Inhibitor of Virus Replication from Protoplasts of a Hypersensitive Tobacco Cultivar Infected with Tobacco Mosaic Virus is Associated with a 23-K Protein Species

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ABSTRACT

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A specific protein with an approximate molecular weight of 23 K was observed consistently by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) from crude preparations of an inhibitor of virus replication (IVR) released into the culture medium from protoplasts of a hypersensitive tobacco cultivar infected with tobacco mosaic virus. Electroeluted protein from SDS-PAGE gels was biologically active, with about a 20-fold increase in specific activity over that of the crude preparation. The active fraction revealed only one protein band at 23 K in PAGE, providing evidence that the 23-K protein is IVR purified to a high degree. A homologous antiserum to the 23-K protein was prepared. The antiserum was highly specific for IVR and efficiently eliminated its antiviral activity. Western blots of IVR extracted from protoplasts or leaf tissue of hypersensitive tobacco cultivar revealed a single 23-K protein band.

Additional keywords: antiviral protein, serology, electroelution.

We have reported that an inhibitor of virus replication (IVR) is released into the medium from tobacco mosaic virus (TMV)-infected protoplasts of a tobacco cultivar in which the infection in the intact plant is localized. IVR-inhibited virus replication in protoplasts from both local lesion-responding, resistant tobacco plants (*Nicotiana tabacum* L. 'Samsun NN') and systemic-responding, susceptible tobacco plants(*N. tabacum* 'Samsun'). IVR was not released from TMV-infected Samsun protoplasts (9). It was suggested that IVR is associated with localization (10). IVR was partially purified using $ZnAc_2$ precipitation from the culture medium of TMV-inoculated Samsun NN protoplast (crude protoplast IVR). Two biologically active compounds were obtained with molecular weights of approximately 26,000 and 57,000 (fractionated IVR-1 and IVR-2), as determined by gel filtration (9). Antisera were prepared against each of these two fractions, which were highly cross-reactive, indicating the presence of identical determinants in both fractions (4).

IVR also was obtained from the intercellular fluid of Samsun NN tobacco infected with TMV (tissue IVR) and from induced resistant tissue (12).

Here we report that the biological activity of IVR is associated with a specific 23-K protein, observed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of crude protoplast IVR. An antiserum specific to the 23-K protein was prepared and used for Western blot analysis.

MATERIALS AND METHODS

Preparation of crude protoplast and tissue IVR. Crude protoplast IVR, fractionated protoplast IVR-1 and IVR-2, were prepared from the culture medium of TMV-inoculated Samsun NN protoplasts as described previously. Control preparations were obtained from sham-inoculated protoplasts (9).

Tissue IVR was obtained from the intercellular fluid of Samsun NN tobacco leaves inoculated with TMV. A preparation obtained from 1 g of leaf tissue was termed one unit. This was found to be roughly equivalent to the amount of IVR obtained from 10^6 protoplasts. One unit of IVR is equivalent to approximately 10 ng of proteins (12). One unit of IVR applied to protoplast or leaf disks 5 hr after inoculation with TMV consistently reduced virus replication 50–60%, in comparison with the respective control, when determined by local lesion assay or by enzyme-linked immunosorbent assay (ELISA) (3,9,12).

PAGE of IVR. For PAGE, 10–20 units of crude protoplast IVR was concentrated to 8 μl mixed with 4 μl of 60 mM Tris-HCl buffer, pH 6.8, containing 3% SDS, 5% 2-mercaptoethanol, 10% glycerol, and a few grains of bromophenol blue (disruption buffer), and boiled for 2 min. Twelve microliters of the SDS-disrupted IVR then was applied to 6–20% polyacrylamide gradient minigels (10 × 7.5 cm, 0.75 mm thick) containing 0.1% SDS and analyzed by electrophoresis. Gels were stained with Coomassie Brilliant Blue (CBB) (1). An equivalent control preparation obtained from the culture medium of sham-inoculated protoplasts was analyzed.

Electroelution of IVR from SDS-PAGE. Ten to twenty units of IVR or control preparations per lane was electrophoresed in 6–20% polyacrylamide gradient minigels containing 0.1% SDS. After electrophoresis, one lane containing IVR and one containing marker proteins were stained with CBB; the other lanes were kept refrigerated until elution. The areas parallel to the stained IVR band were excised and inserted into a dialysis bag containing 1 ml of 50 mM borate buffer, pH 8.0, 20% methanol, and 0.02% mercaptoethanol. The sealed dialysis bag was placed in 4 L of the same solution, and protein was eluted from the gel by electrophoresis at 300 mA at 2 C for 6 hr, using a Hoefer Transphor apparatus (Hoefer Scientific Instruments, San Francisco, CA) attached to a cooling circulator. After electroelution, the electrodes were reversed and the current was applied for another 1 hr to detach the proteins from the dialysis bag (2). Gel pieces were precipitated by low-speed centrifugation. Supernatant was dialysed for 24 hr against three changes of 1 L of distilled water. The eluted protein was freeze-dried and dissolved in 30 μl of distilled water, and the biological activity was assayed with TMV-infected protoplasts, in comparison with an equivalent fraction recovered from the gel used to purify proteins from control preparations. A sample of this preparation was analyzed by SDS-PAGE.

Protein bands corresponding to a molecular weight of 25.5 and 16 K were electroeluted and recovered from the gel. The

biological activity of each protein was tested with TMV-infected protoplasts in comparison with the respective control fraction as described above.

Serology. Antisera were prepared by injecting the gel-purified 23 K protein. From 80 to 120 units of IVR equivalent to 0.8–1.2 μg of protein was recovered from a total of six gel channels. Gel pieces containing the 23-K protein were washed overnight in 10 ml of distilled water, with three or four changes of water. The washed gel pieces were homogenized in 1 ml of phosphate-buffered saline (PBS) and emulsified with an equal volume of Freund's incomplete adjuvant. The emulsion was injected into a rabbit subcutaneously in six places along both sides of the spinal cord. Five additional injections were given similarly at 2-wk intervals, and the rabbit was bled 2 wk after the last injection. The antiserum was absorbed with control preparation isolated from the culture medium of sham-inoculated protoplasts to eliminate possible nonspecific reactions.

Indirect ELISA was performed as described previously (5). Three units of crude IVR or control preparations was dissolved in 50 μl of coating buffer and applied to the ELISA wells. After incubation overnight at 4 C, attachment sites were saturated with 2% bovine serum albumin (BSA) before adding twofold dilutions of the 23-K protein antiserum (1:400–1:1,600). Subsequently, goat anti-rabbit conjugate was used to detect and quantify the reaction.

Agar-gel diffusion tests were done in 55-mm petri dishes containing a 4-mm layer of 0.75% Bacto agar, 0.001 M ethylenediaminetetraacetic acid, 0.85% NaCl, and 0.02% sodium azide at pH 7.8 (4,13). Five units of crude protoplast IVR, fractionated IVR-1, IVR-2, and the corresponding control preparations dissolved in 50 μl of 0.1 M phosphate buffer, pH 7, were tested using the 23-K protein antiserum (diluted 1:5).

Elimination of IVR's biological activity by the 23-K protein antiserum. The possibility that reaction with the 23-K protein antiserum eliminates the biological activity of crude protoplast IVR was tested as described previously (4,5). Polystyrene microplates were coated with either the 23-K protein antiserum or normal rabbit serum diluted 1:20. The plates were incubated for 4 hr at 37 C and washed with PBS containing 0.05% Tween 20. Fifty microliters of crude protoplast IVR was added to triplicate wells. After 3 hr of incubation, the fluid from the wells was collected and its biological activity was tested on TMV-infected tobacco protoplasts (3,9).

Western blot and protein measurement. Proteins were electrophoretically transferred onto nitrocellulose membranes as described by Towbin et al (14). Immunoreactive bands were visualized by incubating the membrane in 50 mM sodium acetate, pH 5.0, containing 0.4 mg/ml of 3 amino-9-ethylcarbazole and 0.015% (v/v) H_2O_2 until color developed. The reaction was stopped by washing the membrane in deionized water.

Proteins were quantified using the Coomassie blue dye binding procedure (7) with slight modifications. Briefly, the dye was recovered from the filter paper by shaking instead of ultrasonic elution. BSA was used as a standard.

RESULTS

PAGE of crude protoplast IVR. A 23-K-specific protein band that was not present in control preparations was consistently observed in PAGE of crude protoplast IVR disrupted in SDS (Fig. 1A). The calculated molecular weight of this protein, based on migratory distances relative to the standard protein markers, was 23 K. As seen in Figure 1A, the amount of protein in the IVR preparation (lane 2) is higher than in the control (lane 3), although both were obtained from the culture media of a similar number of TMV-inoculated and sham-inoculated protoplasts. This is understandable because after infection various pathogenesis-related (PR) proteins are produced (15). Therefore, an additional experiment was done. Fifteen units of IVR was electrophoresed in comparison to 30 units of control preparation. A distinct 23-K protein band was observed in the crude protoplast IVR that was not observed in the control preparation (data not shown).

Electroelution of IVR after PAGE. The biological activity of IVR was retained after electroelution. When an electroeluted preparation, equivalent to 3 units of IVR, a nonsaturating concentration (6), was tested in TMV-infected protoplasts and assayed on *N. glutinosa* L., it reduced virus replication by 68%, compared with 71% for the original crude IVR. The respective control preparations were without inhibitory activity (Table 1).

Electroeluted protein bands corresponding to a molecular weight of 25.5 and 16 K were recovered from the gel. The biological activity of each protein was tested in comparison with the respective control fraction. All four proteins were without inhibitory activity.

Protein contents as determined by the dye method were 0.0253 and 0.646 μg/unit for electroeluted IVR and crude IVR, respectively (averages from two experiments). Apparently, about a 20-fold increase in specific activity was obtained after electroelution.

A single band at 23 K was observed when this preparation (20 units) was analyzed by PAGE (Fig. 1B). Similar results were obtained in three additional experiments.

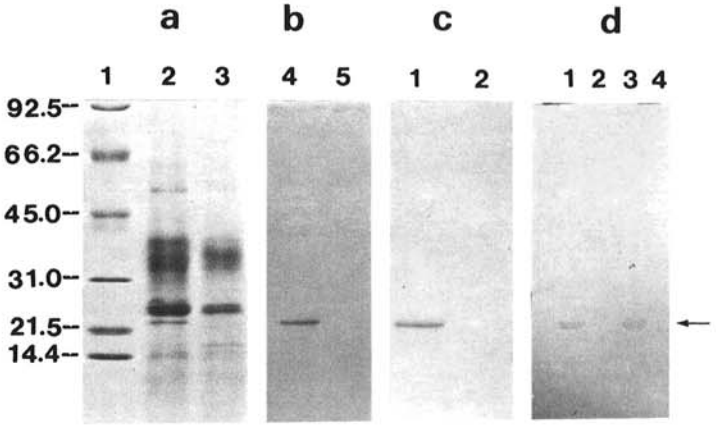

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting of an inhibitor of virus replication (IVR). **A,** SDS-PAGE of crude protoplast IVR; **B,** SDS-PAGE of IVR recovered after electroelution from the 23-K band. Lane 1, polypeptide markers phosphorylase B (92.5 K), bovine serum albumin (66.2 K), ovalbumin (45 K), carbonic anhydrase (31 K), soybean trypsin inhibitor (21.5 K), and lysozyme (14.4 K); lane 2, crude protoplast IVR (10 units); lane 3, control preparation (10 units); lane 4, electroeluted IVR (15 units); lane 5, electroeluted control preparation (15 units). **C,** Immunoblot of electroeluted IVR. Lane 1, electroeluted IVR (15 units); lane 2, electroeluted control preparation (15 units). **D,** Immunoblot of crude protoplast and tissue IVR. Lane 1, crude tissue IVR (10 units); lane 2, crude control from tissue (10 units); lane 3, crude protoplast IVR (10 units); lane 4, control preparation (10 units). IVR band indicated by arrow. Molecular weight markers are in kilodaltons.

TABLE 1. Effect of the 23-K protein electroeluted from sodium dodecyl sulfate-polyacrylamide gel on tobacco mosaic virus (TMV) replication in protoplast from tobacco cultivar Samsun NN

Sample	Infectivity[a] from test protoplast treated with: Inhibitor of virus replication	Control preparation	Percent inhibition
Starting material[b]	7.5 ± 1.5	26 ± 3.8	71
Electroeluted 23-K protein	9.0 ± 1.8	28 ± 4.5	68
Control protoplasts[c]		31 ± 3.5	

[a]Average number of local lesions and standard error from three to four experiments per 10^6 protoplasts 72 hr after inoculation on one-half leaf of *Nicotiana glutinosa* calibrated to a standard TMV (0.5 μg/ml) which yielded about 70 lesions per half leaf.
[b]Equivalent to 3 units.
[c]TMV-inoculated protoplasts with no addition.

Serological evaluation of PAGE antiserum. The 23-K protein antiserum enabled a clear distinction in ELISA between crude protoplast IVR and control preparation. IVR values from indirect ELISA were 1.47, 1.10, and 0.67 (averages from four experiments, two replicates for each sample), compared with 0.214, 0.121, and 0.025 for the control preparation at serum dilutions of 1:400, 1:800, and 1:1,600, respectively.

In agar-gel diffusion tests, clear precipitation lines were obtained between crude protoplast IVR, IVR-1, and IVR-2 and the 23-K protein antiserum (diluted 1:5). The precipitation lines fused without spurs (Fig. 2), indicating that the fractions were antigenically similar.

Elimination of IVR's biological activity by the 23-K protein antiserum. The 23-K protein antiserum decreased the inhibitory activity of IVR by 85% when compared with normal serum (Table 2). When a further cycle of binding was performed, all inhibitory activity in the crude preparation was removed by binding to the antiserum.

Western blotting analysis of IVR. The 23-K protein band observed in PAGE of crude protoplast IVR reacted specifically in Western blots with the 23-K protein antiserum (Fig. 1D). Electroeluted 23-K protein, which exhibits a single band in SDS-PAGE, reacted specifically in Western blot with the 23-K protein antiserum (Fig. 1C). The antiserum reacted specifically also with tissue IVR isolated from the intercellular fluid of hypersensitive tobacco plants, and the active component comigrated with protoplast IVR (Fig. 1D). These results strengthen our previous findings that tissue IVR and protoplast IVR are identical (12).

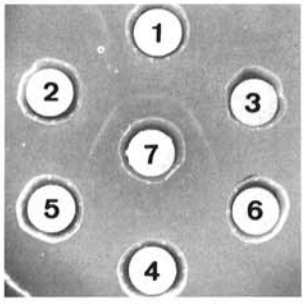

Fig. 2. Agar-gel double diffusion serology of inhibitor of virus replication (IVR). 1, crude IVR; 2, IVR-1; 3, IVR-2; 4, 5, and 6, parallel control preparations to IVR, IVR-1, and IVR-2, respectively; 7, 23-K protein antiserum.

TABLE 2. Effect of the 23-K protein antiserum on the biological activity of the inhibitor of virus replication (IVR)

Wells coated with antiserum[a]	Antigen[b]	Infectivity from test protoplast treated with the respective preparation[c]	Percent inhibition
1. ···	Protoplast IVR	6.5 ± 2.5	71
	Control preparation	22.6 ± 5.2	
2. Normal	Protoplast IVR	7.5 ± 2.8	69
	Control preparation	24.5 ± 5.8	
3. Anti-23-K protein	Protoplast IVR	18.7 ± 3.5	10
	Control preparation	20.8 ± 4.2	
Control protoplasts[d]		26.5 ± 4.5	

[a]Antiserum diluted 1:20 in coating buffer was used to coat enzyme-linked immunosorbent assay plates.
[b]Three units of crude protoplast IVR or control preparation was added.
[c]Average number of local lesions and standard error from three to four experiments per 10^6 Samsun NN tobacco protoplasts 72 hr after inoculation, on one-half leaf of *Nicotiana glutinosa* calibrated to a standard tobacco mosaic virus (TMV) solution (0.5 μg/ml) which yielded about 70 lesions per half leaf.
[d]TMV-inoculated protoplasts in incubation medium with no addition.

DISCUSSION

Our main finding in this study is the association of the 23-K protein with the biological activity of the IVR isolated from the culture medium of Samsun NN tobacco protoplast infected with TMV. The 23-K protein band that was not present in control preparations was observed consistently in SDS-PAGE of crude protoplast IVR. The biological activity could be recovered from the 23-K protein band by electroelution. Apparently, after dialysis to remove the SDS, the IVR molecule retains its biological activity. This biologically active fraction obtained after electroelution revealed a 23-K protein band when analyzed by PAGE. Electroeluted protein fractions above and below the region of the 23-K protein were without inhibitory activity. This together with the increase in specific activity provides evidence that the 23-K fraction is IVR, purified to a high degree.

The amount of protein per unit determined after electroelution is two to three times higher than that determined in preliminary experiments for fractions obtained by high-pressure liquid chromatography (12). This may be due to contaminating proteins in the electroeluted fraction, or inaccuracy of the Coomassie blue dye binding procedure (7), or both. It seems reasonable therefore to assume that the content of IVR protein per unit is within the range of 10–30 ng.

The antiserum prepared from the 23-K band was highly reactive with the two biologically active fractions, IVR-1 and IVR-2, obtained previously from Sephadex 6-75 columns (Pharmacia, Uppsala, Sweden) (9). Precipitation lines fused completely, indicating that IVR-1 and IVR-2 have identical antigenic determinants. This strengthens our previous suggestion that IVR-2 is a dimer of IVR-1 (9). Support for this suggestion is given by the fact that, in Western blots after SDS-PAGE, only one band around 23 K is observed and, under denaturing conditions, the dimer IVR-2 is separated to the monomeric form: IVR-1. Similar results were obtained with IVR-1 and IVR-2 obtained from the intercellular fluid of Samsun NN infected with TMV (12).

The 23-K protein antiserum described in this study was not only immunoreactive with IVR but also efficiently eliminated its antiviral activity.

The antiserum was used further for the detection of tissue IVR isolated from the intercellular fluid of hypersensitive tobacco. Western blots of both protoplast and tissue IVR revealed a single 23-K band, not present in the control preparations. These results are in line with our previous findings that protoplast and tissue IVR are closely related (12).

Some discrepancy between the molecular-weight determination based on SDS-PAGE and on gel filtration was noticed. Using SDS-PAGE, a molecular weight of approximately 23,000 was estimated for IVR, whereas by gel filtration a value of 26,000 was obtained. Similar observations also have been made with other proteins (8). The different molecular-weight estimation may stem from configuration changes under denaturing conditions.

Many proteins, sometimes called pathogenesis-related proteins, are known to be induced in the leaves of tobacco by pathogen infections that induce the hypersensitive response (15). These PR proteins generally occur at much higher concentrations than IVR and are resistant to proteases. No inhibitory activity has been reported for any of them. One of these proteins, PR-R, with a molecular weight near 23,000, resembles thaumatin (11). Preliminary data (unpublished) indicate a marked difference in the amino acid composition of IVR and PR-R. It seems, therefore, that IVR and PR-R are two different proteins.

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