{40}40-60 % ASSF was loaded and eluted with 0.1 M NaCl, it resulted in two peaks, designated as ^{40}40-60/0.1-P I and ^{40}40-60/0.1-P II (results not shown). Similarly, elution with 0.2 M NaCl also gave two peaks, ^{40}40-60/0.2-P I and ^{40}40-60/0.2-P II (results not shown). Elution with 0.3 M NaCl resulted in two very small peaks and thus were not used for bioassay and protein quantification. When step gradient elution of ^{60}60-80 % ASSF was carried out, 0.1 M NaCl and 0.2 M NaCl elutions resulted in single peak in each case (results