Partial Cleavage of Sweet Potato Feathery Mottle Virus Coat Protein Subunit by an Enzyme in Extracts of Infected Symptomless Leaves

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SUMMARY

The coat protein of particles of sweet potato feathery mottle potyvirus (SPFMV) extracted from Ipomoea spp. migrated in SDS–PAGE mainly as bands of Mr 38000 (38K), 36K, 32K and 30K. Trypsin treatment of the particles resulted in the appearance of only one 30K polypeptide. The inclusion of protease inhibitors in the extraction procedure did not alter the heterogeneity of SPFMV coat protein. A partially purified fraction of extracts from recovering, symptomless, but not from healthy leaves of I. nil had a proteolytic activity similar to that of trypsin. Amino acid sequencing showed that the trypsin-cleaved 30K polypeptide had some sequence homology with other potyvirus coat proteins. The site at which the Ipomoea extract cleaved the protein was five amino acids nearer the N terminus than the trypsin cleavage site.

INTRODUCTION

Preparations of the coat proteins of potyviruses are often heterogeneous in size and in the relative concentrations of the various polypeptides (Moghal & Francki, 1976; Hiebert et al., 1984; Moyer & Cali, 1985; Stein et al., 1986; Cohen et al., 1988) and sweet potato feathery mottle potyvirus (SPFMV) is typical in that coat protein from purified virus particles comprises an Mr 38 000 (38K) polypeptide and several minor smaller polypeptides (Moyer & Cali, 1985; Cohen et al., 1988). Although SPFMV-infected sweet potato has some symptomless leaves, Ipomoea nil exhibits more regular symptomless recovery and was therefore selected for this study. The aim of this research was to correlate the emergence of proteolytic activity with symptomless recovery.

METHODS

Plant material. Cotyledons of I. nil seedlings were mechanically inoculated with extracts from SPFMV-infected I. batatas, or 0.05 mg/ml of SPFMV purified from infected I. batatas (Cohen et al., 1988).

The first four or five true leaves emerging within 10 days after inoculation usually showed typical SPFMV symptoms; upper leaves that emerged later were symptomless. The relative concentrations of virus capsid protein in the leaves were estimated by ELISA (Clark & Adams, 1977).

Virus preparation. SPFMV was purified from infected I. batatas leaves as described by Cohen et al. (1988) except that when analysing the effect of protease inhibitors on the viral coat cleavage, a partial purification procedure utilizing polyethylene glycol was used (Alper et al., 1984).

Protease inhibitors. Soybean trypsin inhibitor, aprotinin and PMSF were obtained from Sigma; TLCK was from Boehringer-Mannheim. PMSF was dissolved in 100% methanol, all the other inhibitors were water-soluble. The final concentrations of the protease inhibitors used were 174 µg/ml (1 mM) PMSF, 50 µg/ml for soybean trypsin inhibitor and aprotinin and 100 µg/ml for TLCK.

Extraction of proteolytic activity from I. nil leaves. In each preparation, 3 to 10 g leaves was used. Leaves were homogenized for 30 s with a high speed rotor homogenizer in two volumes (v/w) of 0.5 M-sodium borate buffer pH 8.0, containing 10 mm-disodium EDTA and 0.675% 2-mercaptoethanol, one volume of chloroform and one volume of carbon tetrachloride. The aqueous phase was recovered following 10 min centrifugation at 8000 g in a refrigerated centrifuge and brought to 30% saturation with solid ammonium sulphate by stirring in the cold for 15 min. The ammonium sulphate precipitate was recovered by 10 min centrifugation at 12000 g and suspended in...
The fraction soluble in 30% saturated ammonium sulphate was brought to 60% saturation and the precipitate was treated as above. All the above procedures were carried out at 4°C.

**Peptide mapping.** Acrylamide gels were prepared as described by Cleveland et al. (1977). Digestion was with *Staphylococcus aureus* V8 protease (Miles Laboratories) at a concentration of approximately 0·25 μg enzyme per 5 μg virus protein.

**Protein determination.** Protein determination was performed with Coomassie Brilliant Blue (CBB) on filter paper according to Ghosh et al. (1988) using bovine serum albumin (BSA) as a standard. The dye was eluted from the spots in 300 μl of 1% SDS by gentle shaking for 1 h or more. Protein concentration was determined by absorbance at 570 nm in an automatic ELISA reader.

**Analysis of proteolytic activity.** SPFMV, BSA and bovine casein were used as standard substrates. Products of proteolysis were analysed by SDS—PAGE separation in 8 to 20% gradient polyacrylamide gels. SPFMV (3 to 5 μg) was incubated with 0·25 μg bovine trypsin (Allison et al., 1985; Dougherty et al., 1985). Alternatively, 2 to 6 μg *I. nil* extract in 0·05 m-sodium borate (pH 8·0) was used. Incubation was in 50 μl final volume and 37 °C. Reaction was stopped by boiling for 3 min with 0·5 volume of 0·06 M-Tris-NCI, pH 6·8 containing 3% SDS, 5% 2-mercaptoethanol, 10% glycerol and a few grains of bromophenol blue (Laemmli, 1970).

**Serological assays.** Immunoblotting was performed according to Towbin et al. (1979) with minor modifications. For visualization, peroxidase-conjugated goat anti-rabbit IgG, 3-amino-9-ethyl carbazole and H2O2 were used. The relative concentration of virus capsid protein in the leaves was estimated by ELISA (Clark & Adams, 1977) utilizing rabbit antiserum against purified SPFMV prepared in our laboratory. Our antiserum was compared to samples of antiserum obtained from Dr J. W. Moyer, University of North Carolina, Raleigh, N.C., U.S.A., Dr R. N. Campbell, University of California, Davis, Ca., U.S.A. and Dr C. A. Clark, Louisiana State University, Baton Rouge, La., U.S.A.

**Amino acid sequencing.** Trypsin-treated SPFMV was purified by centrifugation to equilibrium in a CsCl density gradient, dialysed, concentrated and used for amino acid sequencing with an Applied Microsystems 470 A protein sequencer with liquid pulse used according to the manufacturer's instructions. SPFMV protein cleaved by the *Ipomoea* enzyme was transferred to Immobilon membrane (PVDF, Millipore), stained with CBB and the membrane-bound 30K band was used for N terminus sequencing (Matsudaira, 1987).

**RESULTS**

**Proteolytic activity of *I. nil* leaf extracts**

*I. nil* leaves were examined for the presence of SPFMV. ELISA showed that leaf extracts from symptomless leaves contained little virus (about four times background) whereas leaves with symptoms gave values 70- to 80-fold higher than background. Crude extracts from recovered *I. nil* leaves showed more proteolytic activity than extracts from healthy leaves. However, the presence of total soluble leaf proteins masked the specific proteolytic activity; therefore, a partial purification procedure was adopted.

Since it was assumed that the specific proteolytic activity is associated with the virus, organic phase partition was included in the fractionation procedure to resemble the virus purification procedure. The water-soluble fraction showed specific proteolytic activity. Extracts from recovered leaves cleaved the SPFMV coat subunit, while those from healthy leaves showed little activity. The material precipitating from this fraction at 0 to 30% ammonium sulphate saturation (1·76 g/10 ml) had a high level of proteolytic activity but contained only a small quantity of protein and was therefore suspended in 1/10 of the original volume to give 0·2 to 0·3 mg protein/ml. The activity in this fraction was stable for at least 3 months at −80 °C. The precipitate from 30 to 60% ammonium sulphate saturation contained most of the soluble proteins left in the aqueous phase, but showed very little proteolytic activity.

Enzyme activity decreased markedly if the concentration of 2-mercaptoethanol was 0·1% or less. None of the four protease inhibitors included in the purification procedure inhibited cleavage of the SPFMV coat polypeptide.

Fig. 1 shows the cleavage of SPFMV coat polypeptides by the 0 to 30% ammonium sulphate saturation fraction from recovered *I. nil* leaves but not by that from healthy leaves. Under these conditions the extract from recovered leaves cleaved most of the SPFMV coat polypeptides to the size of the smallest one (30K).

Fig. 2 shows a comparison between *I. nil* proteolytic activity and trypsin. Both treatments yielded mainly a 30K product from the SPFMV coat protein. When the extracts from *I. nil*
Cleavage of SPFMV coat subunit

Fig. 1. Time course of SPFMV coat protein subunit cleavage by *I. nil* extract. Approximately 5 μg of SPFMV was incubated at 37 °C with 2 μg of protein in an enzyme extract prepared from *I. nil* leaves, recovered or healthy, for various incubation times. Half of the reaction mixture was resolved by SDS-PAGE on an 8 to 20% polyacrylamide gradient gel and stained with CBB. Lane 1 shows Bio-Rad M, markers with the apparent Mr (× 10−3) indicated. Lanes 2 and 3 represent zero incubation time for SPFMV with extracts of recovered and healthy leaves, respectively. Lanes 4 and 5 are after 30 min incubation, lanes 6 and 7 after 60 min, and lane 8 is a sample of denatured, untreated SPFMV. The Mr values of the four SPFMV coat polypeptides are indicated on the right side of the figure.

leaves were incubated with bovine casein, the extract from recovered leaves digested the casein but that from the healthy leaves did not. In contrast *I. nil* extracts had no effect on BSA.

Immunoblotting of SPFMV polypeptides separated by SDS–PAGE and electrotransferred to nitrocellulose membranes was done with rabbit anti-SPFMV antibodies. Antibodies prepared in this laboratory against purified intact SPFMV particles, as well as antisera from other laboratories, reacted with all the polypeptides obtained from the disrupted SPFMV virion preparation and cross-reacted with the polypeptides resulting from trypsin treatment. Partial digestion of the SPFMV 30K product from SDS–PAGE by *S. aureus* V8 protease showed that the 30K polypeptides obtained from an untreated virus preparation, or from virus particles treated with either *I. nil* extract or trypsin, yielded peptides of the same sizes (Fig. 3).

Amino acid sequencing

The sequence of 10 amino acids from the N-terminal site of trypsin-treated coat protein is shown in Table 1 and compared to the known sequences of the N termini of trypsin-treated coat proteins of other potyviruses. The SPFMV coat protein sequence shares eight or seven of these 10 amino acids with each of the five potyviruses studied (Table 1). Up to 15 amino acids were sequenced from the N terminus of the 30K polypeptide from untreated SPFMV particles. Table 2 shows the N-terminal sequence of the 30K polypeptide made by treatment with *Ipomoea* extract. The cleavage site was located five amino acids from the trypsin cleavage site toward the natural N terminus of SPFMV coat protein. Four of these five amino acids were the same as those in the corresponding sequence of tobacco etch potyvirus coat protein (Allison et al., 1985). One amino acid could not be unequivocally determined and was therefore omitted (Table 2). There was one amino acid shift in some preparations with Asn before the Ala at the cleaved site indicating a non-homogeneously cleaving enzyme.
Fig. 2. Comparison of the products generated when SPFMV is treated with either *I. nil* extract or trypsin. Coat polypeptide products were resolved by SDS–PAGE on a 8 to 20% polyacrylamide gradient gel. Lane 1, Bio-Rad *M, markers*; lane 2, 2.5 μg SPFMV treated with 1 μg of proteins from an extract of a recovered *I. nil* leaf for 60 min at 37 °C; lane 3, 2.5 μg of SPFMV treated with 0.25 μg trypsin for 60 min at 37 °C; lane 4, the same amount of SPFMV but without treatment.

Table 1. Homologies at the N termini of trypsin-treated potyvirus coat proteins

<table>
<thead>
<tr>
<th>Virus</th>
<th>Number of amino acids from N terminus of the coat</th>
<th>Amino acid sequence*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPFMV</td>
<td>+ 70†</td>
<td>D V N V G T V G T F</td>
<td>Allison <em>et al.</em> (1985)</td>
</tr>
<tr>
<td>Tobacco etch virus</td>
<td>+ 29</td>
<td>D V N A G T S G T F</td>
<td>Domier <em>et al.</em> (1987)</td>
</tr>
<tr>
<td>Tobacco vein mottle virus</td>
<td>+ 30</td>
<td>D V N A G T S G T F</td>
<td>Shukla <em>et al.</em> (1986); Rosner &amp; Raccah (1988)</td>
</tr>
<tr>
<td>Potato virus Y</td>
<td>+ 32</td>
<td>D V N A G T S G T H</td>
<td>Dougherty <em>et al.</em> (1985)</td>
</tr>
<tr>
<td>Johnsongrass mosaic virus</td>
<td>+ 69</td>
<td>D V D V G S T G T F</td>
<td></td>
</tr>
</tbody>
</table>

* The amino acids that are different from the SPFMV sequence are underlined.
† Estimated from *M*.
Cleavage of SPFMV coat subunit

Fig. 3. Peptide mapping analysis of the 30K polypeptide from SPFMV. The 30K polypeptide was excised from acrylamide gels. Lanes 1 and 4, 30K from untreated SPFMV; lanes 2 and 5, 30K from SPFMV cleaved with I. nil recovered leaf extract; lanes 3 and 6, 30K of trypsin-treated SPFMV. These samples were mixed with 0.25 µg S. aureus V8 protease (lanes 1 to 3) or untreated (lanes 4, 5, 6); lane 7 contained 0.25 µg S. aureus V8 protease; lane 8 contained Bio-Rad markers which were partially cleaved by overflow of the S. aureus protease. V8 protease polypeptides are indicated by the two adjacent bars. The bars next to lane 1 indicate the four common peptides produced from the 30K polypeptides.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cleavage site*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td></td>
</tr>
<tr>
<td>SPFMV</td>
<td>A S K D - D V N V G T V G T F</td>
</tr>
<tr>
<td>TEV</td>
<td>A S K D R D V N A G T S G T F</td>
</tr>
</tbody>
</table>

* The site of cleavage by trypsin (Table 1) is indicated by the arrow. The SPFMV sequence is compared to that of TEV coat protein. The rule (−) indicates an amino acid that differed in the various sequencing experiments and thus could not be unequivocally determined. The underlined amino acids are those which differ from the SPFMV sequence.

The proteolytic cleavage of a segment from the viral coat protein does not abolish virus infectivity, as judged in mechanical inoculation tests, but may affect translocation by removing some attachment sites from the coat. Mechanical infectivity rates of trypsin-treated and untreated SPFMV were the same, although the appearance of symptoms after inoculation with trypsin-treated SPFMV was slower than in control plants. When recovered I. nil plants were pruned the new growth showed symptoms that then disappeared in the upper leaves much as after the initial inoculation.

DISCUSSION

SPFMV preparations contain four polypeptide products of the coat protein subunit of which three are smaller in size than the full-length coat protein (Moyer & Cali, 1985; Cohen et al., 1988). The proportion of shorter polypeptides varied with time in partially purified
preparations. However, following CsCl density gradient separation, the virus preparation was stable for at least a year (Cohen et al., 1988). These observations indicated a close association between the virus and a protease that cleaves segments from the coat protein, leaving its core intact. This proteolytic activity can be dissociated from the virus by high salt concentrations and thus prevents further cleavage of the protein. Such cleavage may therefore take place only during extraction (Hiebert et al., 1984). The inclusion of protease inhibitors in the extraction buffer did not inhibit the coat cleavage indicating that it may take place in vivo. Such activity was sought in healthy and symptomless recovering I. nil leaves, assuming that SPFMV infection stimulates a specific proteolytic activity.

To learn more of the specificity of this proteolytic activity, digestion of two animal proteins, BSA and casein, was tried. BSA was insensitive to both I. nil extracts, but the phosphoprotein casein was digested to a much greater extent by the fractionated extract from recovered leaves. The use of animal proteins for the study of plant proteases was reported recently to have the advantage of preventing cross-contamination (Felicioli et al., 1988). Trypsin cleavage resulted in a 30K product similar to the one produced by Ipomoea sp. The results indicate the existence of a protease-inaccessible core and proteolysis-sensitive tail of about 70 amino acids (Fig. 2). The sequence of 10 amino acids at the N terminus of the trypsin-treated core protein revealed a sequence very like that of several potyvirus coat proteins (Table 1). Furthermore the trypsin-resistant core of the coat proteins of these potyviruses were similar in size but the proteolysis-sensitive tails differed in length (Shukla et al., 1988). The SPFMV core 30K polypeptide produced by the Ipomoea enzyme showed a cleavage site five amino acids nearer the N terminus from the trypsin cleavage site but this difference in size was too small to be detectable by SDS-PAGE.

This work demonstrates the emergence of a proteolytic activity that cleaves the SPFMV coat protein at the N-terminal tail adjacent to the trypsin cleavage site in I. nil leaves which have recovered from viral infection. No such activity was detected in healthy leaves. A search for proteolytic activity in other plants and its effect on natural spread is in progress.

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REFERENCES


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