Induction of Systemic Resistance in Plants Against Viruses by a Basic Protein from *Clerodendrum aculeatum* Leaves

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ABSTRACT

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A nonphytotoxic, systemic resistance-inducing agent present in *Clerodendrum aculeatum* leaves was purified. A specific basic protein (*C. aculeatum*-systemic resistance inducing [CA-SRI]) with a molecular mass of 34 kDa was observed consistently in leaf extracts by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Treatment of plants with the purified protein preparation induced a very high level of systemic resistance against virus infection. Resistance was detectable a few hours after challenge-inoculation with virus and resulted in lesions that were fewer in number or totally absent. The minimum time required for induction of systemic resistance in nontreated leaves of susceptible host plants was 5 to 30 min depending on the host. The resistance-inducing activity of CA-SRI was not affected by protease treatment. After digestion of CA-SRI with endoproteinase Arg-C, the eluted protein fragments from SDS-PAGE were biologically active. An antiserum to the 34-kDa protein was highly specific for CA-SRI, and Western blots of the purified protein recognized the 34-kDa protein band. The isoelectric point of the protein was 8.65. Treatment of susceptible healthy test hosts with purified CA-SRI consistently resulted in the accumulation of a virus inhibitory agent in the resistant leaves. An extract prepared from resistant leaves reduced the infectivity of added virus, with an average reduction in the number of lesions by more than 90%. The specific 34-kDa protein was observed consistently in the leaves of plants with induced resistance and was highly active in reducing the infectivity of the virus. There seems to be a causal relationship between induced resistance and accumulation of the 34-kDa protein based on gel electrophoresis. The protein was present naturally in very low amounts in nontreated healthy plants.

Higher plants possess endogenous virus inhibitors (30), of which proteinaceous antiviral substances are of particular interest. At present, the proposed mechanism for the antiviral action of the widely occurring proteinaceous inhibitors seems to be a direct result of the inhibitors' ribosome-specific *N*-glycosidase activity in vitro (2). The best characterized ribosome-inactivating proteins (RIPs) from *Phytolacca americana* (10,11), *Dianthus caryophyllous* (22), and *Mirabilis jalapa* (8,9) show antiviral activity when mixed with a virus and inoculated in plants, which presumably allows the entry of RIPs along with the virus. Exactly how these RIPs inhibit viral infection in whole plants is unclear.

An endogenous agent that occurs in *Clerodendrum aculeatum* L. leaves induced a very high degree of systemic resistance (CA-SRI) against virus infection in susceptible plants when lower leaves were treated (sprayed/rubbed) with a *C. aculeatum* (CA) leaf extract (29). The induction of systemic resistance by CA leaf extract was very fast, was reversed by actinomycin D, and was associated with the development of a virus-inhibitory agent (VIA) in the extract-treated susceptible plants. The VIA was present both in treated and nontreated leaves of *C. aculeatum*-treated plants (29). Such endogenously occurring substances from plants, which can function as signal molecules, are of particular interest and deserve greater emphasis, because they are not antiviral themselves but they act by inducing the host to produce VIA(s).

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The present work attempts to isolate the specific agent present in *C. aculeatum* leaves to which systemic resistance-inducing activity can be attributed. The isolation of this agent and some of its properties are reported. In addition, the results of experiments to correlate biological activity with the absolute amount of the purified agent, characterized as a 34-kDa basic protein, are given. Induction of such strong systemic resistance and formation of an antiviral defense protein in susceptible plants by a purified plant protein has not been demonstrated previously.

MATERIALS AND METHODS

Source of inoculum. Cultures of tobacco mosaic virus (TMV, common strain) and sunnhemp rosette virus (SRV) (24,25) were maintained by regular passage in their systemic hosts *Nicotiana tabacum* L. 'NP 31' and *Crotolaria juncea* L, respectively.

Preparation of virus inoculum. Virus inoculum was prepared by grinding 3 to 4 g of fresh diseased leaves in a mortar with distilled water (DW, 1 g/ml). The pulp was squeezed through two layers of muslin cloth, and the filtrate was centrifuged at 5,000 × *g* for 15 min. The supernatant was diluted with DW to obtain 200 to 600 lesions on leaves after inoculation with a virus inoculum.

Host plants. Seeds of *N. tabacum* 'Samsun NN' and *Cyamopsis tetragonoloba* (L.) Taub. were sown in clay pots. The seedlings were transplanted to 12-cm-diameter clay pots filled with compost and transferred to an insect-free greenhouse. For experimental work, Samsun NN and *N. glutinosa* L. plants were used at the 5- to 6-leaf stage, and *Cyamopsis* plants were used at the 4-leaf stage (3 unifoliate leaves and 1 trifoliate leaf). All experi-

ments were performed with a minimum of three replicates (minimum three plants with three to four leaves) per treatment.

Assay for virus inhibition. *Cyamopsis tetragonoloba*/SRV and *N. tabacum* Samsun NN/TMV were the host/virus systems employed in the assay for virus infectivity. The two basal leaves of the host plants were treated with buffer-extracted and centrifuged sap from *C. aculeatum* leaves or purified protein solution. Posttreatment inoculation on treated as well as nontreated upper leaves was done 24 h after the treatment unless otherwise specified. The basal leaves of the control set of plants were treated with DW before virus inoculation. Percent inhibition of virus infectivity was calculated by the formula: percent inhibition = $(C - T/C) \times 100$, where C = average number of lesions on control leaves and T = average number of lesions on treated leaves.

Minimum time for induction of systemic resistance. The two lower leaves of the test plants, *N. glutinosa* and *Cyamopsis tetragonoloba*, were treated with CA leaf extract. After varying treatment intervals, these leaves were removed, and the virus, TMV or SRV, was inoculated on the upper untreated leaves 24 h later. The lesions were counted, and percent reduction in number of lesions was calculated.

TABLE 1. Antiviral activity of *Clerodendrum aculeatum* (CA) leaf extract[a]

Treatment	Average no. of lesions ± SEM[b]		Resistance induced (%)	
	At site	At remote site	LIR[c]	SIR[d]
Control	357.0 ± 28.2	672.0 ± 20.1	...	...
CA leaf extract	16.0 ± 6.3	44.0 ± 5.5	96.0	93.4

[a] Treatment (by rubbing) with distilled water (control) or CA leaf extract (1:10 wt/vol) was done on two lower leaves of Samsun NN tobacco plants. After 24 h, tobacco mosaic virus inoculations were done on the treated and nontreated leaves of the test host.
[b] Standard error of the mean.
[c] Local induced resistance.
[d] Systemic induced resistance.

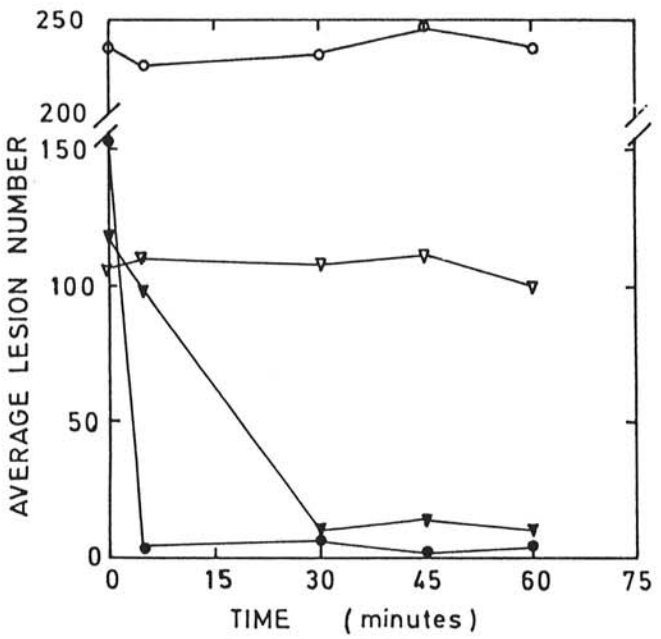

Fig. 1. Minimum time required for induction of antiviral resistance in nontreated leaves by *Clerodendrum aculeatum* leaf extract. Lower leaves of *Cyamopsis tetragonoloba* and *Nicotiana glutinosa* plants were treated with buffer-extracted and centrifuged sap from *C. aculeatum* leaves. At different timed intervals after treatment, the lower leaves were detached, and the upper nontreated leaves were challenge-inoculated with sunnhemp rosette virus (SRV) (control = ○; treated = ● [*Cyamopsis tetragonoloba*/SRV]) and tobacco mosaic virus (TMV) (control = ▽; treated = ▼ [*N. glutinosa*/TMV]), respectively. In control plants, the leaves were treated with distilled water.

Purification of CA-SRI. A 50-g sample of leaves from field-grown *C. aculeatum* plants was washed, frozen, and homogenized in extraction buffer (200 mM sodium acetate, pH 5.2, containing 0.2% β-mercaptoethanol at 1:2 [wt/vol]). The pulp was squeezed through two layers of cheesecloth, and the filtrate was clarified by centrifugation at 6,000 rpm. The resistance-inducing substance was precipitated from the supernatant by 60% ammonium sulfate (wt/vol). The precipitate was collected and suspended in 20 ml of a 1:10 dilution of extraction buffer and was dialyzed against several changes of the same buffer at a low temperature (5°C) for 24 h.

Chromatography on Sephadex G-25. The dialysate (25 ml) was loaded onto a Sephadex G-25 column (40.0 × 2.6 cm) equilibrated with 20 mM sodium acetate buffer, pH 5.2, containing 0.02% (wt/vol) sodium azide and 0.02% (vol/vol) β-mercaptoethanol. The biologically active fraction (void volume [V_o] eluate) was concentrated and dialyzed overnight against three changes of 50 mM sodium acetate buffer, pH 5.2. Precipitate formed during dialysis was removed by centrifugation. The supernatant (10 ml) was used for further purification on an ion-exchange column (Mono S) and then on a Superose-6 column by a fast protein liquid chromatography system (Pharmacia Biotech, Uppsala, Sweden).

Chromatography on a cation-exchange Mono S column. A 0.5 volume (5 ml) of the dialyzed sample was loaded on a prepacked cation-exchange Mono S HR 5/5 column that had been equilibrated with 50 mM sodium acetate buffer, pH 5.2. The bound proteins were eluted with 30 ml of linear NaCl gradient from 0 to 0.25 M in the same buffer at a flow rate of 1 ml/min. V_o fractions of 1 ml were collected, and 100 µl of a 1:5 dilution of the fractions was tested for the ability to inhibit SRV infection on *Cyamopsis tetragonoloba*. Fractions that showed maximum resistance-inducing activity against virus infection were pooled for a second run. V_o fractions eluted from the column were analyzed on 12.5% sodium dodecyl sulfate (SDS) polyacrylamide gels. The pooled fractions from the second run were subjected to molecular sieving on a Superose-6 column.

Chromatography on Superose-6. A 500-µl sample of the protein concentrate, obtained by the previous step, was applied to a Superose-6 HR 30/10 column previously equilibrated with 20 mM sodium acetate buffer, pH 5.2, containing 200 mM sodium chloride and 0.02% (wt/vol) sodium azide. The column was eluted with the same buffer at a flow rate of 0.2 ml/min. V_o fractions (100) of 200 µl were collected. Resistance-inducing activity against TMV was tested on Samsun NN plants with 100 µl of a 1:5 dilution of the V_o fractions. Fractions also were analyzed on 12.5% SDS polyacrylamide gels. The native molecular weight of the purified protein (CA-SRI) was determined from its mobility in relation to standard proteins.

Estimation of native molecular weight. The molecular weight of CA-SRI was calculated by gel-filtration chromatography on native gels (1). The column was calibrated with blue dextran, and standard proteins were obtained from Pharmacia (ribonuclease A, M_r = 13.7 kDa; chymotrypsinogen A, M_r = 25 kDa; ovalbumin, M_r = 43 kDa; and bovine serum albumin [BSA], M_r = 67 kDa).

Estimation of protein and sugar content. Protein concentration was determined by the method of Lowry et al. (15) with BSA as the standard. Sugar concentration was estimated by the phenol sulfuric acid method (4) with glucose as the standard.

Isoelectric focusing (IEF). The Pharmacia Phast Gel system was used for IEF of CA-SRI. Premade IEF gels from 3 to 9 pI were used according to the instructions supplied by the manufacturer. The gel was stained in 0.025% Phast Gel blue R-350 in 30% methanol and 10% acetic acid and was destained in 30% methanol and 10% acetic acid.

Protease sensitivity. Pure CA-SRI (1 mg/ml) was dialyzed against 50 mM Tris-HCl buffer (pH 7.5). Aliquots (100 µl) of the sample were incubated separately with 5 µg of proteinase K (Stratagene, La Jolla, CA), pronase, and trypsin (Sigma Chemical Company, St. Louis) in the presence of 5 mM $CaCl_2$ for 24 h at 37°C. The

proteases alone and the CA-SRI alone served as controls. The incubated samples were screened for biological activity against TMV on Samsun NN plants.

Digestion of CA-SRI with endoproteinase Arg-C. A 100-µg sample of CA-SRI was digested with endoproteinase Arg-C. Fragments of CA-SRI generated from incubation with the protease were separated on a 10% preparative SDS polyacrylamide gel. Fragments were cut from the gel, and the gel pieces were extracted individually in elution renaturation buffer (ERB). The ERB consisted of Triton X-100 (1%); EDTA (1 mM); HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.6 (20 mM); NaCl (100 mM); BSA (5 mg/ml); DTT (dithiothreitol) (2 mM); and PMSF (phenylmethylsulfonyl fluoride) (0.1 mM). After overnight incubation in ERB, the supernatant was dialyzed against 10 mM Tris-HCl, pH 8.0, and the dialyzed samples were concentrated in a Speed-Vac (Brinkman Instruments, Westbury, NY) concentrator before testing for biological activity against TMV on Samsun NN plants.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and determination of molecular weight. Electrophoresis was performed with 12.5% SDS polyacrylamide gels according to the method of Laemmli (13). The slab gel was electrophoresed at 25 to 30 mA constant current and 150 to 200 V for 3 to 4 h. The gel was fixed in 12% trichloroacetic acid for 1 h and stained with Coomassie blue R-250 or silver nitrate (17). Molecular weight under denaturing conditions was determined by SDS-PAGE with markers obtained from Pharmacia (α-lactalbumin, M_r = 14.4 kDa; soybean trypsin inhibitor, M_r = 20 kDa; carbonic anhydrase, M_r = 30 kDa; ovalbumin, M_r = 43 kDa; BSA, M_r = 67 kDa; and phosphorylase b, M_r = 94 kDa).

Preparation of antiserum. Antiserum against CA-SRI was prepared in rabbits by injecting purified protein (350 µg) in 0.5 ml of 50 mM sodium acetate buffer, pH 5.2. Five intravenous injections at a timed interval of 1 week were given. After 6 weeks, the animals were bled, and the serum was tested for cross-reactivity with the purified antigen by immunoblotting.

Immunoblotting. The procedure of Towbin et al. (23) was adopted for the electrophoretic transfer of protein with a Bio-Rad transblot apparatus (Bio-Rad Laboratories, Richmond, CA). After SDS-PAGE, the CA-SRI protein band was transblotted onto a nitrocellulose membrane in a Tris-glycine-methanol buffer system. The antiserum raised against CA-SRI was diluted to 1:500 in Tris-buffered saline (TBS) with 0.1% Tween 20. Anti-rabbit antibodies conjugated with alkaline phosphatase were diluted to 1:2,000 in TBS. Color development was carried out with the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate system. Prestained markers were obtained from Gibco-BRL, Gaithersburg, MD.

Preparation of crude and partially purified VIA induced in CA-SRI–treated healthy plants. Two basal leaves of *Cyamopsis tetragonoloba* were rubbed with CA-SRI (60 µg/ml) or DW. After 24 h, the upper leaves (nontreated) were harvested and ground in a grinder with an equal volume of 200 mM sodium acetate buffer, pH 5.2, containing 0.1% (vol/vol) β-mercaptoethanol. The resulting homogenate was passed through cheesecloth, and the filtrate was centrifuged at 8,000 rpm for 30 min. The supernatant was chromatographed on a Sephadex G-25 column as described earlier. The V_0 fraction was assayed for inhibition of SRV infection on *Cyamopsis tetragonoloba.* For purification, the V_0 fraction was dialyzed against 50 mM sodium acetate buffer prior to fractionation on a cation-exchange matrix (SP Sepharose fast flow, Pharmacia). The column (10.5 × 1.5 cm) was equilibrated with 50 mM sodium acetate buffer, pH 5.2, and the bound basic proteins were eluted with a 0 to 250 mM NaCl gradient in 400 ml of the equilibration buffer. A flow rate of 4 ml/min was maintained during elution.

A preparation from the same amount of leaves harvested from DW-treated healthy plants, constituting the noninduced controls, was chromatographed under identical conditions. Basic protein fractions (each of 4 ml) from induced and noninduced plants were

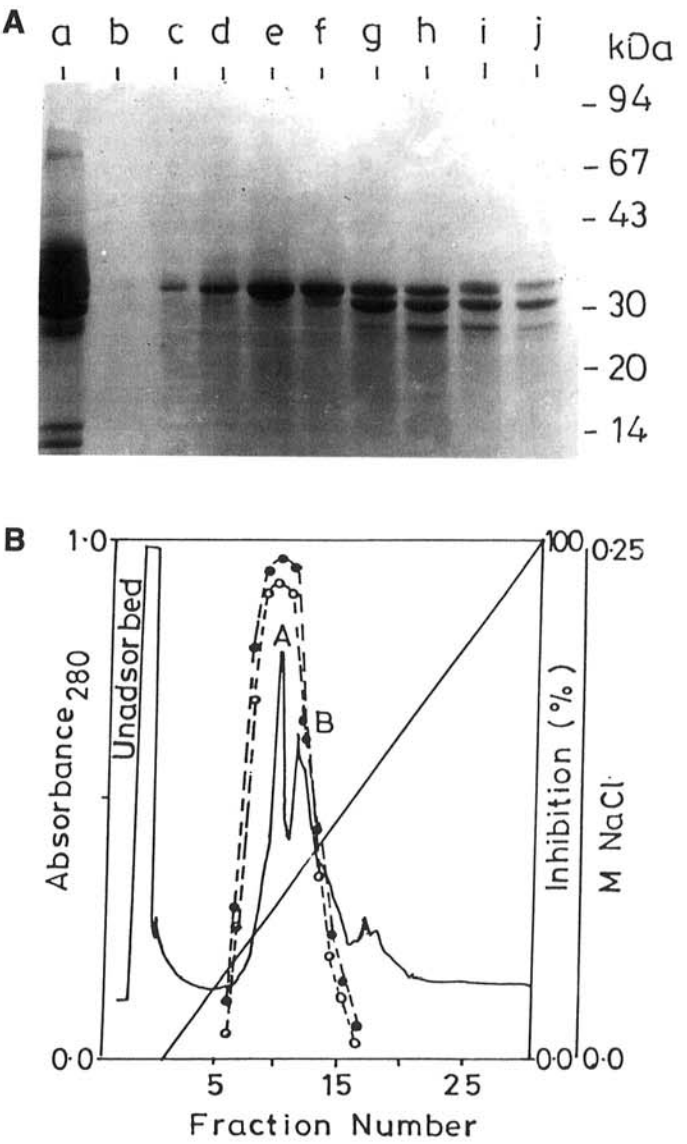

Fig. 2. A, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of Mono S chromatographed fractions. Lane a, void volume (V_0) eluate from a Sephadex G-25 column. Lanes b through j, fraction numbers 6 through 14 eluted from the Mono S column. Values to the right refer to the molecular masses of the standard proteins. The arrow to the left indicates the position of the *Clerodendrum aculeatum* resistance-inducing protein (CA-SRI). **B,** Cation-exchange chromatography on a Mono S column. The Sephadex G-25 fraction (V_0 eluate) was applied to a Mono S column equilibrated with 50 mM sodium acetate buffer, pH 5.2, and eluted with a 30-ml linear NaCl gradient from 0 to 250 mM in the same buffer. Fractions (1 ml) were collected, and 100 ml of a 1:5 dilution of the fractions was tested for the ability to inhibit sunnhemp rosette virus (SRV) infection of *Cyamopsis tetragonoloba.* Inhibition of SRV on site leaves (●) and on remote site leaves (○). The absorbance (—) was monitored at 280 nm.

TABLE 2. Purification of the *Clerodendrum aculeatum* resistance-inducing protein CA-SRI[a]

Fraction	Total protein (mg)	Protein concentration (µg/ml)	No. of active units/mg[b]	Total activity (units)
Sephadex G-25	40	20	50	2,000
Mono S	4.5	4.0	250	1,125
Superose-6	3.2	3.8	263.1	842

[a] Data refer to 50 g of *C. aculeatum* leaves.

[b] A unit of activity is defined as the amount of inhibitor that induces 50% inhibition of tobacco mosaic virus lesion counts on Samsun NN tobacco plants.

assayed for inhibition of SRV infection on *Cyamopsis tetragonoloba*. Fractions 27 to 32 eluted after cation-exchange chromatography of the sap from induced plants were screened for biological activity and also analyzed by SDS-PAGE. Fraction 30, which showed maximum virus inhibitory activity, was concentrated to 500 µl on C-10 Centricon concentrators (Amicon, Beverly, MA), and the protein profile was analyzed by SDS-PAGE.

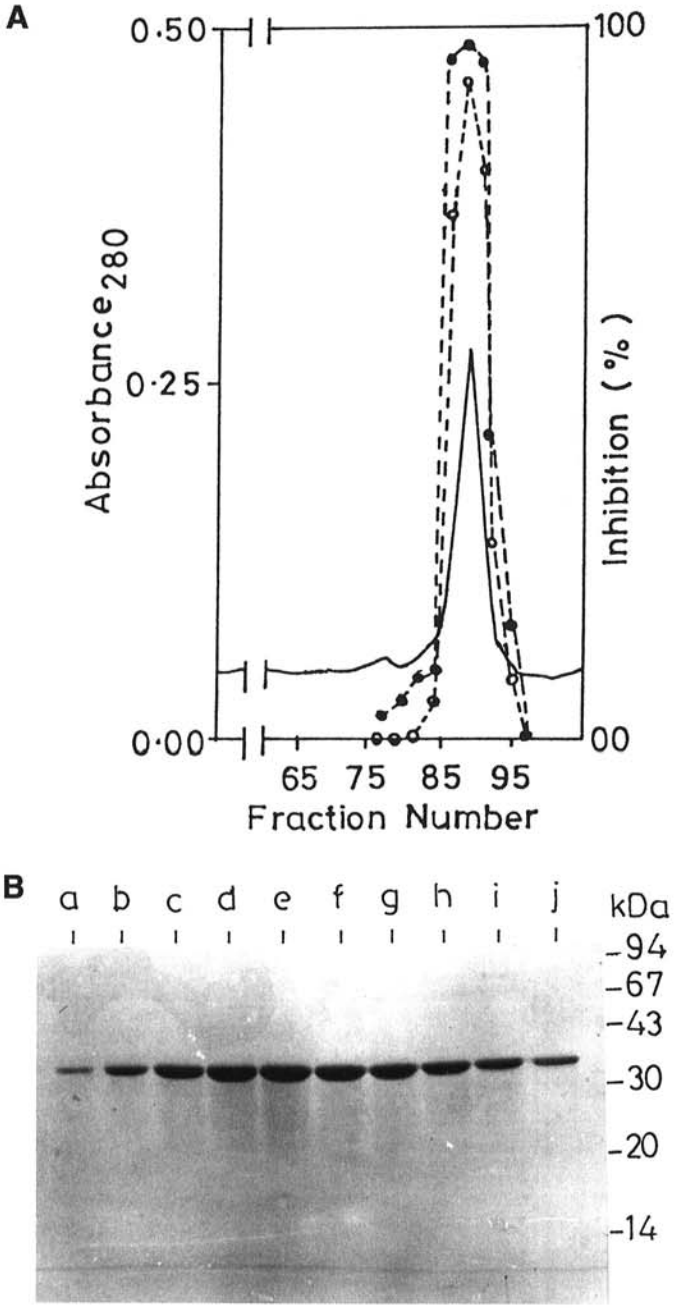

Fig. 3. A, Gel-filtration chromatography on a Superose-6 column. Concentrated peak A fractions from the rerun on a Mono S column (2.22 mg of protein in 500 ml of 50 mM sodium acetate buffer, pH 5.2) were applied to a Superose-6 column equilibrated with 20 mM sodium acetate buffer, pH 5.2, containing 0.02% (wt/vol) sodium azide and 200 mM NaCl. Fractions (200 ml each) were collected. The *Clerodendrum aculeatum* protein (CA-SRI) that induced resistance against tobacco mosaic virus (TMV) was tested on Samsun NN tobacco plants with 100 ml of a 1:5 dilution of the fractions. Percent inhibition of TMV on site leaves (●) and remote site leaves (○). The absorbance (—) was monitored at 280 nm. **B,** Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis analysis of protein CA-SRI after gel-filtration chromatography on Superose-6. Lanes a through j, fraction numbers 85 through 94 eluted from the Superose-6 column. Values to the right represent the molecular masses of the marker proteins. CA-SRI migrated as a single band on 12.5% SDS polyacrylamide gels with a molecular mass of 34 kDa.

Test for activity. The virus inoculum (SRV) was mixed with an equal volume of leaf extract, partially purified preparations from noninduced plants and induced plants, or DW. The mixture was incubated at room temperature (26°C) for 2 h and applied to the leaves of a uniform group of test host plants (*Cyamopsis tetragonoloba*). The lesion counts were statistically analyzed, and percent inhibition was calculated.

RESULTS

Induction of systemic resistance. Treatment (spray/application) of CA leaf extract to lower leaves of Samsun NN plants affected subsequent infection by TMV throughout the plant. Induced systemic resistance was detectable when the number of lesions was decreased by about 94% in upper nontreated leaves (Table 1).

Minimum time for induction of systemic resistance. Infection by SRV in nontreated upper leaves of *Cyamopsis tetragonoloba* was prevented completely when lower leaves were removed 5 min after treatment with CA leaf extract. *N. glutinosa*, however, required 30 min for the induction of resistance in nontreated upper leaves of plants (Fig. 1). Thus, the minimum time for induction of systemic resistance was 5 to 30 min in the two hosts tested.

Purification of CA-SRI. The bulk of the resistance-inducing activity was recovered after precipitation of crude CA leaf extract with 60% ammonium sulfate. After chromatography on Sephadex G-25, the material separated into two major peaks, as seen by absorbance at 280 nm. The resistance-inducing activity was found with peak 1 (V_o eluate). This fraction when bioassayed on Samsun NN plants inhibited TMV infection by about 95% on both the protein-treated leaves and the upper nontreated leaves, to a dilution of 1:10. The second peak, containing the low molecular weight substances, showed traces of resistance-inducing activity. SDS-PAGE revealed that the peak 1 fraction contained several proteins (Fig. 2A).

After chromatography on a cation-exchange Mono S column, the bound basic proteins resolved into two major peaks (peaks A and B), as seen by absorbance at 280 nm (Fig 2B). The bioassay tests revealed that the major resistance-inducing activity was found within the first protein peak, which consisted of fractions 9 and 10. Fractions 11 and 12, constituting peak B, also showed considerable biological activity. The unabsorbed fraction did not possess any virus resistance-inducing activity.

Fractions 6 to 14 eluted from the Mono S column were analyzed on SDS polyacrylamide gels. The resistance-inducing activity coincided with a 34-kDa protein (Fig. 2A) that was present as a major protein in peak A and minor protein in peak B. A 32-kDa protein that was a major protein in peak B was sometimes present in peak A. Hence, the pooled fractions of peak A were repurified on a Mono S column and put on a Superose-6 column. A 50% reduction in the

TABLE 3. Effects of varying *Clerodendrum aculeatum* protein CA-SRI concentrations on resistance induced in Samsun NN tobacco plants[a]

Concentration (µg/ml)	Average no. of lesions ± SEM[b]		Resistance induced (%)	
	At site	At remote site	LIR[c]	SIR[d]
0.0	682.0 ± 16.1	698.0 ± 14.4	...	...
4.0	264.6 ± 23.7	786.0 ± 42.0	61.2	0.0
8.0	128.0 ± 20.1	282.6 ± 15.3	81.2	59.5
16.0	28.6 ± 1.3	86.0 ± 19.0	95.8	87.6
32.0	4.3 ± 1.2	59.3 ± 24.3	99.3	91.3
64.0	0.0 ± 0.0	0.0 ± 0.0	100.0	100.0

[a] Dilutions of CA-SRI were prepared in distilled water. Samples (100 µl) of solutions containing CA-SRI at different concentrations were applied on two basal leaves of Samsun NN plants. The treated and nontreated upper leaves were inoculated with tobacco mosaic virus after 24 h of treatment.
[b] Standard error of the mean.
[c] Local induced resistance.
[d] Systemic induced resistance.

number of lesions was observed when pooled fractions were applied on Samsun NN leaves at a concentration of 4 mg/ml (Table 2).

After molecular sieving of Mono S pooled fractions on Superose-6, a single major peak was seen by absorbance at 280 nm (Fig. 3A). Fractions 85 to 94, corresponding to the active peak, were analyzed on SDS polyacrylamide gels. A single protein band of approximately 34 kDa could be seen in all the lanes (Fig 3B). The ability to induce resistance in Samsun NN plants against TMV by different fractions followed the protein elution profile. Fractions 85 and 94 (lanes 1 and 10), which contained a faint band, showed low resistance-inducing activity, whereas lanes 4 through 8, which contained the maximum amount of protein, showed a very high degree of resistance-inducing activity (Fig. 3A and B).

The peak fraction (89) decreased TMV lesion production by 99% in treated leaves of Samsun NN plants and by 93% in remote site leaves (Fig. 3A). Nearly 70 to 80% of the protein applied to the column was recovered after gel-filtration chromatography. The V_e/V_0 ratio for the inhibitory peak was 2.25, which corresponded to a molecular mass of 35 kDa (data not shown). The resistance-inducing activity of CA-SRI was directly correlated with the concentration of protein in the sample, although induced resistance on the challenged leaf was obtained with a lower concentration of the protein than that required for systemic induced resistance (Table 3). At 64 µg of protein per ml, the degree of resistance induced was of a very high order, and a 100% decrease in the number of lesions was observed on nontreated leaves (Table 3).

Immunoblotting and IEF. CA-SRI reacted specifically in the immunoblots with the anti–CA-SRI serum (Fig. 4). A stained band at the 34-kDa position could be visualized on the immunoblots. On IEF, the purified protein (CA-SRI) produced a single band with an isoelectric point of 8.65 (Fig. 5).

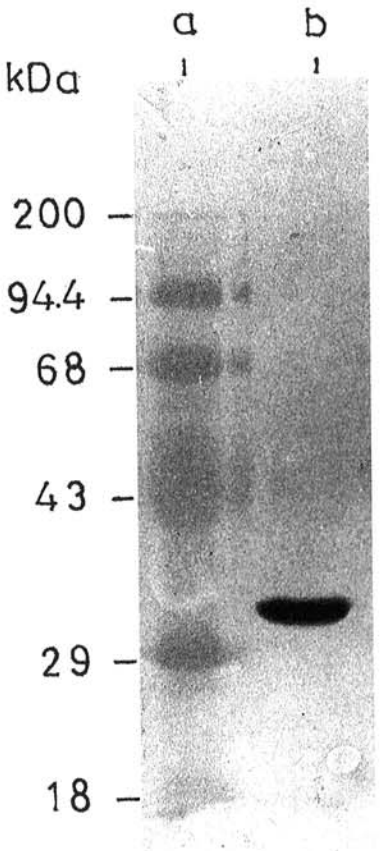

Fig. 4. Immunodetection of the *Clerodendrum aculeatum* resistance-inducing protein (CA-SRI). Electrophoresis was performed on denaturing gel (12.5%) followed by electrophoretic transfer onto a nitrocellulose sheet. Anti–CA-SRI serum was diluted 250-fold in Tris-buffered saline. Lane a, prestained markers; lane b, CA-SRI.

Effect of proteases. The biological activity of CA-SRI remained conserved after incubation with proteases. The protease-incubated samples inhibited TMV infection on Samsun NN to approximately the same extent as the undigested CA-SRI (Table 4). After incubating CA-SRI with endoproteinase Arg-C, 14-, 16-, 20-, and 28-kDa fragments were generated and showed varying amounts of biological activity (Table 5).

Induction of a VIA. The crude extract prepared from leaves of CA-SRI–treated plants when incubated with a virus decreased virus infectivity markedly, whereas extracts from DW-treated plants did not affect virus infectivity under similar conditions (Table 6). The results of this experiment suggest the presence of a strong VIA in the CA-SRI–treated plants.

To identify the VIA, leaf extracts from noninduced and induced plants were desalted on a Sephadex G-25 column and separately chromatographed on SP Sepharose fast flow (Fig. 6A). The bound

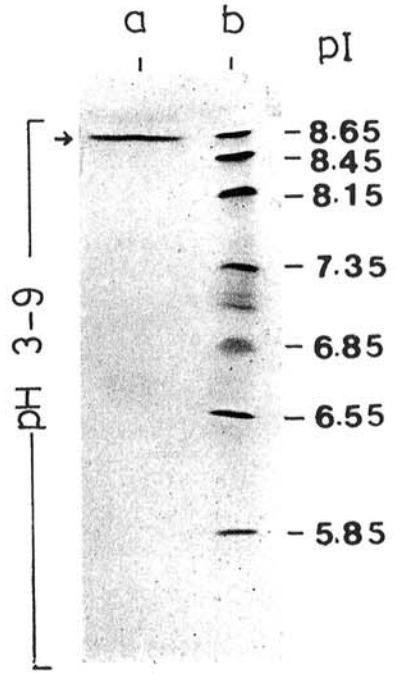

Fig. 5. Isoelectric focusing on Phast Gel (pH 3 to 9) (Pharmacia). Gel was loaded with 15 µl of the *Clerodendrum aculeatum* resistance-inducing protein (CA-SRI). The proteins were stained with Phast Gel blue R. Lane a, CA-SRI (indicated by an arrow); lane b, isoelectric point (pI) markers. CA-SRI was focused at pH 8.65.

TABLE 4. Effect of proteases on the resistance-inducing activity of *Clerodendrum aculeatum* protein CA-SRI[a]

Treatment	Average no. of lesions ± SEM[b]		Resistance induced (%)	
	At site	At remote site	LIR[c]	SIR[d]
Distilled water	212.0 ± 17.40	206.0 ± 8.71	...	...
CA-SRI	0.3 ± 0.33	1.0 ± 0.57	99.8	99.5
PK control	229.0 ± 7.81	256.0 ± 5.29	0.0	0.0
CA-SRI + PK	1.0 ± 0.57	0.3 ± 0.33	99.5	99.8
P	207.0 ± 6.24	184.0 ± 5.29	2.3	10.6
CA-SRI + P	0.6 ± 0.66	0.3 ± 0.33	99.7	99.8
T control	231.0 ± 1.73	166.0 ± 11.53	0.0	19.4
CA-SRI + T	1.3 ± 1.33	1.3 ± 1.33	99.3	99.3

[a] CA-SRI was incubated with pronase (P), trypsin (T), and proteinase K (PK) in a 20:1 ratio by weight under conditions specified in text. Distilled water, proteases alone, and CA-SRI served as the controls. A 100-µl sample from each treatment was applied to two leaves of Samsun NN tobacco plants. Tobacco mosaic virus inoculations were done on treated as well as nontreated leaves of the test host.
[b] Standard error of the mean.
[c] Local induced resistance.
[d] Systemic induced resistance.

basic protein fractions that eluted when screened for biological activity showed a fraction from induced plants (number 30), which when mixed with virus and inoculated on test hosts markedly decreased virus infectivity, whereas a corresponding fraction from noninduced plants showed no such virus inhibitory activity (Table 7). Fraction 30 from both CA-SRI– and DW-treated plants, after concentration to 500 µl (eightfold concentration), were chromatographed on SDS-PAGE. Fraction 30 from the extract from CA-SRI–treated leaves showed a predominant protein band at the 34-kDa position (Fig. 6B, lane a). However, an extremely faint band of the same molecular weight also could be seen in the lane loaded with the fraction from control leaves (Fig. 6B, lane b). Fraction numbers 27 to 32 from the extracts from CA-SRI–treated plants, which also had SRV-inhibiting activity, also showed the 34-kDa protein. The decrease in virus infectivity was proportional to the size (width) of the band formed in the SDS polyacrylamide gels (Fig. 6A and C).

DISCUSSION

Treatment of lower leaves of *N. tabacum* Samsun NN with a purified basic 34-kDa protein (64 µg/ml) isolated from field-grown *C. aculeatum* leaves resulted in complete protection of nontreated upper leaves against TMV infection. The systemic protective effect of the protein (CS-SRI), however, was evident in leaves even with as low a quantity of protein as 10 µg/ml. That the resistance-inducing activity resided in the protein was substantiated by the finding that treatment of the protein with proteases yielded small resistance-inducing fragments. The amount of CA-SRI that prevented virus infection was nonphytotoxic to the plant, so it seems likely to be a useful biological control agent for viruses. A systemic resistance inducer (also proteinaceous) that occurs in *Boerhaavia diffusa* also induces strong local and systemic resistance in several susceptible hosts (26,27,28). A comparison with reported data on various systemic resistance inducers (biotic or abiotic) revealed that CA-SRI was the most potent resistance inducer known to date. Viruses or TMV protein having similar activity induce only partial protection (14,20).

TABLE 5. Resistance-inducing activity of endoproteinase Arg-C–digested fractions of *Clerodendrum aculeatum* protein CA-SRI[a]

Fragment (kDa)	Average no. of lesions ± SEM[b]		Resistance induced (%)	
	At site	At remote site	LIR[c]	SIR[d]
Control	352.5 ± 22.51	334.0 ± 74.0	...	...
14	159.5 ± 15.67	245.0 ± 19.0	54.7	26.6
16	254.6 ± 3.52	332.0 ± 4.0	27.0	0.5
20	88.5 ± 11.59	157.0 ± 17.0	74.8	52.9
28	145.0 ± 14.20	248.0 ± 12.0	58.8	25.7

[a] CA-SRI (100 µg) was digested with endoproteinase Arg-C, and the generated fragments were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The peptides were eluted from the gel and renatured in appropriate buffer as detailed in text. A 100-µl sample of each renatured peptide was applied on two basal leaves of Samsun NN tobacco plants. After 24 h, tobacco mosaic virus inoculations were made on treated and nontreated leaves of the test host. The control consisted of renaturation buffer alone.
[b] Standard error of the mean.
[c] Local induced resistance.
[d] Systemic induced resistance.

TABLE 6. Inhibition of virus infectivity by extracts from *Clerodendrum aculeatum* resistance-inducing protein (CA-SRI)– and distilled water (DW)-treated (control) *Cyamopsis tetragonoloba* plants[a]

Treatment	Average no. of lesions ± SEM[b]	% reduction in virus infectivity
Virus + DW	200.6 ± 8.4	...
Virus + sap from DW-treated plants	206.0 ± 8.2	...
Virus + sap from CA-SRI–treated plants	24.8 ± 1.9	87.6

[a] Extracts from CA-SRI– and DW-treated plants were mixed with sunnhemp rosette virus inoculum in equal volumes, and the mixture was inoculated on leaves of the test host. Inoculum mixed with DW served as an additional control.
[b] Standard error of the mean.

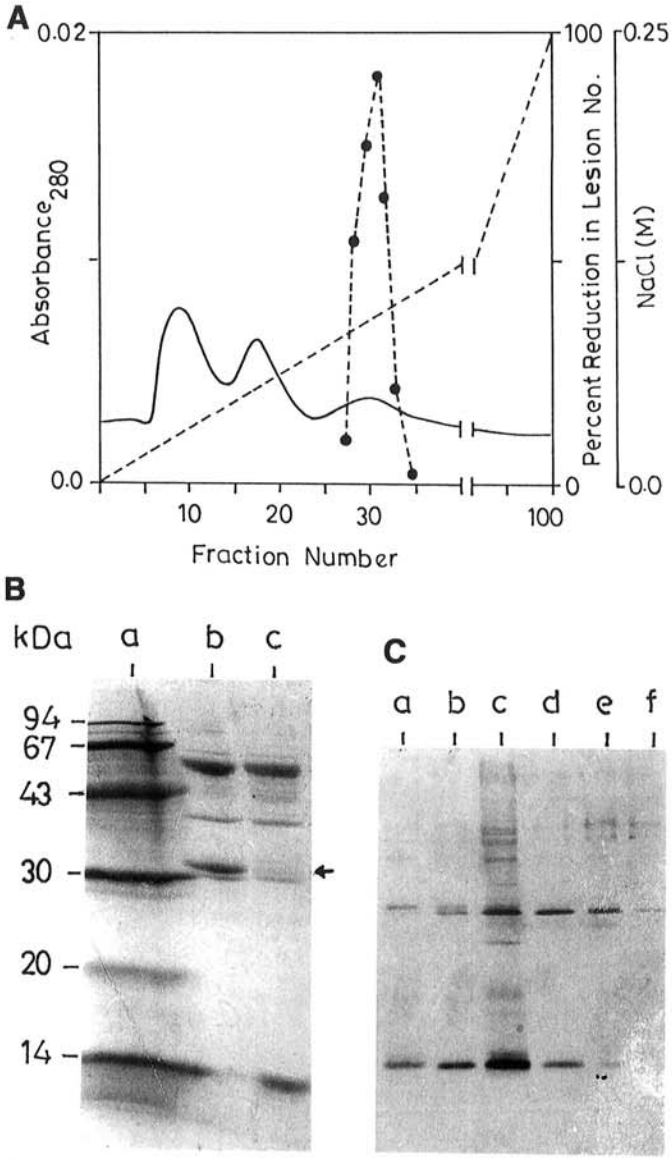

Fig. 6. A, Cation-exchange chromatography on a SP Sepharose column of desalted *Clerodendrum aculeatum* leaf extract from the resistance-inducing protein (CA-SRI)–treated *Cyamopsis tetragonoloba* plants. Two basal leaves of *Cyamopsis tetragonoloba* plants were rubbed with CA-SRI. After 24 h, the upper nontreated leaves were harvested and homogenized in 200 mM sodium acetate buffer, pH 5.2, containing 0.1% (vol/vol) β-mercaptoethanol. The leaf homogenate was desalted on a Sephadex G-25 column prior to fractionation on SP Sepharose. The SP Sepharose column was equilibrated with 50 mM sodium acetate buffer, pH 5.2, and eluted with a 0 to 250 mM NaCl gradient (dashed line) in the equilibration buffer. Sunnhemp rosette virus infection on *Cyamopsis tetragonoloba* was inhibited by fraction numbers 27 through 32 (line with solid circles). The absorbance (—) was monitored at 280 nm. **B,** Comparison of electrophoretic patterns of CA-SRI– and distilled water (DW)-treated *Cyamopsis tetragonoloba* leaf homogenate. SP Sepharose-eluted fraction number 30 from the two sets was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after eightfold concentration. Lane a, molecular mass markers; lane b, CA-SRI–treated (induced); lane c, DW–treated (noninduced). The arrow indicates the increased amount of a 34-kDa protein in the treated set. **C,** SDS-PAGE of virus inhibitory fractions obtained after chromatography on SP Sepharose of CA-SRI–treated *Cyamopsis tetragonoloba* leaf homogenate. Lanes a through f, fraction numbers 27 through 32.

Experiments with radioactively labeled TMV protein showed that the systemic protective effect was not due to movement of protein in the plants (14). Systemic induced resistance in virus-infected or TMV protein-treated plants (20 mg/ml) is due to the development/accumulation of some induced VIA, such as antiviral factor (AVF) (18), antiviral principle (AVP) (16), or inhibitor of virus replication (IVR) (7), in treated plants. Such factors did not occur in noninfected or nontreated plants. Infective virus inocula when mixed in vitro with the induced VIA lost most of their infectivity. The present work also provides evidence of stimulated formation of a VIA in leaves showing resistance after treatment with purified CA-SRI. The induced VIA that occurred in leaves showing systemic resistance was presumably a protein of 34 ± 1 kDa, because the amount of stimulated protein was related to the degree of resistance induced. Accumulation of this protein in low amounts in the cells of the host appears sufficient to protect these cells from virus infection. However, how it prevents virus infection in vivo has yet to be determined.

Parallels may be drawn between the CA-SRI–induced virus inhibitory protein and the glyco-proteinaceous components of AVF (5,6) or IVR (7) that accumulate in virus-infected plants or protoplasts. The pathogenesis-related proteins that accumulate in susceptible plants are not antiviral (12) but do inhibit fungal and bacterial development (21). It appears that accumulation of new proteins is a general defense response of plants against infection, and different heterogeneous agents evoke the triggering process to various degrees against different pathogens. Until data are available, it will be difficult to resolve the relationship of CA-SRI–induced antiviral protein with other induced virus inhibitors isolated from resistant plant tissues. The genome of susceptible hosts appears to carry the information for virus resistance in a cryptic form. Virus multiplication or a component of a virus (coat protein) alone may not be necessary for the induction of systemic resistance or production of VIA. Proteins from a few exotic plants also may be assumed to have this ability.

The proteinaceous virus inhibitors from plants are similar with respect to molecular mass (25 to 35 kDa), basic nature, resistance to exogenous proteases, and high temperature tolerance, but they appear to act in different ways. The widely occurring RIPs apparently enter cytosol along with the virus, disrupt cellular protein synthesis, and, thereby, prevent viral replication (19). However, it is doubtful that antiviral action in whole plants depends on the inhibition of host ribosomes (3). The systemic resistance-inducing protein (CA-SRI) possibly exerts virus inhibitory activity by stimulating the systemic formation of an antiviral protein in the plant, and this induced protein presumably has a role in the defense of plants against viruses.

TABLE 7. Fractionation of virus inhibitory activity due to *Clerodendrum aculeatum* protein CA-SRI after cation-exchange chromatography on SP-Sepharose[a]

Fraction	Average no. of lesions ± SEM[b]	
	Noninduced	Induced
15	378.6 ± 11.6	400.0 ± 3.4
20	316.6 ± 14.5	432.0 ± 11.7
25	318.0 ± 12.4	124.6 ± 5.2
30	323.3 ± 13.5	40.0 ± 1.1
35	315.3 ± 13.8	414.0 ± 7.5
Control[c]	369.3 ± 16.2	421.3 ± 15.2

[a] Desalted extracts from CA-SRI–treated (induced) and distilled water (DW)-treated (nontreated) *Cyamopsis tetragonoloba* plants were separately chromatographed on SP-Sepharose with a 0 to 0.25 M NaCl gradient in 400 ml of buffer (50 mM sodium acetate, pH 5.2). Eluted fractions were mixed with an equal volume of sunnhemp rosette virus (SRV) inoculum, and after 2 h of incubation at 26°C, the mixture was inoculated on leaves of *Cyamopsis tetragonoloba* plants.

[b] Standard error of the mean.

[c] SRV inoculum mixed with DW served as an additional control.

Work is in progress to obtain a homogenous preparation of CA-SRI–induced virus-inhibitory protein in sufficient quantities to characterize the protein. Further work also is needed to discover the relationship between this and other induced VIAs: AVF, AVP, and IVR.

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