Partial Degradation of the Protein in Tobacco Rattle Virus during Storage

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SUMMARY

When purified preparations of the CAM isolate of tobacco rattle virus were stored the protein in the virus particles underwent limited proteolysis. Using polyacrylamide gel electrophoresis, a change was observed in the mol. wt. of the virus polypeptide from 28,500 to 23,500, and in some instances a possible intermediate of 26,000 mol. wt. was detected. Polypeptides of mol. wt. 13,500, 11,500 and 9,000 were also detected after prolonged storage. Virus protein was not degraded when virus preparations containing sodium azide or antibiotics were stored, but it was degraded in the presence of azide when incubated with the supernatant fluid obtained by centrifuging preparations of virus which had been stored for several months without bacteriostatic additive. The proteolysis was probably caused by microbial exoenzyme(s). The conversion of polypeptide from mol. wt. 28,500 to 23,500 did not affect sedimentation coefficient, behaviour when centrifuged to equilibrium in caesium chloride, appearance in the electron microscope, serological reactivity, infectivity, heat stability or resistance of infectivity to pancreatic ribonuclease.

Purified virus was not affected by treatment with trypsin or chymotrypsin whereas the virus polypeptide was degraded by pronase and by papain. Papain had effects similar but not identical to those of storage. These enzyme treatments did not greatly affect infectivity or alter the appearance of virus particles in the electron microscope.

Isolate PRN was affected by storage and by papain treatment in the same way as isolate CAM, but more slowly.

INTRODUCTION

Tobacco rattle virus (TRV) is one of the better-characterized plant viruses and there are estimates of the mol. wt. of the coat protein of several isolates. Estimates obtained using electrophoresis in polyacrylamide gels of protein denatured with SDS have been in the range 27,000 to 29,000 (Lesnaw & Reichmann, 1970; Carpenter, Cook & Gibbs, 1971; Cooper & Mayo, 1972) whereas those derived from amino acid analyses have been consistently lower (Offord & Harris, 1965; Miki & Okada, 1970; Semancik, 1970). When we examined the protein from virus particles, using polyacrylamide gel electrophoresis, different estimates of mol. wt. were obtained when the same preparation was tested on successive occasions. In this paper we show how these differences can arise.
METHODS

Virus purification. The CAM isolate (R/1:2:5/5+0:7/5:E/E:S/) and the PRN isolate (R/1:2:5/5+1/5:E/E:S/Ne) of TRV (Cooper & Mayo, 1972) were purified from systemically infected leaves of Nicotiana clevelandii Gray. Infected leaves were minced with an equal weight of 0·006 M-phosphate buffer (Sörenson's, sodium and potassium salts, pH 7) and the expressed sap was stored at −15 °C for at least a month. Frozen sap was thawed overnight and virus was purified by three or four cycles of differential centrifuging. Virus pellets were resuspended in 0·03 M-phosphate buffer (pH 7) and stored at 4 to 6 °C. Except where otherwise indicated, the results quoted are for isolate CAM.

Infectivity assay. Virus infectivity was determined by lesion counts following manual inoculation of the expanded leaves of Chenopodium amaranticolor Coste & Reyn. with inocula prepared in 0·02 M-phosphate buffer and mixed with Celite (Johns-Manville Ltd.). Treatments were inoculated to comparable leaves and lesions were counted 5 days after inoculation.

Polyacrylamide gel electrophoresis. Protein samples were prepared by heating virus (1 to 2 mg/ml) in 1 % SDS + 1 % 2-mercaptoethanol in 0·01 M-sodium phosphate (pH 7) for 90 s in boiling water. Unless otherwise stated, electrophoresis was in 10 % acrylamide + 0·25 % methylene bis-acrylamide gels in 0·1 M-sodium phosphate + 0·1 % SDS, with 0·1 % 2-mercaptoethanol in the buffer reservoirs. Current (4 V/cm, 8 mA/gel) was passed for 60 min through rectangular cross-section (4 mm x 8 mm) gels and the samples were then electrophoresed for 4 to 5 h. Alternatively, the system described by Swank & Munkres (1971) was used: 12·5 % acrylamide + 1·25 % methylene bis-acrylamide gels were prepared containing 0·1 M-tris-phosphate (pH 6·8) + 8 M-urea + 0·1 % SDS. Samples prepared as above were run using this buffer + 0·1 % 2-mercaptoethanol. Electrophoresis was at 4 V/cm for 16 to 18 h. Protein was detected by staining with 0·25 % Coomassie blue in methanol: water:acetic acid (5:5:1, v/v/v) and the stained gels were scanned using a microdensitometer (Joyce Loebl, Mk. III C).

Mol. wts. were estimated by comparing the mobilities of bands of virus protein with the mobility of proteins of known mol. wt. In 10 % gels, bovine serum albumin (Calbiochem) (66000), ovalbumin (45000), carbonic anhydrase (29000), chymotrypsinogen (Sigma) (25700) and tobacco mosaic virus (TMV) protein (17400) were used as standards. In 12·5 % gels containing urea, a linear relation between mol. wt. and mobility was only valid for proteins of low mol. wt. and TMV protein, cytochrome C (Koch–Light) (12000) and insulin (Sigma) (5700) were used as markers in this system. Mol. wts. were taken from Dunker & Rueckert (1969) or Weber & Osborn (1969).

Enzyme incubation. Trypsin (Sigma, Type XI; DCC-treated), α-chymotrypsin (Sigma, Type II), papain (Sigma, 2 x crystallized) and pronase (Calbiochem) were used. Virus was made 5 to 10 mg/ml in 0·015 M-sodium phosphate (pH 7·0) and warmed to 30 °C. Freshly prepared solutions of enzymes (1 mg/ml in 0·015 M-sodium phosphate, pH 7) were added to bring the final enzyme concentration in the virus suspensions to 0·1 mg/ml; 0·01 % 2-mercaptoethanol was added to papain solutions. Virus–enzyme mixtures were incubated at 30 °C, and at intervals samples were removed and 0·01 M-sodium phosphate added to give a virus concentration of 1 mg/ml in a solution containing 1 % SDS and 1 % 2-mercaptoethanol; the samples were then heated at 100 °C for 90 s and electrophoresed in gels. When preparing samples for 12·5 % gels containing urea, the final virus concentration was 2 to 3 mg/ml.

Electron microscopy. Virus preparations were diluted in water and stained on carbon
coated grids with sodium phosphotungstate, pH 6·8, or uranyl formate in sodium hydroxide (Barnett & Murant, 1970). Grids were examined in a Siemens Elmiskop 1 A at × 40000 and 80 kV. Particle dimensions were measured as described by Cooper & Mayo (1972).

Analytical centrifugation. Virus was centrifuged to equilibrium in caesium chloride at 30000 rev/min for 18 h (22 to 27 °C) in the AnD rotor of a Beckman Model E ultracentrifuge. Buoyant densities were determined by measuring Schlieren diagrams (Szybalski & Szybalski, 1971). Sedimentation coefficients \( (s_{20,w}) \) were calculated from Schlieren diagrams of virus sedimenting in 0·03 M-phosphate buffer, pH 7·3, using the same rotor.

Serological tests. Antiserum was prepared and precipitin tests were made as described by Cooper & Mayo (1972).

RESULTS

Protein size changes during storage

Protein prepared from samples of the CAM isolate of TRV taken immediately after purification consisted of a single polypeptide with a mol. wt. of 28500, occasionally with small amounts of a polypeptide with an estimated mol. wt. of 13000. However, after storage at 5 °C for 2 to 5 weeks the size estimate of the predominant polypeptide altered to 23500, and a less abundant, smaller polypeptide was also detected in addition to the mol. wt. 13000 polypeptide (Fig. 1). After intermediate periods both 28500 polypeptide and 23500 polypeptide were found (Fig. 1 b). Virus purified using heat clarification underwent similar changes, although when virus was stored in buffer containing 0·02 % sodium azide or 0·01 % chloramphenicol + 0·01 % cycloheximide, no size changes were detected. Alkylation with 0·4 M-iodoacetamide in 0·07 M-tris-HCl pH 8·3 + 1 % SDS + 1 % 2-mercaptoethanol for 20 min at 37 °C did not alter size estimates of either of the principal polypeptide species found.

In some experiments, evidence was obtained for the transient existence of a polypeptide intermediate in size between the two commonly found polypeptides; three principal polypeptides could be resolved (Fig. 2), the fastest and slowest migrating bands of the three corresponding to mol. wt. of 23500 and 28500, respectively. These size changes were confirmed by electrophoresing mixtures of samples of protein obtained after different periods of storage at 5 °C. Fig. 3 shows densitometer tracings of gels containing samples of protein taken from a preparation of virus at intervals after purification. All samples contained some polypeptide material of mol. wt. 13000 but the changes discussed relate to the larger polypeptide of mol. wt. 23500 to 28500. The main polypeptide in the initial sample taken after 8 days storage was of mol. wt. 28500 (Fig. 3d), and after 12 days storage a faster migrating shoulder was resolved from the main peak (Fig. 3b). However, both these polypeptides in the 12-day sample were smaller than 28500 mol. wt. because they were resolved when a 1:1 mixture of the two samples was examined (Fig. 3c). Similarly two polypeptides in almost equal amounts were resolved in the main peak of a sample taken after 13 days storage (Fig. 3d) and both polypeptides were resolved from the 28500 mol. wt. polypeptide in the first sample (Fig. 3e). In a sample taken after 14 days storage only the smallest of the three polypeptides, which was the relatively stable 23500 mol. wt. polypeptide, was found (Fig. 3f), and this was well resolved from the 28500 mol. wt. polypeptide in the initial sample (Fig. 3g).

Estimates of the size of the small polypeptides illustrated in Fig. 1d, made using 10 % acrylamide gels, were not reliable because the pores of this gel are too large for such small molecules to be separated accurately according to size. Gels prepared using 12·5 % acrylamide with a high concentration of cross-linking agent, and containing 8 M-urea, have much
smaller pores and appear to be capable of separating protein molecules in the mol. wt. range \(6 \text{ to } 20 \times 10^3\) such that the log of the protein mol. wt. is linearly related to the electrophoretic mobility. This system resolved the two small polypeptide bands found in protein samples containing 23,500 polypeptide (Fig. 1d) into three bands (Fig. 4a). Mol. wt. estimates were 13,500, 11,500 and 9,000, with a probable error of \(\pm 15\%\) (Swank & Munkres, 1971). In some preparations kept for several months before sampling, only these small polypeptides were found (Fig. 5).

Preparations of CAM which had been stored for several months without bacteriostatic agent were centrifuged to sediment bacteria and virus particles. When the supernatant liquid was mixed with an equal volume of freshly purified CAM in 0.03 M-phosphate buffer containing 0.02% sodium azide and incubated at 25 °C the mol. wt. of the virus protein changed from 28,500 to 23,500. In one test, approximately half the virus protein was affected following incubation for 4 days, and in another test more than half the virus protein was
Fig. 3. Densitometer tracings of gels of samples of protein from a virus preparation stored at 5 °C and sampled at intervals after purification. (a) Purified virus after 8 days storage; (b) after 12 days storage; (c) a 1:1 mixture of (a)+(b); (d) after 13 days storage; (e) a 1:1 mixture of (a)+(d); (f) after 14 days storage; (g) a 1:1 mixture of (a)+(f). Electrophoresis is from left to right.

degraded after 1 day and no unaltered protein was detected after 4 days. No micro-organisms were detected in a virus preparation containing 0.02% sodium azide when samples were spread on 1% agar in tap water and incubated at 33 °C for 4 days, whereas approximately 6 x 10^5 bacteria/ml were detected when a virus preparation which had been stored without azide was examined. Bacteria were isolated from these plates and cultured for 10 days at 33 °C in 3% malt + 1% peptone (Oxoid) broth; none of the three cultures tested affected CAM protein when mixed with virus and incubated for 4 days at 25 °C. No further attempts were made to isolate the micro-organisms producing the presumed exoenzyme.

Protein prepared from isolate PRN which had been stored for several months was mainly of 23,500 mol. wt. whereas that from freshly purified virus was of 28,500 mol. wt. PRN protein was, however, much more stable in size than CAM protein, some preparations of PRN being unaffected after 6 months storage without additive whereas the size of CAM protein was always changed after 1 month, and frequently after 2 weeks of storage without bacteriostatic additive.

Isolate SP 5 of pea early browning virus, a virus allied to tobacco rattle, was also found to undergo a similar partial degradation during storage without bacteriostatic additive. It was estimated that the mol. wt. of the polypeptide altered from 24,000 to approximately 21,000.

Properties of virus differing in polypeptide size

Tests were made to compare the properties of virus with a 23,500 mol. wt. polypeptide and virus with a 28,500 mol. wt. polypeptide. A newly purified preparation of virus was divided into two parts; antibiotics were added to one part and both parts were then stored
Table 1. The effect of heating on the infectivity of CAM containing either undegraded or partially degraded protein

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Infectivity*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Undegraded virus</td>
</tr>
<tr>
<td>55‡</td>
<td>212</td>
</tr>
<tr>
<td>65</td>
<td>284</td>
</tr>
<tr>
<td>70</td>
<td>56</td>
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<td>75</td>
<td>32</td>
</tr>
<tr>
<td>80</td>
<td>4</td>
</tr>
<tr>
<td>20</td>
<td>232</td>
</tr>
</tbody>
</table>

* Total number of local lesions in four leaves of Chenopodium amaranticolor.
† Virus with a main polypeptide mol. wt. of 23 500.
‡ Purified virus of either type was kept at the indicated temperature for 10 min and then diluted with distilled water and inoculated immediately.

Table 2. The effect of pancreatic ribonuclease on the infectivity of CAM containing either undegraded or partially degraded protein

<table>
<thead>
<tr>
<th>Expt.</th>
<th>No RNase</th>
<th>RNase †0 h</th>
<th>RNase 2 h</th>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partially degraded virus‡</td>
<td>2860</td>
<td>148</td>
<td>102</td>
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<tr>
<td>Undegraded virus</td>
<td>3160</td>
<td>228</td>
<td>254</td>
</tr>
<tr>
<td>Expt. 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partially degraded virus</td>
<td>2598</td>
<td>2175</td>
<td>1888</td>
</tr>
<tr>
<td>Undegraded virus</td>
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<td>3355</td>
<td>3030</td>
</tr>
<tr>
<td>Expt. 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partially degraded virus</td>
<td>623</td>
<td>564</td>
<td>519</td>
</tr>
<tr>
<td>Undegraded virus</td>
<td>602</td>
<td>587</td>
<td>551</td>
</tr>
</tbody>
</table>

* Total number of local lesions in eight leaves of Chenopodium amaranticolor.
† Purified virus was inoculated immediately after mixing with ribonuclease, after incubating for 2 h at 20 °C, or without adding enzyme. RNase concentrations were: Expt. 1, 0.01 mg/l; Expts. 2 and 3, 0.002 mg/l.
‡ Purified virus was incubated for 2 h at 20 °C, or without adding enzyme. RNase concentrations were: Expt. 1, 0.01 mg/l; Expts. 2 and 3, 0.002 mg/l.

Virus in the two samples did not differ in sedimentation coefficient or in behaviour when centrifuged to equilibrium in caesium chloride. The two density components previously reported in bulk cultures of CAM (Cooper & Mayo, 1972) were found in both virus samples, and only these two components were detected when a mixture of the samples was centrifuged to equilibrium. Virus particles in the two samples were of similar modal length and width in electron micrographs. Electron microscopy also showed that a virus preparation in which only the small polypeptides (mol. wt. 8000 to 13 000) were detected (Fig. 5) contained the characteristic straight tubular particles of TRV, although some were aggregated end-to-end. Virus in the two samples used in the sedimentation tests reacted to the same antisem erum end point in serological tests, and the infectivity of both was similarly affected by heat treatment (Table 1). The infectivity of virus in both samples was equally resistant to low concentrations of pancreatic ribonuclease (Table 2).
Partial degradation of TRV

Fig. 5. 7.5% gels in which the bands have migrated further. (a) Sample of protein from freshly purified virus. (b) Samples of protein from a virus preparation kept for several months before sampling.

Fig. 6. Ten per cent gels containing samples of protein from virus treated with papain for different times. (a) Before the addition of the enzyme; (b) after 10 min incubation; (c) after 1 h incubation; (d) after 5 h incubation.

Fig. 7. 12.5% gels as in Fig. 4. (a) Samples of virus protein after degradation to mol. wt. 23,500. (b) Protein from virus incubated with papain for 1 min. (c) Proteins of known mol. wt. as in Fig. 4(b).

Effect of proteolytic enzymes on virus protein

The protein size, the appearance of virus particles in the electron microscope and the infectivity of virus preparations were unaffected by incubation with 0.1 mg/ml trypsin or 0.1 mg/ml α-chymotrypsin in 0.02 M-sodium phosphate (pH 7.0) at 30 °C for 24 h. However, when 0.1 mg/ml papain in buffer + 0.01% 2-mercaptoethanol was used the protein size was altered. The polypeptide was changed from mol. wt. 28,500 to 23,500 and this was further degraded to produce faster running bands (Fig. 6). These changes appeared similar to those occurring during storage and neither the appearance of virus particles in the electron microscope nor infectivity were affected despite the proteolytic attack. However, the smaller polypeptides produced by prolonged papain proteolysis were not all the same size as those produced during storage (Fig. 7). Estimated mol. wts. were 14,500, 11,500 and 8,500.

The rate of papain digestion was variable and the enzyme was rapidly inactivated in the absence of 2-mercaptoethanol. PRN protein in virus particles was also digested by papain to produce apparently similar end products to those from CAM, but when CAM and PRN were compared using the same enzyme preparation the effect on PRN was very much slower than that on CAM.

In some experiments the effect of pronase was investigated. The size of the polypeptides obtained from treated virus altered with the period of incubation, polypeptides with a range of sizes being produced. Pronase-treated virus which mainly contained polypeptides
of mol. wt. less than 20,000 and apparently none of mol. wt. 28,500 looked normal in the electron microscope, and was at least 25% as infective as untreated virus.

**DISCUSSION**

Our results indicate that when sodium azide or antibiotics were omitted from preparations of TRV the size of the virus polypeptide was altered during storage apparently from 28,500 mol. wt. to approximately 26,000 and 23,500. The 23,500 mol. wt. polypeptide was the most stable of these, although some protein in all virus preparations was degraded further, resulting in polypeptides with mol. wt. of about 13,500, 11,500 and 9,000. In contrast to the spontaneous degradation of adenovirus protein during storage (Pereira & Skehel, 1971), the degradation of TRV was prevented by sodium azide.

Changes in the charge on a polypeptide can alter electrophoretic mobility in SDS-acrylamide gels (Tung & Knight, 1970) and one possible interpretation of our data is that the charge on the virus polypeptide is altered during storage either by loss of a few amino acids or by masking of charged groups. However, some proteolysis must have occurred when polypeptides of apparent mol. wt. of 8,000 to 13,000 were produced, and when bacteria and virus particles were centrifuged from a preparation containing degraded virus, the supernatant fluid caused degradation of virus protein in the presence of sodium azide. We therefore conclude that proteolytic activity was present in stored virus preparations.

Changes in the chemical composition of plant viruses caused by enzymes of host plant origin have been observed with TMV (Rees & Short, 1965), cowpea mosaic and bean pod mottle viruses (Niblett & Semancik, 1969) and potato virus X (Koenig et al. 1970). However, it seems probable from our observations that proteolysis of TRV during storage of purified preparations is due to exoenzymes produced by micro-organisms.

Although the presumed microbial enzyme and papain caused similar changes in the protein of CAM and PRN, both enzymes degraded CAM protein much more rapidly than PRN protein. These strains belong to separate serotypes (Harrison & Woods, 1966), which suggests that the proteins are dissimilar, and the difference in the rate of enzyme attack may reflect this difference. However, the production of similar sizes of polypeptides suggests either some degree of homology in amino acid sequence or that the folding of the polypeptides in the virus subunits of the different isolates may be similar, allowing the same region of polypeptide to become exposed to proteolytic attack.

When the mol. wt. of the subunit protein was altered to 23,506 there was no evidence of any change in gross structure detectable by electron microscopy, or of any more subtle changes such as increased susceptibility to ribonuclease or alteration of the heat-stability of the virus particles. Virus consisting of protein substantially cleaved into small peptides of 8,000 to 12,000 mol. wt. also retained its characteristic appearance in electron micrographs. Similarly, degraded preparations resulting from papain or pronase digestion also retained infectivity, and a preparation degraded as a result of protracted storage was found to contain RNA molecules of the sizes characteristic of TRV. Clearly, an intact polypeptide subunit is not required for the virus functions examined. However, it remains to be established whether the small fragments of peptide or possibly single amino acids produced by the change of mol. wt. from 28,500 to 23,500 are retained in the structure of the virus or whether they are released.

The finding that the protein of TRV is degraded during storage unless microbial growth is prevented may help to explain some of the differences in the reported estimates of mol. wt. of the virus protein. Loss of some polypeptide from the protein subunits could have
Partial degradation of TRV resulted in the lower estimates of mol. wt. obtained from amino acid analyses compared with those from polyacrylamide gel electrophoresis. If the fragments were retained in the virus structure, however, no great difference in amino acid composition should result, unless protein were prepared from purified virus before hydrolysis (Miki & Okada, 1970) when a small peptide might well be lost.

The results show that although tobacco rattle is a ‘stable’ virus with a protracted longevity in sap (Cadman & Harrison, 1959) the subunit proteins of two isolates of the virus are readily degraded during storage. Retention of infectivity, therefore, does not indicate that a virus preparation is unchanged by storage, and antimicrobial agents should be added to purified preparations of TRV when these are being stored.

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