COLLECTION, ESTABLISHMENT AND MAINTENANCE

The mosaic disease on *S. grandiflora* was collected (Solunke, et al., 1983) and the same virus culture was maintained in 60 mesh wire house by mechanical inoculation to 10 day old healthy *S. grandiflora* leaves. The virus was further subjected to single lesion isolation technique using *Cyamopsis tetragonoloba* cv. local as a local lesion host. The virus was propagated in the wiremesh house by periodical sap inoculation to *S. grandiflora* for further studies.

VIRUS PURIFICATION

The virus was initially purified from systemically infected *S. grandiflora* leaves according to the procedure given by Sreenivasulu and Nayudu (1982) and later improved. The SMV infected leaves with mosaic symptoms were collected 3 weeks after inoculation, and homogenized in 0.05 M sodium
acetate buffer, pH 5.6 containing 0.2% sodium thioglycolate (SAT buffer) (3 ml buffer/g fresh leaf), and strained through 2 layers of muslin cloth. The supernatant was emulsified for 30 min with 8% (v/v) butanol at 4°C. The top, dark coloured organic phase was carefully removed by subjecting it to 10,000 rpm for 15 min in RC5C Sorvall centrifuge with SS34 rotor. Virus was precipitated from the aqueous phase by the addition of ammonium sulphate (50% w/v). The virus was collected by centrifugation at 10,000 rpm for 20 min and was dissolved in a small volume of extraction buffer, clarified at 10,000 rpm for 15 min and subjected to rate-zonal density gradient centrifugation. The gradient columns were prepared by layering 4, 7, 7 and 8 ml of 10, 20, 30, and 40% sucrose in SAT buffer and then stored overnight at 4°C. Six ml of virus preparation was layered on each gradient column and centrifuged at 26,000 rpm for 2 h in OTD-combi ultracentrifuge with AH 629 rotor. The gradient columns were observed under a narrow beam of light in the dark for light scattering zones. The distance from the bottom of the tube to the light scattering zone was recorded. The virus zone was collected by using a glass pasteur pipette, diluted with SAT buffer and pelleted at 35,000 rpm for 2 h in a OTD-combi ultracentrifuge with T865 rotor. The virus pellet was resuspended in minimal volume of SAT buffer. All the centrifugation steps were carried out at 4°C.

UV absorbance

The purified virus sample was scanned from 200 to 320 nm in Shimadzu UV-Visible recording spectrophotometer to calculate $A_{260}/280$ ratio and $A_{max/min}$ ratios.
Quantitation of purified virus

Purified virus was quantified spectrophotometrically measuring the absorbance at 260 nm assuming a specific extinction coefficient of $6 \text{ cm}^2 \text{ mg}^{-1}$ (uncorrected for light scattering).

ELECTRON MICROSCOPY

The virus samples were prepared for electron microscopy by following the procedure described by Milne (1984). A clean glass-slide was dipped into a solution of 0.35% Formvar (Polyvinyl formaldeyde) in chloroform. The slide was withdrawn vertically and allowed to dry in air. The edges of the slide were scraped to cut the film. The slide was lowered carefully into glass distilled water in a beaker and the film was allowed to detach from the slide and float on the water. A few copper grids (Type G-300, 3 mm) were placed on the floating film and the film along with the grids was taken onto a fresh ivory-paper with the film covering the grids. The paper was allowed to dry in dust free area.

Purified virus (density gradient column-light scattering zone) preparation was used for particle morphology studies. A drop of virus suspension was placed on a piece of parafilm. The formvar coated grid was placed on the virus sample (formvar film facing the virus sample). After one minute the grid was removed using a fine forceps, the excess solution was removed with a filter paper and the thin film of virus solution was allowed to dry. A drop of 0.1% phosphotungstic acid in water was then placed on the grid. The excess stain was removed and the grid was allowed to dry. After drying the grids were observed with a JEOL 100 C X II Electron Microscope operating at 80 kV.
SEROLOGY

a) Production of antiserum

Polyclonal antibodies were produced against purified virus (sucrose gradient zone) in a New Zealand white inbred rabbit by giving intramuscular injections with 1 mg of purified virus emulsified with Freund's incomplete adjuvant (Difco laboratories, Detroit, Michigan, USA) at weekly intervals for four weeks. The rabbit was bled one week after the last injection. The blood was incubated for 3-4 h at 4°C. The serum was collected by centrifugation at 6,000 rpm for 10 min. 2 ml antiserum fractions were lyophilized (Hindhivac, Bangalore) and stored at -20°C for further use.

b) Cross absorption of antiserum

i. Purification of healthy leaf proteins: Healthy leaf proteins were purified from S. grandiflora leaves by procedure similar to that for virus purification up to clarification step and the aqueous phase pelleted at 35,000 rpm for 2 h. The pelleted healthy proteins were suspended in minimal volume of 0.02 M potassium phosphate buffer, pH 7.2 and clarified at 6,000 rpm for 10 min. The amount of protein was quantified by the method of Lowry et al. (1951).

ii. Cross absorption of antiserum: One ml of antiserum was mixed with 2.6 mg of healthy proteins. After 1 h incubation at 37°C, the antiserum samples were centrifuged at 4,000 rpm for 15 min. The supernatant antiserum was collected and used for further serological studies.

c) Determination of antiserum/antigen titres

i. Antiserum titre: Agar gel double diffusion test (AGDDT) (Purcifull and Batchelor, 1977) and Direct antigen coating ELISA (DAC-ELISA)
(Hobbs et al., 1987; Mowat and Dawson, 1987) were performed to determine the titre of antiserum.

a) Agar gel double diffusion test

The medium was prepared by using 0.8% agarose melted in PBS (0.01M potassium phosphate buffer, pH 7.0 + 0.85% sodium chloride). The molten agarose at 45-50°C was poured onto a glass plate, with the help of a pipette and allowed to solidify at 6°C for 2 h. The wells were cut in the solidified medium using a template and cork borer (8 peripheral wells at a distance of 3 mm from the edge of the central well). The agarose plugs were taken out with the help of a pasteur pipette connected to a suction pump. The bottom of the wells was sealed with a 15 µl of molten agarose.

To determine the antiserum titre, the central wells were filled with 30 µl of purified virus and the peripheral wells were filled with 30 µl of different dilutions of antisera (2-fold dilutions up to 1/2048). Healthy and buffer controls were included in the tests. The plates were incubated in a moist chamber at room temperature for 36 to 48 h.

b) Enzyme-linked immunosorbent assay (ELISA)

DAC ELISA (Hobbs et al., 1987; Mowat and Dawson, 1987) was used to determine antiserum titre.

i. Preparation of reagents (Clark and Bar-Joseph, 1984)

**Phosphate buffer-saline (PBS) pH 7.4**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>: 8.0 g</td>
<td></td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>: 1.44 g</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>: 0.2 g</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>: 0.2 g</td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>: 800.0 ml</td>
<td></td>
</tr>
</tbody>
</table>

pH adjusted to 7.4 with 1 N HCl and made up to 1,000 ml with distilled water.
PBS-Tween (PBS-T)

Tween - 20 : 0.5 ml
PBS : 999.5 ml

PBS-TPO

Polyvinyl pyrrolidone : 20.0 g
(Mr 40,000, Sigma)
Ovalbumin (Sigma) : 2.0 g
PBS-T : 1,000 ml

Stirred on a magnetic stirrer for 20 min

Carbonate (coating) buffer, pH 9.6

\[\text{Na}_2\text{CO}_3 : 1.59 \text{ g}\]
\[\text{NaHCO}_3 : 2.93 \text{ g}\]

Dissolved in 800 ml distilled water, pH adjusted to 9.6 with 1 N HCl and made up to 1,000 ml with distilled water.

Diethanolamine (substrate) buffer, pH 9.8

Diethanolamine : 9.7 ml
Distilled water : 80.0 ml

pH adjusted to 9.8 with 1 N HCl and made up to 100 ml with distilled water

Protein A-labeled alkaline phosphatase (Sigma P-9650)

Phosphatase substrate (p-nitrophenyl phosphate):

\[\text{p-Nitrophenyl phosphate (Sigma 104)} : 10 \text{ mg}\]

Substrate buffer : 20 ml

Prepared freshly just before use
Purified virus dilutions (1 μg and 500 ng/ml) were prepared in carbonate buffer containing 0.01 M DIECA and the dilutions were added to microtitre plate (200 μl/well) (Laxbro, R. Bhagvandas and Bros. Pune, India). The plates were incubated at 37°C in a humid box for 2 h. After incubation, the plates were washed thrice at 3 min intervals with PBS-T. The antiserum dilutions were made with PBS-TPO, and dispensed (200 μl/well) and incubated at 37°C for 2 h. The plates were washed thrice with PBS-T (3 min each time). Then 200 μl/well of protein A labeled with alkaline phosphatase (0.25 units/ml in PBS-TPO) was added and incubated at 37°C. After 2 h, the plates were washed thrice with PBS-T at 3 min interval. Finally, freshly prepared p-nitrophenyl phosphate (PNP) was added (200 μl/well). The appearance of yellow colour was observed. The reaction was terminated by adding 50 μl of 3 M NaOH. The results were recorded by reading the plate at 405 nm in a Vmax Microplate Reader. Wells filled with buffer were used as negative controls (blanks) of the reaction. Samples with absorbance values two times greater than buffer controls were considered as positives. The experiment was conducted twice with three replicates.

ii. Antigen titre

DAC-ELISA (Hobbs et al., 1987; Mowat and Dawson, 1987) and Dot immunobinding assay (DIBA) (Banttari and Goodwin, 1985) were performed to determine antigen titres of purified virus.

In DAC-ELISA, initially the antigen dilutions (from 1 μg to 1 ng/ml) were prepared in carbonate buffer and coated (200 μl/well) to the microtitre plate, incubated at 37°C for 2 h, and washed thrice at 3 min interval. Then
antiserum dilutions (1:10,000 and 1:20,000) were prepared in PBS-TPO and were dispensed (200 µl/well) and incubated at 37°C for 2 h. The other steps were as mentioned in the above.

**Dot immunobinding assay (DIBA)**

The basic procedure of Banttari and Goodwin (1985) was used to determine the antigen dilution/titre in purified virus, and SDS-dissociated virus.

Antigen dilutions from purified virus and dissociated virus were prepared in coating buffer and spotted onto nitrocellulose membrane, air dried and incubated in 5% commercial milk powder in tris-buffered saline for 1 h at room temperature. Then the nitrocellulose membrane was incubated in 1:10,000 dilution of homologous antisera in TBS (0.02 M Tris, 0.5 M NaCl) with 0.05% tween-20 (TBS-T) containing 5% milk powder ('Everday' by Nestle) (TBS-TM). Washed thrice with TBS-T (each time 5 min) and the membrane was incubated for 1 h in 1:1,000 dilution of horseradish peroxidase labeled goat antirabbit Fc-specific antibodies in TBS-TM. The membrane was washed thrice with TBS-T and transferred to a substrate solution (sodium citrate 0.05 M pH 4.8 containing 0.03% H₂O₂) containing 3 mg/5 ml of DAB (diaminobenzidine, Sigma). Colour development was recorded visually and stopped the reaction by washing the membrane in distilled water.

**Serological relationships**

Serological relationships of SMV with antisera to certain other reported sobemoviruses, tomatobushy stunt virus (TBSV), narcissus tip necrosis virus...
(NTNV) and elderberry latent virus (ELV) were determined by AGDDT and DAC-ELISA.

a. AGDDT: This test was carried out as described earlier. The central well was filled with 30 μl of purified virus preparation and the peripheral wells with 50 μl antisera of SBMV-type strain (procured from J.W.Demski, Georgia Experiment station, USA); SBMV-Cp strain (procured from O.P.Sehgal, Dept. of Plant Pathology, Columbia, USA; and A. Fuentes, Canada) and NTNV, TBSV, ELV (procured from G.P. Martelli, Italy). Homologous, healthy and buffer controls were maintained.

b. DAC-ELISA: The plates were coated with 1 μg/ml (200 μl/well) of purified virus in carbonate buffer and incubated at 37°C. After 2 h the plates were washed thrice at 3 min intervals with PBS-T. 1:1,000 dilutions of different antisera (homologous/heterologous) were coated (200 μl/well) and incubated for 2 h, washed thrice with PBS-T (at 3 min interval) and coated with 200 μl/well of protein A labeled with alkaline phosphatase (0.25 units/ml in PBS-TPO) was added and incubated at 37°C. After 2 h the plates were washed thrice with PBS-T at 3 min interval. Finally freshly prepared p-nitrophenyl phosphate (PNP) was added (200 μl/well). The appearance of yellow colour was noted and the reaction was terminated by adding 50 μl of 3 M NaOH. The results were recorded at 405 nm in a Vmax Microplate reader.

Wells filled with antigen extraction buffer were used as negative controls (blank) of the reaction instead of leaf extracts. The homologous antiserum was included in each plate as controls. Samples with absorbance
values two times greater than blank were considered as positives. The experiment was conducted twice with three replicates.

**VIRUS COAT PROTEIN**

Virus coat protein was analyzed using purified virus (sucrose density gradient zone)

**Molecular weight of virus coat protein**

It was determined by 12% SDS-PAGE according to the method of Laemmli (1970).

**A. Stock solutions**

i. **30% Acrylamide**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>30.0 g</td>
</tr>
<tr>
<td>N,N'-methylene bisacrylamide</td>
<td>0.8 g</td>
</tr>
</tbody>
</table>

Dissolved in 60 ml of distilled water and made up to 100 ml with distilled water, filtered and stored at 4°C in an amber-coloured bottle.

ii. **Resolving gel buffer** (1.5 M Tris-C1, pH 8.8)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-hydroxymethyl aminomethane (Tris base)</td>
<td>18.15 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>80.0 ml</td>
</tr>
</tbody>
</table>

pH adjusted to 8.8 with 1 N HCl and made up to 100 ml with distilled water, filtered and stored at 4°C.

iii. **Stacking gel buffer** (0.5 M Tris-C1, pH 6.8)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>6.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>70.0 ml</td>
</tr>
</tbody>
</table>
pH adjusted to 6.8 with 1 N HCl and made up to 100 ml with distilled water, filtered and stored at 4°C.

iv. 10% Sodium dodecyl sulphate (SDS)

One gram of SDS dissolved in 10 ml of distilled water with gentle stirring and stored at room temperature.

v. Sample buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M Tris-Cl, pH 6.8</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>1.00 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>2.00 ml</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>0.50 ml</td>
</tr>
<tr>
<td>0.05% Bromophenol blue (w/v)</td>
<td>0.25 ml</td>
</tr>
</tbody>
</table>

made up to 10 ml with distilled water and stored at room temperature.

vi. Electrophoresis (Tank) buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>1.80 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>8.62 g</td>
</tr>
<tr>
<td>SDS</td>
<td>0.60 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>500.00 ml</td>
</tr>
</tbody>
</table>

pH adjusted to 8.3 with 1 N HCl and made up to 600 ml with distilled water and stored at room temperature.

vii. 10% Ammonium persulphate (APS)

100 mg APS dissolved in 1 ml distilled water just before use.
B. Gel composition

<table>
<thead>
<tr>
<th>Solution</th>
<th>Resolving gel (20 ml)</th>
<th>Stacking gel (10 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10%</td>
<td>12%</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>6.6 ml</td>
<td>8.0 ml</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>8.1 ml</td>
<td>6.7 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>20 µl</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

C. Gel preparation

Gel (1 mm thick) was cast in the gel mold consisting of two glass plates, spacers and well forming comb. The glass plates were sealed in the sides by applying wax and in the bottom by pouring molten agar. The resolving gel solution was prepared according to the table and pipetted immediately into the gel mold gently from the top. Distilled water was overlaid on the top of the resolving gel to eliminate air bubbles and to get a uniform gel surface. After polymerization, the water layer was decanted carefully. Freshly prepared stacking gel was poured without air bubbles and the comb was inserted into the stacking gel. The stacking gel solution was allowed to polymerize at room temperature for 30 min. After polymerization the comb was removed gently and the wells were washed with tank buffer. Then the glass plates were clamped to the electrophoresis setup.
with the help of screw clips. Then the lower and upper reservoirs were filled with electrophoresis buffer without air bubbles on the bottom of the gel.

D. Sample preparation

Purified virus suspended in 0.05 M sodium acetate buffer, pH 5.4 was diluted with sample buffer (1:1 v/v). The samples were heated at 100°C for 3 min and then cooled rapidly on ice-bath. Phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and β-lactalbumin (14.4 kDa) (Pharmacia) were used as Mr standards.

E. Sample application and electrophoresis

The digested purified virus samples equivalent to 10-12 µg were loaded into the gel slots by means of a Hamilton microsyringe. A constant 50 V was applied and electrophoresed for 7 h at room temperature. The run was terminated when tracking dye was 0.5 cm above the bottom of the gel. After completion of the run, the gel mold was carefully dismantled and the gel was immersed in a glass tray containing 0.2% coomassie brilliant blue-R 250 (w/v) in methanol:acetic acid:distilled water (40:7:53, v/v/v) for 1 h. Then the gel was destained in methanol:acetic acid:distilled water (20:7:73 v/v/v) until the background of the gel became clear.

The distance migrated by virus proteins and marker proteins was recorded. The Mr of virus protein was calculated by plotting distance migration of protein against Mr on a semi-log graph paper. The experiment was conducted thrice to confirm the results.
Electroblot Immunoassay

The basic procedure for immunological electroblot detection of viral proteins was the same as that described by Burgermeister and Koenig (1984). Approximately 40 μg of virus coat protein was boiled with sample buffer (1:1 v/v) for 5 min in boiling water and electrophoresed by SDS-PAGE as described earlier. Electroblotting was carried out using Broviga electroblot apparatus. Three Whatman No.3 filter papers were cut 1 cm excess to the gel size, wetted with transfer buffer (0.025 M Tris-Cl; 0.0192 M glycine and 20% methanol), pH 8.3, and placed on the sponge pads. The nitrocellulose paper (Bio-rad, 0.45 μm pore size) equal to the gel size was kept on the wet blotters, on which acrylamide gel was placed. Three more wet Whatman filters were kept on the gel, on which wet sponge pads were kept and clamped. The clamped unit was immersed into the transfer buffer in the transfer unit and the electrodes were connected to a power pack (Aplab, Bombay) and electroblotted at 50 V for 90 min.

After the electrophoretic transfer of proteins, the nitrocellulose membrane was incubated on a shaker with 5% commercial milk powder in Tris buffered saline (TBS, 20 mM Tris-Cl, 500 mM NaCl, pH 7.5) for 2 h at room temperature. Afterwards the nitrocellulose membrane was incubated for 1 h in a 1:5,000 dilution of antisera (homologous and SBMV-Cp) in TBS with 0.05% tween-20 (TBS-T) containing 5% milk powder (TBS-TM). After 3 washings, each 5 min in TBS-T, the membrane was incubated for 1 h in a 1:1000 dilution of horseradish peroxidase labeled goat antirabbit Fc-specific antibodies (Genie, Bangalore) in TBS-TM. The nitrocellulose membrane was washed thrice, 5 min each in TBS-T and transferred to a substrate solution containing 25 ml TMB (3,3', 5, 5'-tetramethyl benzidine) membrane peroxidase
substrate solution, 25 ml H₂O₂ and 5 ml TMB membrane enhancer (Kirkegaard and Parry laboratories Inc.). Colour development was recorded visually and stopped by washing the membrane in distilled water.

**VIRUS NUCLEIC ACID**

The virus nucleic acid was isolated from purified virus preparations (sucrose gradient zone) by the method of Sambrook et al. (1989).

**A Glassware and reagents preparation**

All the glassware and centrifuge tubes were washed initially with 0.1 M NaOH followed by sterile distilled water and finally with sterile distilled water containing 0.1% diethyl pyrocarbonate (DEPC-RNase inhibitor). The glassware and buffers were sterilized in an autoclave at 15 lbs for 15 min. Disposable sterile gloves were used while preparing the solutions, glassware and while isolating the RNA.

1. **10% Sodium dodecyl sulphate (SDS)**

   SDS : 1.0 g
   Sterile distilled water : 10.0 ml

   Dissolved, no autoclaving, stored at room temperature.

2. **200 mM EDTA, pH 8.0**

   Na₂ EDTA : 3.72 g
   Dis. water : 50.00 ml

   Dissolved in 30 ml of sterile distilled water, pH adjusted to 8.0 with 3 N NaOH and made up to 50 ml with sterile water, autoclaved and stored at 4°C.
3. 2.5 M sodium acetate, pH 5.2

Sodium acetate : 10.25 g
Sterile distilled water : 50.00 ml

Dissolved in 30 ml of sterile distilled water, pH adjusted to 5.2 with acetic acid and made up to 50 ml with sterile water, autoclaved and stored at 4°C.

4. Proteinase K

Proteinase K : 20.0 mg
Sterile dis. water : 1.0 ml

No autoclaving, stored at -20°C.

5. 1 M Tris-Cl buffer, pH 8.0 (containing 0.2% 2-ME)

Tris base : 121.1 g
Distilled water : 800.0 ml

pH adjusted to 8.0 with conc. HCl and made up to 1000 ml. Autoclaved and then added 2 ml of 2-mercaptoethanol. Stored at 4°C.

6. 0.1 M Tris-Cl buffer, pH 8.0 (containing 0.2% 2-ME)

Autoclaved 1.0 M Tris-Cl buffer pH 8.0 : 10.0 ml
Sterile distilled water : 90.0 ml
2-ME : 0.2 ml

Stored at 4°C.
7. Absolute ethanol stored at -20°C

8. Chloroform: isoamyl alcohol (24:1 v/v)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>24.0 ml</td>
</tr>
<tr>
<td>Isoamyl alcohol</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

Stored at 4°C.

9. Phenol

Buffer saturated phenol was prepared as described by Sambrook et al. (1989). 8-Hydroxyquinoline was added to 0.1% to liquid phenol. The phenol was extracted several times with equal volume of 1 M Tris-Cl buffer pH 8.0 until pH of the aqueous phase was >7.6. After the phenol was equilibrated, final aqueous phase was removed, 0.1 volume of 0.1 M Tris-Cl buffer pH 8.0 containing 0.2% 2-mercaptoethanol was added, and stored in an amber coloured bottle at 4°C.

10. 50 mM KPO₄ buffer, pH 7.0

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₂HPO₄</td>
<td>0.212 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.106 g</td>
</tr>
</tbody>
</table>

Sterile dis. water: 40,000 ml

pH adjusted to 7.0, made up to 50 ml, autoclaved and stored at 4°C.

11. Distilled water containing 0.1% DEPC

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>999.0 ml</td>
</tr>
<tr>
<td>DEPC</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

Dispensed into aliquots, autoclaved, stored at 4°C.
B. Isolation Procedure

The final purified virus pellets were suspended in minimal volume of potassium phosphate buffer, pH 7.0; 0.05 M. SDS to 1% and EDTA to 20 mM were added to the purified virus suspension from the stock solutions. The contents of the tubes were gently mixed and incubated at 65°C for 5 min. The tubes were cooled to room temperature, 1 mg/ml proteinase K was added (from 20 mg/ml stock) and incubated at 37°C for 10 min. To this equal volume of phenol: chloroform-isoamyl alcohol (24:1, v/v) was added. The tubes were covered with parafilm and gently shaken by inverting the tubes for 2-3 min. Tube contents were centrifuged at 6,000 rpm for 5 min. The upper aqueous phase was collected with the help of a sterile pasteur pipette into another sterile tube kept in ice box. The phenol phase was reextracted with 1-2 ml of 0.05 M potassium phosphate buffer, pH 7.0. Aqueous phase was collected after centrifugation at 6,000 rpm for 5 min. The pooled aqueous phases were reextracted with equal volume of phenol: chloroform mixture and centrifuged at 6,000 rpm for 5 min. The upper aqueous phase was reextracted with equal volume of chloroform-isoamyl alcohol mixture. To the upper aqueous phase 2.5 M sodium acetate pH 5.2 was added to 250 mM concentration. To this, 2.5 volumes of ice-cold absolute ethanol was added and the tubes were covered with parafilm and kept at -20°C overnight.

The nucleic acid was pelleted by centrifugation at 5,000 rpm for 10 min. The nucleic acid pellet was washed twice with 70% ethanol. The pellet was vacuum dried and dissolved in a minimal volume of sterile distilled water and dispensed into 50 to 100 µg aliquots in eppendorf tubes, and precipitated by adding sodium acetate to 250 mM and 2.5 volumes of
ethanol. UV absorption spectrum was taken (200-320 nm) in a Hitachi 150-2u UV-Visible spectrophotometer. $A_{260/280}$ ratio was calculated. RNA was quantified by assuming $1 \text{ OD} = 40 \mu g/mL$.

C Nucleic acid type

The type of nucleic acid present in the virus was determined by orcinol and diphenylamine tests.

1. Orcinol test (Schneider, 1957)

Virus nucleic acid 50-200 $\mu g$ was taken into different tubes and made up to 1 ml with 0.5 M perchloric acid. To the test tubes 1.5 ml of freshly prepared orcinol reagent (one gram orcinol dissolved in 100 ml of conc. HCl containing 0.5 g ferric chloride) was added and heated at $70^\circ C$ for 20 min. The colour of solution in the tubes was recorded visually. Blank was prepared by taking 0.5 M perchloric acid. Appearance of green colour was regarded as positive.

2. Diphenylamine test (Burton, 1956)

Preparation of diphenylamine reagent

One gram of diphenylamine was dissolved in 100 ml glacial acetic acid and 2.75 ml conc. sulphuric acid and mixed well. It was stored at $4^\circ C$ and warmed to room temperature before use.

Viral nucleic acid (100-300 $\mu g$) was taken into test tubes and made up to 2 ml with 5% trichloroacetic acid. To each tube 4 ml diphenylamine reagent was added and boiled for 10 min in a waterbath and cooled rapidly. The colour of the solution in the tubes was recorded visually. Calf thymus
DNA was also similarly tested. Appearance of blue colour was regarded as positive.

D Infectivity assay

The purified viral RNA was checked for its infectivity on C. tetragonoloba. The purified viral RNA suspended in potassium phosphate buffer, pH 7.0, 0.05 M, to get 25, 50, 75 and 100 μg/ml and containing 0.5% bentonite was inoculated onto carborundum dusted C. tetragonoloba plants (kept in dark for 24 h prior to inoculation). After inoculation, the leaves were washed with sterile water. The inoculated plants were observed for 5-6 days for local lesion development and the number of local lesions produced were recorded.

Agarose gel electrophoresis of viral RNA

Horizontal agarose slab gel electrophoresis was performed according to the procedure given by Sambrook et al. (1989).

The gel boat was prepared by covering the edges of a mold with scotch tape and placed it on a level surface. The comb was put on the gel boat groove. 250 mg agarose (Bio-Rad) was dissolved in 25 ml of sterile TBE buffer (Tris 0.089 M; boric acid 0.089 M and EDTA 0.002 M). The agarose was cooled to 45°C and poured into the gel boat. After solidification of the agarose (10-15 min at room temperature) the comb and scotch tapes were removed carefully.

The gel boat was transferred on to a horizontal electrophoresis gel tank. TBE buffer was added slowly, just enough to cover the gel to a depth
of about 1 mm. Approximately 1 µg of RNA was mixed with gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 15% Ficoll Type 400: Pharmacia) and loaded into the wells using a Hamilton microsyringe. The tank was closed with a lid and the electrical leads were connected to an electrophoresis power pack (Aplab, Bombay). Electrophoresis was at 60 volts for 2 h. After the run was over, the gel was stained with ethidium bromide (0.5 µg/ml in water) for 10-15 min at room temperature. The gel was viewed on UV-transilluminator (Haake Buchler, UVT) and photographed by Pentax ME Super with SMC orange filtered (49 mm) camera. TMV-RNA and E. coli ribosomal RNA were coelectrophoresed as Mr standards. The distance migration of each RNA band was recorded. The Mr of RNA was calculated by plotting distance migration of RNA against Mr on a semilog graph paper. The experiment was conducted thrice to confirm the results.

**Double-stranded RNA (dsRNA)**

DsRNA was isolated from SMV infected *C. tetragonoloba* primary leaflets by LiCl fractionation method of Diaz Ruiz and Kaper (1978).

**Reagents**

1. 10 x STE buffer, pH 8.0

\[
\begin{align*}
\text{Tris (0.1 M)} & : 1.211 \text{ g} \\
\text{NaCl (0.6 M)} & : 3.507 \text{ g} \\
\text{EDTA (0.03 M)} & : 1.116 \text{ g}
\end{align*}
\]

Dissolved in 80 ml of distilled water and pH adjusted to 8.0 with 1 N HCl and made up to 100 ml, stored at 4°C.
2. **10 x SSC buffer, pH 7.0**

   Sodium chloride (0.15 M) : 8.766 g  
   Sodium citrate (0.015 M) : 4.411 g  

   Dissolved in 80 ml of distilled water and pH adjusted to 7.0 with 0.1 N HCl and made upto 100 ml, stored at \(4^\circ\text{C}\).

3. **4 M Lithium chloride**

   Lithium chloride : 4.248 g  
   Dis. water : 25.000 ml  

   Dissolved the LiCl in 20 ml of distilled water and made up to 25 ml, prepared just before use.

4. **Kirby's mixture**

   Phenol (recrystallised) : 90.0 ml  
   m-Cresol : 10.0 ml  
   8-Hydroxyquinoline : 0.1 g  

   Saturated with 1 x STE buffer (containing 0.2% 2-ME) thrice and stored in an amber coloured bottle, at \(4^\circ\text{C}\).

5. **PK-D buffer, pH 7.5**

   Tris (0.1 M) : 0.605 g  
   NaCl (0.1 M) : 0.290 g  
   MgCl\(_2\) (0.1 M) : 1.016 g
Dissolved in 40 ml of distilled water, pH adjusted to 7.5 and made up to 50 ml with distilled water.

6. PK-D mixture (0.1% proteinase K; 0.1% DNAse 1:1)

- Proteinase K : 5.0 mg
- DNAse : 5.0 mg
- PK-D buffer : 5.0 ml

7. 0.4 M Sodium acetate, pH 4.7

- Sodium acetate : 3.281 g

Dissolved in 80 ml of distilled water, pH adjusted to 4.7 with acetic acid and made up to 100 ml with distilled water.

8. Stop mix

- 10% SDS : 1.0 ml
- Bromophenol blue : 0.25 mg
- Sucrose : 4.0 g
- TBE buffer : 10.0 ml

Isolation procedure

Forty eight hours SMV inoculated C. tetragonoloba primary leaves were collected, washed with tap water followed by distilled water, blotted with filter pads. Homogenized the cut leaves in chilled 2 x STE buffer containing 0.2% 2-ME (1 g/2 ml). To the homogenate equal volume of kirby's mixture and 1% SDS was added, emulsified for 30 min, centrifuged at 10,000 g for 20 min at 4°C. 2.5 volumes of chilled alcohol was added to the homogenate
and the total nucleic acids (TNA) were precipitated and left at -20°C for overnight. Total nucleic acids were pelleted at 4,500 rpm, resuspended in 1 x SSC buffer and equal volume of 4 M LiCl was added, shaken well, left at 4°C for 12 h. Later this was centrifuged at 4,500 rpm for 20 min. To the aqueous phase, 2.5 volumes of cold ethanol was added and left at -20°C for 12 h. The nucleic acids were pelleted at 4,500 rpm for 15 min and suspended in minimal volume of 1 x SSC buffer and treated with PK-DNAse (10 µl/500 µl) and incubated at room temperature for 30 min. Reprecipitated with 2.5 volumes of cold ethanol at -20°C for 2 h. Centrifuged at 4,500 rpm for 10 min and resuspended the dsRNA pellet in 1 x SSC buffer for analysis. Quantified the absorbance of dsRNA at 260 nm and calculated the concentration (1 OD = 40 µg).

Similar to this, dsRNA's were extracted from healthy C. tetragonoloba leaves as control.

**Agarose gel electrophoresis of dsRNA**

Horizontal agarose slab gel electrophoresis was done. 2% and 1% agarose gels were prepared as described earlier. 1-2 µg of dsRNA in 20 µl of stop mix was loaded into the slots and co-electrophoresed with CMV-B-A.P., India dsRNA's (from banana source in the laboratory) (Kiranmai et al., 1992) and λ DNA Hind III digest markers. Electrophoresed at 60 V for 3 h and stained with ethidium bromide. Visualized on UV-transilluminator and photographed with Pentax ME Super with SMC orange filter (49 mm).

The distance migrated by the virus dsRNA and marker dsRNAs were recorded. The Mr of the dsRNA was calculated by plotting distance of
migration of dsRNA and Mr of marker dsRNAs on a semilog graph paper. The experiment was conducted thrice on 1 and 2% agarose gels to confirm the results.

**Effect of pancreatic RNase A on dsRNA**

Viral double stranded nucleic acid (100 µg/ml) in 3.5 x SSC and 0.1 x SSC was treated with RNase A (Sigma R-5125) to 2 µg/ml, incubated at 37°C for 30 min. Afterwards, the samples were electrophoresed in 2% agarose gels along with untreated dsRNA sample. The gels were stained with ethidium bromide and visualized on UV transilluminator and photographed.

**VIRUS COAT PROTEIN**

**Isolation of SMV coat protein**

SMV coat protein was isolated according to the procedure of Tsugita and Hirashima (1972) with cold glacial acetic acid.

Two and half volumes (66% v/v) of cold glacial acetic acid was added slowly to an ice cold, aqueous solution of SMV (10-20 mg/ml) in sodium acetate buffer, pH 5.4, 0.05M. The mixture was allowed to stand for 1 h at 4°C with occasional stirring. The denatured RNA was removed by centrifugation at 10,000 rpm for 15 min. The supernatant was extensively dialyzed (5-6 changes) against distilled water. During dialysis, the protein precipitates as the pH of the dialyzate reaches the isoelectric point of the protein. The precipitate is then collected by low speed centrifugation, 10,000 rpm for 15 min.
Reduction and carboxymethylation of SMV coat protein

Recrystallization of iodoacetic acid

A saturated solution of iodoacetic acid in carbon tetrachloride was passed through a sintered-glass funnel to remove insoluble material and left at 0°C for 4 h. During this period iodoacetic acid crystallised and settled at the bottom. The supernatant liquid was discarded and recrystallization was repeated two more times by dissolving the crystals in fresh carbon tetrachloride to yield saturated solutions. Recrystallized iodoacetic acid was stored at -20°C in a brown bottle.

Reduction and carboxymethylation

The SMV coat protein was reduced with DTT and cysteine residues were carboxymethylated using iodoacetic acid (Allen, 1981).

The lyophilized SMV protein (200 mg) in a capped polypropylene tube, was dissolved in 10 ml of 0.3 M Tris-Cl buffer, pH 8.3 containing 6 M Guinidine-HCl (Gm) and 2 mM EDTA. A stream of nitrogen gas was blown gently over the surface of the solution for 3 min, incubated at 50°C for 30 min to denature the protein completely, cooled and flushed with nitrogen for 10 min. To this solution, 2 mM DTT was added. The tube was flushed with nitrogen, capped and sealed with parafilm and incubated at 37°C for 4 h to convert cystine residues to cysteine residues. The reaction mixture was cooled and flushed with nitrogen for 2 min. Carboxymethylation of the cysteine residues was initiated by addition of 5 mM iodoacetic acid (5-fold molar excess over cystein residues). A stock solution of iodoacetic acid was prepared by dissolving 50 mg of iodoacetic acid in 500 μl of water and the
pH of the solution was adjusted to 8.0 by the addition of a speck of solid tris base and stored at -20°C. The sample was once again flushed with nitrogen, stoppered and sealed with parafilm and incubated in the dark at 37°C for 1 h; 100 µl of 2-mercaptoethanol was added to the sample and dialysed against 0.1 M N-ethylmorpholine-acetate buffer, pH 8.2. After dialysis the protein suspension was lyophylized and stored at -20°C.

Checking of coat protein homogeneity

The homogeneity of reduced and carboxymethylated coat protein was checked on 15% SDS-PAGE according to the procedure of Laemmli (1970).

Gel composition

<table>
<thead>
<tr>
<th></th>
<th>Resolving gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 ml</td>
<td>10 ml</td>
</tr>
<tr>
<td><strong>Solution</strong></td>
<td>15%</td>
<td>4%</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>10.0 ml</td>
<td>1.3 ml</td>
</tr>
<tr>
<td>(30:0.8 Acrylamide/bis)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>5.0 ml</td>
<td></td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>-</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.2 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>4.68 ml</td>
<td>6.04 ml</td>
</tr>
<tr>
<td>APS 10%</td>
<td>0.1 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>20.0 µl</td>
<td>10.0 µl</td>
</tr>
</tbody>
</table>
Gel was cast as described earlier in virus coat protein parameter. The resolving and stacking gels were prepared according to the above table. Approximately 50 μg of coat protein lyophilized powder (equivalent to 150 μg protein) was dissolved in 100 μl of Laemmli's sample buffer, digested for 5 min in boiling water, cooled and loaded into the gel slots with the help of a Hamilton syringe. Bovine serum albumin (66 kDa); ovalbumin (44 kDa), carbonic anhydrase (30 kDa); β-lactalbumin (18.4 kDa); lysozyme (14.3 kDa) from Sigma were used as Mr standards. Electrophoresis was carried out at 50 V initially and 100 V after 15 min until the dye front reaches the bottom of the resolving gel. The gel was stained and destained as described earlier.

DETERMINATION OF AMINO ACID COMPOSITION OF SMV COAT PROTEIN

The amino acid composition of SMV coat protein was determined after hydrolysis with 6 N HCl.

Acid hydrolysis of carboxymethylated SMV coat protein
200 μg of carboxymethylated and lyophilized SMV coat protein was taken into three 15 ml borosilicate tubes with a constriction in the middle. To this, 300 μl of constant boiling HCl (5.7 N) containing 0.1% (w/v) phenol and 0.05% (v/v) 2-mercaptoethanol were added and dissolved the protein. The tubes were attached to vacuum stop-cocks through rubber corks. The solution was frozen in liquid nitrogen and the tubes were connected to the vacuum line through the stop-cocks. The tubes were evacuated for 5 min and the process of freezing and evacuation repeated again. The evacuated tubes were sealed on a flame and the hydrolysis was performed at 110°C for 24, 48 and 72 h. After hydrolysis, the tubes were centrifuged briefly to sediment the contents and cut open. The hydrolysates were transferred to
1.5 ml eppendorf tubes and lyophilized over P2O5 and KOH and the contents are dissolved in 200 μl of sample preparation buffer (5 N LiCl), pH 2.2. The tubes were centrifuged at 10,000 rpm for 5 min in a microfuge and from each time point 50 μl aliquots were analyzed on LKB 4150 Alpha amino acid analyzer. The peaks were integrated on a Hewlett packard (USA) recording integrator.

Ten n moles of protein hydrolysate calibration mixture (LKB) was also analysed to compute amino acid composition of virus coat protein.

The n moles of each amino acid in virus protein was calculated using the following formula:

\[
n\text{moles of amino acid} = \frac{\text{Peak area in sample}}{\text{Peak area in standard}} \times 10
\]

The total number of amino acid residues in virus protein was calculated by the following formula:

\[
\text{Total number of residues in virus protein} = \frac{\text{Protein Mr}}{\text{Average Mr of each amino acid}} = \frac{31,000}{110}
\]

Average n moles of amino acids released per residue was calculated from the following formula:

\[
\text{Average n moles per residue} = \frac{\text{Amount of all amino acid (n moles)}}{\text{Total number of residues}}
\]
The number of each amino acid residue was calculated by using the formula:

\[
\text{Number of residues of each amino acid} = \frac{\text{n moles of each amino acid}}{\text{average n moles per residue}}
\]

The results were expressed as number of residues of each amino acid per polypeptide as 31 kDa, Val, Ileu and Leu were determined based on values at 72 h hydrolysis.

**N-terminal analysis of carboxymethylated SMV coat protein**

The carboxymethylated SMV coat protein was subjected to N-terminal analysis by DABITC/PITC double coupling method (Chang et al., 1978) of sequencing.

**Purification of organic solvents/chemicals**

Analytical grade solvents/chemicals were purified as follows:

**Pyridine:** Pyridine was distilled (b.p 115-116°C) 3 times over (i) KOH (10 g/l); (ii) ninhydrine (1 g/l) and (iii) KOH (10 g/l) (Chang et al., 1978).

**n-Butyl acetate:** To remove peroxides, n-butyl acetate was passed through a column of activated alumina and then redistilled (b.p 126°C) (Allen, 1981).

**Trifluoroacetic acid (TFA):** TFA was refluxed over CrO₃ (2 g/100 ml) for 3 h and redistilled (b.p 72°C). TFA was also purified by redistillation over
CaSO₄·0.5H₂O 1 g/100 ml) which was heated to 150°C overnight before use. Distilled TFA was stored in a double stoppered bottle at 4°C.

Heptane (b.p 98°C), ethyl acetate (b.p 76.3°C), ethanol (b.p 78°C) and diethylamine (b.p 55°C) were distilled twice.

DABITC was recrystallized as follows (Chang, 1983). 50 mg of DABITC was dissolved in 7.5 ml of boiling acetone and the insoluble material was removed by passing through a sintered glass funnel. The clear solution was left at -20°C overnight, and the crystals of DABITC were collected.

All the purified solvents were stored in aliquots under a nitrogen atmosphere at -20°C.

Preparation of reagents

DABITC is unstable in pyridine; therefore, it was dissolved in pyridine just before use. A stock solution of recrystallized DABITC (2.82 mg/ml) in acetone was prepared. Portions of this stock solution (40 µl) were pipetted in to 0.5 ml eppendorf tubes, dried under vacuum over P₂O₅ and KOH, and stored at -20°C. Just before use each dried portion was redissolved in 40 µl of distilled pyridine and 10 µl aliquots were used for each reaction.

Solvents used for extraction

A mixture of heptane/ethyl acetate 2:1 (v/v) saturated with 67% pyridine was used for extraction of the reaction mixture. Heptane/ethyl acetate mixture was shaken with an equal volume of 67% aqueous pyridine. The top layer was collected and stored at -20°C.
Preparation of internal standard

The internal standard DABITC-diethylamine was prepared according to Chang (1983). Diethylamine (10 µl) was diluted with 100 µl of water and added to 1 ml of DABITC solution (1 n mole/µl in ethanol). The sample was mixed thoroughly and stored at -20°C. It was used without further dilution.

Procedure

Manual DABITC/PITC degradation

All reactions were carried out in stoppered glass centrifuge tubes (about 7 cm in length) made from borosilicate B10119 ground-glass joints. The tubes were soaked overnight in chromic acid and washed with liquid detergent and dried at 100°C for at least 10 h before use.

I Coupling of the protein with DABITC

Five n moles of carboxymethylated protein was dissolved in 20 µl of 50% aqueous pyridine, vortexed gently and treated with 10 µl of DABITC solution in pyridine (prepared fresh), vortexed again gently, centrifuged briefly to sediment the mixture and flushed with nitrogen for about 10 sec. The tubes were stoppered, sealed with parafilm and incubated at 55°C for 20 min.

II Coupling of protein with PITC

PITC (2 µl) was added to the earlier DABITC coupled fraction, vortexed gently, centrifuged briefly, and flushed with nitrogen, stoppered and sealed with parafilm. The coupling reaction was continued for another 20 min at 55°C.
Washing

Excess reagents and by-products were extracted into 200 μl of n-heptane/ethyl acetate mixture saturated with 67% pyridine by vortexing thoroughly and centrifuged for 2 min at 3,000 rpm to separate the layers. The upper phase was carefully withdrawn along the walls of the tube, using a bent pasteur pipette. Care was taken not to disturb the inter-phase and the process of washing was repeated twice. The aqueous phase was dried over P_2O_5 and KOH in a vacuum dessicator for 20 min using a rotary vacuum pump (Hind High Vacuum Co., Bangalore).

Cleavage

Fifty μl of anhydrous TFA was added to the coupled protein, vortexed gently, centrifuged briefly, flushed with nitrogen, capped and sealed with parafilm. The tubes were incubated at 55°C for 10 min, centrifuged briefly and dried over P_2O_5 and KOH in vacuum for 10 min.

Extraction of thiozoline derivative

To the coupled and cleaved protein, 25 μl of degassed water was added, vortexed thoroughly prior to the addition of 50 μl of n-butyl acetate. The mixture was vortexed thoroughly, centrifuged for 2 min at 3,000 rpm. The upper butyl acetate phase was carefully transferred into a fresh centrifuge tube using a pasteur pipette without disturbing the interphase and butyl acetate extraction was repeated twice. The combined extracts and the aqueous phase were separately dried _in vacuo_ over P_2O_5 and KOH for 20 minutes. The dried water phase containing the residual peptide was used for the next degradation cycle.
Conversion to thiohydantoin derivative

To the dried organic phase, 50 µl of 50% TFA was added, vortexed thoroughly, centrifuged briefly and flushed with nitrogen before stoppering and sealing the tubes with parafilm. These tube contents were transferred into 0.5 ml eppendorf tubes, dried in vacuum over P₂O₅ and KOH and DABTHs were stored in dry state at - 20°C until analysis.

Identification of DABTH-amino acid derivatives

DABTH-amino acid derivatives were identified by two dimensional TLC on polyamide sheets (Chang et al., 1978). The 15 x 15 cm polyamide sheets (Schleicher and Schuell, W.Germany) were cut into 3 x 3 cm pieces using long scissors.

On 3 x 3 cm piece polyamide sheet, at the right corner, a pencil mark was made on to which 50 p moles of internal marker, DABITC-diethylamine was spotted using a glass capillary tube, followed by 0.1-0.2 µl of sample dissolved in 2-5 µl of 80% alcohol. The size of the spot was kept as small as possible (usually about 1 mm in diameter) and was 0.6 cm from each edge of the polyamide sheet.

The plates were first developed in 33% aqueous acetic acid in a 50 ml beaker covered with a petridish, the height of the solvent was about 3-4 mm. After the first run, the plates were dried initially in a stream of moderately hot air followed by air at room temperature. The plates were developed a second time perpendicular to the first run using toluene/n-hexane/acetic acid, 2:1:0.9 (by vol) as a second solvent system in another
50 ml beaker. At the end of the run the plates were dried with a hot air blower. The first run takes about 6 min and the second one about 4 min.

The separation of a mixture of standard DABTHs by two dimensional TLC, as described above, is schematically represented in Fig. 3. The amino acid derivatives and the by-products are initially yellow in colour on polyamide sheets. However, after exposure to HCl vapours, the amino acid derivatives turned orange red and the by-products blue. The amino acid derivatives could be identified by relating their position to the internal marker(e), thiourea derivative (U) and excess reagent (DABITC), the last two appeared almost always on the plates.

Enzymatic cleavage of coat protein

Carboxymethylated SMV coat protein was cleaved with enzymes like trypsin, chymotrypsin, clostripaine and V8 protease.

Tryptic digestion

Carboxymethylated SMV coat protein (20 mg) suspended in 1 ml of 0.1 M N-ethylmorpholine-acetate, pH 8.2 was incubated with TPCK-treated trypsin at 37°C for 4 h with continuous stirring. Enzyme to substrate ratio was initially 1:100 (w/w) and after 2 h of digestion another aliquot of the enzyme was added. Trypsin was added from a stock solution of 10 mg/ml in 0.1 mM HCl, stored at -20°C. At the end of the digestion, the insoluble fraction was collected by centrifugation. The insoluble fraction was washed with distilled water (3 x 200 μl) and the washings were pooled with the soluble fraction. Both soluble and insoluble fractions were separately lyophilized (Allen, 1981).
Fig. 3: Schematic representation of the two-dimensional TLC of DABTHs on a polyamide sheet. The colors (after exposure to HCl vapours) are represented by solid areas (red), dotted areas (blue) and hatched areas (purple). A, alanine; Cm, carboxymethyl-cysteine; Cy, cysteic acid; D, aspartic acid; e, DABTC-diethylamine; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K1, \(\alpha\)-DABTH - e-DABTC-Lysine; k2, \(\alpha\)-PTH- e-DABTC-lysine; k3, \(\alpha\)-DABTH- e- PTC-lysine; Q, glutamine; R, arginine; S, serine; S\(\Delta\), dehydroserine; S\(\beta\), rehydroserine; S\(\beta\), polymerization product of serine; T, threonine; T\(\Delta\), dehydro-threonine; T\(\beta\), polymerization product of threonine; U, N-dimethylaminoazobenzene-N-phenylthiourea; V, valine, W, tryptophan; Y, tyrosine.
Chymotryptic digestion

Reduced, carboxymethylated SMV coat protein was digested with chymotrypsin (Allen, 1981).

20 mg of coat protein was dissolved in 2 ml of 0.1 M N-ethylmorpholine-acetate, pH 8.2, and digested with TLCK-treated chymotrypsin (from a stock of 10 mg/ml). Enzyme to protein ratio was 1:50 (w/w). The digestion was carried out at 37°C for 90 min. with continuous stirring on a magnetic stirrer and the reaction was stopped by freezing in liquid nitrogen, followed by lyophilization.

Clostripaine digest

Carboxymethylated SMV coat protein was cleaved at arginine residues by clostripaine.

20 mg of SMV coat protein was suspended in 2 ml of 50 mM potassium phosphate buffer, pH 7.6 containing 1 mM CaCl₂ and 2.5 mM DTT. 200 μg of clostripaine (5 mg/ml) activated 1 h earlier in the above said buffer was added to the protein suspension and digested at 37°C for 1 h. After the completion of digestion it was immediately frozen in liquid nitrogen and lyophilized.

V₈ protease digestion

20 mg of carboxymethylated SMV coat protein was dissolved in 2 ml of 0.1 M ammonium bicarbonate buffer, pH 7.8, allowed to stir for 30 min to dissolve the protein. 200 μg of V₈ protease was added to the protein suspension and vortexed on a magnetic stirrer at 37°C for 2 h. After
completion of the digestion, the reaction was stopped by freezing in liquid nitrogen and lyophilized.

**Peptide purification**

**HPLC system:** Dual-pump HPLC system with a two channel recorder (model 2210), a Hewlett Packard recording integrator (model 3390A, Hewlett packard, PA, USA) and a variable wavelength UV visible model monitor (Model 2151) with 10 min path-length and 10 µl volume flow cell obtained from LKB-producer AB, Bromma, Sweden was used in this study.

**Solvent systems used for purification of peptides by HPLC**

**Solvent system I**

Solvent A : 0.1% TFA in water

Solvent B : 0.1% TFA in 100% acetonitrile.

(Mahoney and Hermodson, 1980; Hermodson and Mahoney, 1983)

**Solvent system II**

Solvent A : 25 mM ammonium acetate pH 6.0

Solvent B : 30% of 50 mM ammonium acetate, pH 6.0 + 70% acetonitrile. (Yang et al., 1981)

The water used in the preparation of solvents was filtered through a 0.25 µm millipore filter and degassed. Aqueous solvents were degassed for
10 min and the organic solvents for 5 min using a vacuum pump. TFA was added to solvent system I after degassing the solvents.

Purification of tryptic/chymotryptic soluble fractions

Gradient programme for tryptic and chymotryptic digests

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow rate (ml/min)</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.7</td>
<td>000</td>
</tr>
<tr>
<td>5</td>
<td>0.7</td>
<td>000</td>
</tr>
<tr>
<td>55</td>
<td>0.7</td>
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</tr>
<tr>
<td>60</td>
<td>0.7</td>
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<tr>
<td>80</td>
<td>0.7</td>
<td>100</td>
</tr>
<tr>
<td>90</td>
<td>0.7</td>
<td>000</td>
</tr>
</tbody>
</table>

Chromatography of tryptic/chymotryptic peptides

The lyophilized soluble fractions of the tryptic digest, chymotryptic digest were dissolved in 1 ml of 0.1% TFA containing 6 M Gm-HCl. Aliquots of 100 μl (approximately 250 μg) were loaded onto Aquapore RP-300 reverse phase column. Aquapore RP-300, a macroporous, less hydrophobic column, reported to be equivalent to C8 column with a pore size of 300 Å diameter. It is known that the yield of peptides particularly the longer ones are usually high in columns with bigger pore size (Hermodson and Mahoney, 1983). The column was washed with 0.1% TFA (solvent A) for five minutes and the bound peptides were eluted using solvent system I at a flow rate of 0.7 ml/min and the elutes were monitored at 225 nm and 1.28 absorbance units of full scale (AUFS). The peak fractions were collected into polypropylene tubes. At the end of the gradient, solvent B was raised to
100% in 5 min and allowed to wash for 20-30 min and the column was reequilibrated with solvent A for 20 min before loading the next aliquot. All the peak fractions were concentrated in a SAVANT speed vac concentrator.

Rechromatography of individual peak fractions

Concentrated individual tryptic/chymotryptic peak fractions were dissolved in 200 μl of 0.1% TFA and loaded on to the same Aquapore RP-300 column and eluted the individual peak fraction with solvent system II.

100 μl fraction of the peptide was injected into the column. The column was washed with 25 mM ammonium acetate, pH 6.0 (Sol.A) for 5 min and the bound peptides were eluted using solvent system II at a flow rate of 0.7 ml/min with different time programmes. The elutes were monitored at 225 nm and around 0.64 AUFS. All the peak fractions of tryptic/chymotryptic peptides were rechromatographed and the peak fractions were collected into a 1.5 ml eppendorf tubes separately, labelled and concentrated in SAVANT speed vac concentrator. They were used in manual/automated sequencing.

Chromatography of Vg and clostripaine digests

Time programme of the gradient for clostripaine and Vg digests.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow rate (ml/min)</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.7</td>
<td>000</td>
</tr>
<tr>
<td>5.00</td>
<td>0.7</td>
<td>000</td>
</tr>
<tr>
<td>105.00</td>
<td>0.7</td>
<td>100</td>
</tr>
<tr>
<td>120.00</td>
<td>0.7</td>
<td>100</td>
</tr>
<tr>
<td>125.00</td>
<td>0.7</td>
<td>000</td>
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</tbody>
</table>
The lyophilized fractions of the clostripaine and V₈ digests were dissolved in 500 μl of 0.1% TFA containing 6 M Gm-HCl. Aliquots of 100 μl (approximately 200 μg) were loaded onto Vidac RP-18 column. The column was washed with 0.1% TFA (solvent A) for five minutes and the bound peptides were eluted using solvent system I as for the above given time programme, monitored at 225 nm and at 0.64 AUFS. The peak fractions were collected into polypropylene tubes. At the end of the gradient, solvent B was raised to 100% and washed the column for 20 min, and equilibrated with solvent A for another 20 min and loaded another aliquot. All the peak fractions were concentrated in a SAVANT speed vac concentrator.

Rechromatography of clostripaine and V₈ protease peptides

Concentrated peak fractions were dissolved in about 200 μl of 0.1% TFA and injected 100 μl and rechromatographed with solvent system II on the same Vidac RP-18 column. The column was washed with 25 mM ammonium acetate, pH 6.0 for 5 min and the bound peptides were eluted using solvent system II at a flow rate of 0.7 ml/min and the elutes were monitored at 225 nm and at 0.32 AUFS. The peak fractions were collected into different 1.5 ml eppendorf tubes and concentrated in SAVANT speed vac concentrator.

Electrophoretic separation of peptides

Tricine gel electrophoresis (Schagger and Van Jagow, 1987) was performed to separate tryptic, clostripaine and V₈ protease digests. The major advantages of the tricine SDS-PAGE were a) resolution of small proteins between 5-20 K at lower acrylamide concentrations; b) the omission of Gly and Urea prevented interference which might occur during subsequent
sequencing; c) the electrophoresis system was reproducible even if ionic strength and pH were changed marginally; d) no-need of casting gradient gels; at high protein concentrations, application of high amounts of protein did not cause over loading effects (Schagger and Van Jagow, 1987).

Stock solutions for Tricine SDS-PAGE

<table>
<thead>
<tr>
<th></th>
<th>Tris (M)</th>
<th>Tricine (M)</th>
<th>pH</th>
<th>SDS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anode buffer</td>
<td>0.2</td>
<td>-</td>
<td>8.90</td>
<td>-</td>
</tr>
<tr>
<td>Cathode buffer</td>
<td>0.1</td>
<td>0.1</td>
<td>8.25</td>
<td>0.1</td>
</tr>
<tr>
<td>Gel buffer</td>
<td>3.0</td>
<td>-</td>
<td>8.45</td>
<td>0.3</td>
</tr>
</tbody>
</table>

1. Acrylamide/bisacrylamide for separating gel (46.5:3 w/v)
   Acrylamide : 46.5 g
   Bisacrylamide : 3.0 g
   Dissolved the chemicals in about 60 ml distilled water and made up to 100 ml, filtered and stored in an amber colour bottle at 4°C.

2. Acrylamide/bisacrylamide for separating gel (48:1.5 w/v)
   Acrylamide : 48.0 g
   Bisacrylamide : 1.5 g
   Dissolved the chemicals in about 60 ml of distilled water and made up to 100 ml, filtered and stored in an amber colour bottle at 4°C.

3. Gel buffer (pH 8.45)
   Tris base : 36.33 g
   SDS : 0.30 g
Dissolved in about 70 ml of distilled water, pH adjusted to 8.45 with 0.1 N HCl and made up to 100 ml with distilled water, stored at room temperature,

4. Anode buffer (pH 8.9)

Tris base : 6.055 g

Dissolved in about 200 ml of distilled water, pH adjusted to 8.9 with 0.1 N HCl and made up to 250 ml, stored at room temperature.

5. Cathode buffer (pH 8.25)

Tris base : 4.844 g
Tricine : 7.168 g
SDS : 0.400 g

Dissolved the chemicals in about 350 ml distilled water, pH adjusted to 8.25, made up to 400 ml, and stored at room temperature.

6. Sample buffer (pH 6.8)

Tris-Cl (50 mM) : 12.1 mg
Glycerol (12%) : 2.4 ml
2-ME (2%) : 0.4 ml
SDS (4%) : 0.4 g

Dissolved the chemicals in 15 ml of distilled water, pH adjusted to 6.8 with dilute HCl and the volume made up to 20 ml with water. A speck of bromophenol blue was added.
<table>
<thead>
<tr>
<th>Gel composition:</th>
<th>Seperating gel</th>
<th>stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>46.5% Acrylamide/3% bis-acrylamide</td>
<td>10 ml</td>
<td>-</td>
</tr>
<tr>
<td>48% Acrylamide/1.5% bis-acrylamide</td>
<td>-</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Gel buffer</td>
<td>10 ml</td>
<td>1.55 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>4 g (3.17 ml)</td>
<td>-</td>
</tr>
<tr>
<td>Water</td>
<td>6.0 ml</td>
<td>4.2 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>50 μl</td>
<td>10 μl</td>
</tr>
<tr>
<td>APS 10%</td>
<td>150 μl</td>
<td>50 μl</td>
</tr>
</tbody>
</table>

The separating gel was poured and allowed to polymerize between two uniform glass plates sealed at the bottom with agar. The spacers and comb used were of 1 mm thickness. The stacking gel was overlaid on the separating gel. About 1 mg of tryptic, clostripaine and V₈ protease digests were dissolved in 20 μl of distilled water and 180 μl of sample buffer, digested for 5 min in boiling water, cooled and about 50 μl of each of the samples were loaded into the wells. The electrophoresis was performed at room temperature. The electrophoresis was started at 30 V (approximately 20 mA Current). After the sample completely entered the stacking gel, the voltage was increased to 90 V. The electrophoresis was continued 6 h after the dye migrated out (approximately 28 to 30 h). Half of the gel was stained with 0.2% coomassie brilliant blue R 250 and later destained, and the other unstained half used in electrotransfer.
Activation of PVDF membrane

The membrane cut to the required size (slightly larger than the gel) was soaked in methanol (HPLC grade) for 5 min before use. After wetting in methanol, the membrane was soaked in transfer buffer (25 mM tris-glycine, pH 8.3) containing 15% methanol for 10 min.

Electrotransfer of peptides

Immediately after the gel electrophoresis, the unstained gel was rinsed in transfer buffer for 15 min. Semi-dry electroblotting was carried out in Novablot (Pharmacia). The surface of the bottom graphite electrode was moistened with transfer buffer. Three strips of whatman 3 filter papers were soaked in transfer buffer was placed on the electrode. The size of the filter paper was half cm larger on each side of the membrane. The membranes were carefully placed on the paper strips. The gel was placed on the membrane. During these manipulations, air bubbles between the layers were avoided. Care was taken that the gel was at the centre of the apparatus, where the electric field was uniform. The membrane was on the anode side of the chamber. The transfer was carried out for 3 h using a current of 1 mA/cm² of filter paper.

Detection of peptides on membranes

After transfer, the membrane was stained for 5 min with 0.5% coomassie brilliant blue R-250 in 50% methanol and destained with 50% methanol. Care was taken not to use acetic acid during staining or destaining the membrane. The destained membrane was extensively washed with water (5 changes), air dried and stored at -20°C.
Methods for sequencing of peptides

1. Manual DABITC/PITC double coupling method

A few of the shorter tryptic peptides were sequenced by DABITC/PITC double coupling method of manual sequencing (Chang et al., 1978). The method of sequencing is similar, which was discussed at N-terminal analysis of coat protein. The separation of a mixture of standard DABTHs by two dimensional TLC are shown in Fig. 3. The amino acid derivatives obtained at the end of each cycle are identified by relating their position to the internal marker(e), the thiourea derivative (U) and the excess reagent (DABITC). Ser, Thr, and Lys appeared as multiple spots on the polyamide sheets. (Fig. 3).

2. Automated gas phase sequencing

Tryptic/chymotryptic peptides were sequenced on an automated gas phase protein sequenator PSQ-1 from Shimadzu. This sequenator carried out Edman degradation by supplying a gaseous reagent for the coupling and cleavage reactions. The peptides are (approximately 100 p mol) dissolved in 5% acetic acid was spotted on TFA treated glass fibre disc coated with polybrene. Coupling was by moistening the glass fibre disc with PITC (R1), in the presence of trimethyl amine (R2). Excess reagents and by-products were washed with n-heptane (S1) and ethyl acetate (S2). The cleavage reaction was carried out with gaseous TFA to form an anilinothiozolinol (ATZ) derivative. Both the coupling and cleavage reactions were made in a temperature controlled reaction chamber. The free ATZ amino acid was extracted into the conversion flask by n-butyl chloride (S3). The ATZ amino acid was converted to the more stable PTH-amino acid by reaction with 25%
TFA (R₄). The PTH-amino acid was dissolved in acetonitrile (S₄) and injected into the HPLC. The residual PTH-amino acid was collected in a sample tube by a fraction collector. The PTH-amino acid was separated by RP-HPLC. The PTH-amino acid was identified and quantified. A standard PTH-amino acid profile is shown in Fig. 4. A flow diagram of the steps involved in automated sequencing is shown in Fig. 5.

The automated method for determining amino acid sequences of proteins and peptides offers several major advantages over previous procedure. They are a) the sample amount required was about 100 p moles b) volume of reagents required was low, c) the time required for each cycle was approximately 50 min and d) ideally suited for sequencing of longer fragments and hence the elucidation of the entire sequence required smaller number of fragments than do conventional methods.
Fig. 4: Separation of PTH amino acid standards on the automated protein sequenator PSQ-I.
Fig. 5: Diagram of the reactions occurring in the gas phase sequenator, PSQ-1.
LOCAL LESION STRAIN OF TRSV

COLLECTION, ESTABLISHMENT AND MAINTENANCE

A local lesion isolate of TRSV naturally infecting brinjal was collected from farmers fields around Tirupati (Sastry and Nayudu, 1976). The same virus isolate was established in the wiremesh house and maintained on cowpea (Vigna unguiculata), plants which develop brown necrotic local lesions on inoculated primary leaves. Single lesion isolate was passed five successive generations and maintained on local cowpea varieties. For all experiments the virus was maintained on 7-day old cowpea plants which develop necrotic local lesions on the inoculated primary leaves and in some cases systemic necrotic lesions on stems, petioles and trifoliate leaves.

PURIFICATION OF TRSV

Initially to purify the virus the author followed the procedure of Sastry and Nayudu (1976), but found proteolytic degradation of the virus. So, to purify the virus intact, several buffer systems were used and successfully purified the virus intact.
Purification of the virus

100 g of 48 h TRSV infected cowpea primary leaves showing necrotic local lesions were harvested, washed in tap water followed by distilled water. The leaf material was macerated (2 ml/g leaf material) in an electric blender in cold bicarbonate buffer, pH 9.0, 0.05 M, containing 0.02 M 2-ME, 0.01% MgCl₂, 20% glycerol and 1 mM PMSF. The sap was strained through muslin cloth. To the sap chloroform to 10% was added, emulsified for 30 min, subjected to centrifugation at 10,000 rpm for 30 min in Sorvall RC5C centrifuge. The sediment was discarded, the supernatant was centrifuged at 27,000 rpm for 2 h in AH 629 rotor in OTD Combi ultracentrifuge (Sorvall). The pellets were suspended in 12 ml of 0.05 M phosphate buffer, pH 7.2 containing 1 mM PMSF. The partially purified virus was subjected to low speed centrifugation at 8,000 rpm for 10 min and layered onto preformed 10 to 40% linear sucrose density gradient columns in 0.05 M KPO₄ buffer, pH 7.0 (as described in SMV purification) and centrifuged at 26,000 rpm for 2 h. After centrifugation the tubes were examined in dark by passing a beam of light vertically. The position of the light scattering virus zones were noted and collected with a pasteur pipette. The virus zone was diluted (1:1) with KPO₄ buffer pH 7.2, 0.05 M and subjected to ultracentrifugation at 35,000 rpm for 2 h in T 865 fixed angle rotor. The final pellets were dissolved in minimal volume of KPO₄ buffer pH 7.2 containing 1 mM PMSF and 10 mM MgCl₂.

Quantitation of purified virus

Purified virus was quantitated spectrophotometrically by measuring the absorbance at 260 nm assuming a specific extinction coefficient of 10 cm² mg⁻¹ (uncorrected for light scattering).
UV absorption

The purified virus (middle, bottom and unfractionated) samples were scanned from 200-320 nm in Shimadzu UV-visible recording spectrophotometer, (UV-260) to calculate $A_{260}/280$ ratio and $A_{\text{max/min}}$ ratios.

ELECTRON MICROSCOPY

Purified virus (sucrose density gradient zone, middle and bottom components separately) preparation was used for particle morphology studies. EM studies were performed similar to that of SMV-EM section, except that the preparations were stained with 1% aqueous uranyl acetate. The grids were observed in Phillips 301 transmission electron microscope, operating at 60 kV.

SEROLOGY

(a) Production of polyclonal antiserum

Polyclonal antibodies were produced against purified virus (sucrose gradient zone, unfractionated). The procedure was same as described under SMV antiserum production section.

(b) Cross absorption of antiserum

One ml of antiserum was mixed with 2.6 mg of healthy proteins. After 2 h incubation at 37°C, the antiserum samples were centrifuged at 4,000 rpm for 15 min. The supernatant was collected and used for other serological studies.

(c) Antiserum/Antigen titres

Direct antigen coating ELISA (DAC-ELISA) (Hobbs et al., 1987, Mowat and Dawson, 1987) was used to determine the antiserum/antigen titres.
Antiserum titre

To determine the antibody dilutions the microtitre plates were coated with 1 µg, 500 ng, 250 ng and 125 ng/ml of purified virus (200 µl/ml). The plates were incubated in a humid box at 37°C. After 2 h the plates were washed thrice with PBS-T at 3 min interval. Then the plates were coated with different dilutions of antiserum (5,000 to 3,200,000) (200 µl/well) and incubated at 37°C for 2 h. The plates were washed thrice at 3 min interval. Then the plates were coated with different dilutions of antiserum (5,000 to 3,200,000) (200 µl/well) and incubated at 37°C for 2 h. The plates were washed thrice at 3 min interval. Then 200 µl/well of protein A labeled with alkaline phosphatase (0.25 units/ml in PBS-TPO) was added and incubated at 37°C. After 2 h the plates were washed thrice with PBS-T at 3 min interval. Finally freshly prepared p-nitrophenyl phosphate (PNP) was added (200 µl/well). The appearance of yellow colour was observed, the reaction was terminated by adding 50 µl of 3 M NaOH. The results were read at 405 nm in a Vmax Microplate Reader.

Antigen titre

To determine antigen dilutions the plates were initially coated with different dilutions (125 ng to 2,000 ng/ml: 200 µl/ml) of purified virus. Then plates were incubated in a humid box at 37°C for 2 h. Then the plates were coated with 1:5,000, 1:10,000, 1:20,000/ml dilutions of antiserum. In the third step the plates were coated with 200 µl/well of protein A labeled with alkaline phosphatase and finally developed with PNP. The reactions were terminated with 50 µl of 3 M NaOH and read at 405 nm in a Vmax Microplate reader.

Wells filled with antigen extraction buffer were used as negative controls (blank) of the reaction instead of leaf extracts. Samples with absorbance values two times greater than buffer controls were considered as positives.
Serological relationships

DAC-ELISA was used to evaluate serological relationships of TRSV to other reported nepoviruses. The procedure for DAC-ELISA was the same as described under SMV-serological relationship section.

Antisera used were TRSV (Gladiolus strain- from R.I.B. Francki) TRSV-Stace-Smith, TRSV-'G' and 'F' isolates from Squash (from O.W. Barnett), TRSV-Cherry (from R.I. Hamilton), TRSV peruvian, arabis mosaic virus, tomato black ring virus, tomato ringspot virus (from Danish government Institute of seed pathology for developing countries, Denmark).

VIRUS PROTEIN

Virus coat protein Mr was determined by using purified virus preparations as described under SMV section. The gels were photographed and the distance migration of virus protein and marker proteins (Sigma MW-SDS-70) Bovine albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa), B-lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa) were recorded. The Mr of virus protein was calculated from marker proteins as described under SMV coat protein section.

VIRUS NUCLEIC ACID

a. Isolation from purified virus

The virus nucleic acid was isolated from purified virus preparations (sucrose gradient zone) by the method of Sambrook et al. (1989), as described under SMV nucleic acid section.
b. Nucleic acid type

The type of nucleic acid present in the virus was determined by diphenylamine and orcinol tests as described in SMV section.

c. Infectivity assay

The purified viral RNA was checked for its infectivity on \textit{V. unguiculata} primary leaves. The purified viral RNA was suspended in sterile K \textit{PO}_4 buffer, pH 7.0, 0.05 M to get 10, 25, 50 and 100 \mu g/ml and containing 0.5\% bentonite was inoculated on to carborundum dusted \textit{V. unguiculata} plants (kept in dark for 24 h prior to inoculation). After inoculation, the leaves were washed with sterile water. The inoculated plants were observed for 48 h. The number of local lesions produced were recorded.

\textit{Agarose gel electrophoresis of viral RNA}

Horizontal slab gel electrophoresis was done as described in SMV-RNA section. TMV-RNA and \textit{E.coli} ribosomal RNA were used as marker RNAs.

\textit{Double-stranded RNA isolation}

dsRNA was isolated according to the procedure of Diaz Ruiz and Kaper (1978) with slight modifications.

Ten grams of 24 h TRSV post inoculated cowpea primary leaves were collected, washed in tap water followed by distilled water and macerated in 20 ml of 2 x STE buffer (Tris 0.1 M; NaCl 0.6 M, EDTA 0.03 M; pH 8.0) containing 0.2\% 2-ME and 200 mg SDS. To the homogenate 1:1 volume (v/v)
of Kirby's mixture (as described in SMV-dsRNA section) and chloroform were added, emulsified for 30 min and centrifuged at 10,000 rpm for 20 min, and isolated the dsRNA as described in SMV-dsRNA section.

Similar to the infected tissues, dsRNAs were prepared from healthy cowpea primary leaves.

**Agarose gel electrophoresis of dsRNA and Mr determination**

dsRNAs were electrophoresed as described in SMV dsRNA section. CMV dsRNAs (Kiranmai et al., 1992) and λ DNA Hind III digest markers were used as standards.

**Nuclease treatments**

dsRNA was subjected to pancreatic RNAse A as described in SMV dsRNA section and the gels were photographed.

**Virus coat protein isolation**

TRSV native coat protein was isolated by 66% cold glacial acetic acid method of Tsugita and Hirashima (1972) as described in SMV coat protein section.

**Reduction and carboxymethylation of TRSV coat protein**

The isolated coat protein was reduced with DTT and carboxymethylated with iodoacetic acid as described in SMV coat protein section.
ACID HYDROLYSIS AND AMINO ACID COMPOSITION

The reduced and carboxymethylated TRSV coat protein was hydrolysed and the amino acid composition was determined. The results were expressed as number of residues of each amino acid per polypeptide Mr as 60 kDa: Val, Ileu and Leu were determined based on values at 72 h hydrolysis, as described in SMV-amino acid composition section.