The Tryptic Peptides and Terminal Sequences of the Protein from the Cowpea Strain of Tobacco Mosaic Virus

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The use of ion exchange chromatography, gel filtration, and paper electrophoresis for the separation of the peptides obtained by tryptic digestion of the cowpea strain of tobacco mosaic virus protein is described. The amino acid compositions of the 13 tryptic peptides obtained were determined and accounted for 159 residues compared with the 158 found for type tobacco mosaic virus protein. The only tryptic peptide that cowpea tobacco mosaic virus protein and type tobacco mosaic virus protein have in common is asparaginyl-arginine despite the fact that the former is very similar to type tobacco mosaic virus protein in a number of its properties. The amino terminal sequence was found to be acetyl alanyl-tyrosine and the carboxyl terminal sequence was confirmed as alanine preceded by threonine.

INTRODUCTION
Lister and Thresh (1955) isolated a strain of tobacco mosaic virus (TMV) which, unlike all other strains of TMV, produced a systemic mosaic disease in cowpea, Vigna unguiculata (L) Walp. Bawden (1956, 1958) confirmed the identity of the virus, which is called the cowpea strain of TMV (cowpea TMV), and studied its electrophoretic mobilities and serological properties. The amino acid composition of the protein of the cowpea TMV and its C-terminus obtained by carboxypeptidase digestion has been reported by Rees and Short (1965). The present study describes the terminal sequences of the cowpea TMV protein and the separation and amino acid composition of the peptides produced by tryptic digestion.

MATERIALS AND METHODS
The source of virus and the preparation and purification of the cowpea TMV and its protein were as described by Rees and Short (1965).

The trypsin used for the hydrolysis of the protein was 3 × crystallized, and the chymotrypsin was chromatographically pure (both enzymes from Worthington Biochemicals). The trypsin was pretreated with L-(1-tosylamide-2-phenyl)ethyl-chloromethyl ketone by the method of Kostka and Carpenter (1964). For the amino acid analyses of the purified peptides suitable samples were dried and hydrolyzed for 24 hr and 72 hr by refluxing with constant boiling HCl. The excess HCl was removed at 40°C in a rotary evaporator, and the amino acid analyses were done on a Beckman Spinco amino acid analyzer by the method of Spackman et al. (1958). All pyridine used was redistilled from ninhydrin (Hill and Delaney, 1967).

Tryptic Digestion
The precipitated protein was weighed into a small glass homogenizer and a 1% solution of NH₄HCO₃ was added to give a suspension of 1% of protein. The mixture was homogenized to make a fine suspension and a portion of a freshly prepared solution of trypsin in 0.001 N HCl was added to give a protein to enzyme ratio of 50:1 and the mixture was incubated at 37°C. The suspended protein became completely soluble 10 min after the addition of the trypsin and digestion was...
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allowed to proceed for 4 hr; the digest was then lyophilized.

Chymotryptic Digestion

The protein or peptide was treated as above and a portion of a freshly prepared solution of chymotrypsin in water was added to give a protein or peptide to enzyme ratio of 50:1. The mixture was incubated for 16 hr at 37°C and was then lyophilized.

Two-dimensional separations of the digests were made by the methods described by Rees et al. (1970).

Chromatographic Separation of Tryptic Peptides

Ion exchange chromatography. The tryptic peptides were fractionated on a 1.0 × 150 cm column of AG50W × 2 cation exchange resin, 200–400 mesh (AG50) (Bio-Rad Laboratories). Preparation of the column and composition of the pyridine–acetic acid buffers used were as described by Schroeder et al. (1962). The lyophilized digest was extracted with 4 ml of the starting buffer (pH 3.1, 0.2 N pyridine–acetic acid) at 40°C. Insoluble material was removed by centrifugation, and the supernatant liquid was loaded onto the column and washed in with further extracts (4 × 2 ml) of the pellet. The peptides were chromatographed at a flow rate of 15 ml per hour and fractions of 2.6 ml were collected. At fraction 240 the fraction size was doubled. Development was made with a linear gradient consisting of 680 ml of pH 3.1, 0.2 N pyridine–acetic acid and an equal volume of pH 5.0, 2 N pyridine–acetic acid buffer.

After fraction 367, a further 500 ml of 2 N pyridine–acetic acid buffer (pH 5) was passed through the column.

The peptides from the column separations were located by drying down 0.1 ml portions of each fraction. After redrying from aqueous solution in high vacuum, the residues were dissolved in 0.01 ml of 0.2 N ammonia. The fractions were spotted across buffer-wetted Whatman 3 MM paper (32 × 63 cm) 40 at a time at 8-mm intervals and were electrophoresed at pH 6.5. High voltage electrophoresis was carried out on a cooled plate apparatus at 40 V/cm for 2 hr using pyridine–acetic acid–water buffer, pH 6.5 (100:4:896 by volume) as solvent. Peptides were located by the staining method of Reindel and Hoppé (1954), and appropriate fractions were pooled and taken to dryness on a rotary evaporator. Pyridine–acetic buffers were removed by repeated drying after the addition of water, and were finally dried in a desiccator over P2O5 and KOH. Pooled fractions that contained more than one peptide were further separated by chromatography on Sephadex G-25 superfine and/or paper electrophoresis.

Sephadex chromatography. The peptide separations were made on a column of Sephadex G-25 superfine made up of four 150 × 0.9 cm sections with standard glass screw threads at each end (SQ13, Quickfit and Quartz Ltd.). The columns were connected in series with 0.58 mm i.d., 0.96 mm o.d. polythene tubing by means of screw caps and connectors.

The Sephadex suspended in pyridine–1 N ammonia solution (7:3) was packed into the columns in four or five sections using a Beckman Spinco Accum pump at flow rates in excess of those used for the separation of the peptides.

The last section of each column was packed with the aid of an extension tube. When the Sephadex had ceased to compress in the extension piece, it was removed and the completely filled column was sealed with a screw cap and adaptor.

The peptides were dissolved in 2 ml of the pyridine–1 N ammonia solution, loaded onto the column, and washed in with (3 × 2 ml) of the same solvent. The column was developed with the pyridine–ammonia solution at a flow rate of 6 ml per hour, and 2.5 ml fractions were collected. One liter of the solvent was required to ensure that all peptides and any free amino acids present were eluted from the column.

Paper Electrophoresis

The electrophoretic separations of the pooled fractions were made on 27 × 63 cm sheets of Whatman 3 MM paper using the pyridine–acetic acid–water buffer, pH 6.5, previously described and electrophoresis at 40 V/cm for 2 hr. The peptides were applied
as a line across the paper 1.5 cm in from each side after the paper had been dipped in the buffer. After location by staining of side strips, the separated components were eluted with 0.2 N ammonia and lyophilized.

RESULTS AND DISCUSSION

Figure 1 (a) shows the electrophoretic pattern of the fractions obtained from chromatography of the tryptic peptides soluble in pH 3.1, 0.2 N pyridine-acetic acid buffer when the mixture of peptides was separated on AG50W × 2 resin.

The fractions were pooled as indicated by the brackets in Fig. 1 and further purification was done as described in Materials and Methods.

Table 1 gives the amino acid compositions of the peptides purified from the pooled chromatographic fractions. The purification methods and percentage yield of each purified peptide are also given. This table deals with peptides obtained in significant amounts only, as judged from amino acid analyses.

The peptides listed in Table 1 account for 159 amino acid residues as compared to the 155 residues found from amino acid analysis of the cowpea TMV protein. Peptide AG50 11A gave the same analysis as peptide AG50 11. The difference in the electrophoretic mobility of the peptide resulted from the loss of an amide group from the asparaginyl residue. Peptides 13A, 13B, 13C, and 13D all arose from the chymotryptic-like activity of the trypsin on peptide 13 in which the peptide bonds formed by the carboxyl groups of tyrosine and isoleucine were hydrolyzed. When tryptic digests of the cowpea TMV were extracted with pH 3.1, 0.2 N pyridine-acetic acid buffer, as previously described, 90% of the weight of peptides was soluble. The remaining 10% was extracted with pyridine-1 N ammonia solution and chromatographed on Sephadex G25 superfine as previously described.

Two major peptides were found. One (fractions 4–16) gave the same analysis as obtained for AG50 1 and the other (fractions 44–62) had a similar analysis to peptide AG50 3. The two purified peptides accounted for 50% of the weight of the pH 3.1, 0.2 N pyridine-acetic acid insoluble fraction. Thus, overall, 95% of the starting weight of protein was accounted for.

Tryptic peptides containing tyrosine and/or phenylalanine were further digested with chymotrypsin and the resulting peptides investigated.

From the chymotryptic digest of tryptic peptide AG50 1, a peptide that stained with the chlorination technique and possessed a highly negative charge was isolated. After hydrolysis, this peptide was found to be a dipeptide consisting of alanine and tyrosine. No terminal amino acid was obtained by the dinitrophenylation technique of Sanger (1945); when the peptide was digested with carboxypeptidase A and analyzed without hydrolysis, the only ninhydrin-positive peak was tyrosine.

As almost all of the plant virus proteins previously investigated have been found to contain an N acetyl amino terminus, it was concluded from this evidence that the cowpea TMV protein probably had an N-terminal acetyl alanine followed by tyrosine. This was confirmed by mass spectrometry (unpublished work).

In a previous paper on the protein of cowpea TMV Rees and Short (1965) concluded on a basis of experiments using carboxypeptidase A, that the end carboxyl amino acid was alanine and that this was preceded by threonine. The only peptide that contained these two amino acids and did not contain an arginine or lysine, was peptide AG50 4, a dipeptide consisting of equimolar amounts of threonine and alanine. After one subtractive Edman degradation, 90% of the threonine was removed confirming the previously reported carboxyl-terminal sequence.

The sum of the amino acid residues from the analyses of the tryptic peptides of cowpea TMV is 159 as opposed to the 155 residues reported previously from the amino acid analyses of the protein and compared with the 158 residues found from the sequence studies of type TMV (Tsugita et al., 1960; Tsugita and Fraenkel-Conrat, 1963; Anderer et al., 1960; Anderer and Handshuh, 1962). More detailed investigations of the tryptic peptides may show that the number of amino acid residues corresponds to that of type TMV.

The amino acid residues obtained from the tryptic peptides of cowpea TMV protein show 27 differences from type TMV protein,
Fig. 1. Electrophoretic peptide patterns of cowpea TMV tryptic digest. (a) pH 3.1, 0.2 N pyridine acetic acid-soluble peptides separated on AG 50 × 2 resin. (b) Peptides insoluble in pH 3.1, 0.2 N pyridine acetic acid but soluble in pyridine-1 N NH₃ (70:30) separated on Sephadex G-25.
### TABLE 1

**Amino Acid Composition of Tryptic Peptides Obtained from Cowpea Strain of Tobacco Mosaic Virus Protein**

<table>
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<th>Amino Acid</th>
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<th>AG50 2</th>
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<th>AG50 7</th>
<th>AG50 8</th>
<th>AG50 9</th>
<th>AG50 10</th>
<th>AG50 11 &amp; 11A</th>
<th>AG50 12</th>
<th>AG50 13</th>
<th>Sum of Residues from Tryptic Peptides</th>
<th>Amino Acid Analysis of Protein Rees &amp; Short 1965</th>
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<td>(1)</td>
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<td>(1)</td>
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<td>(1)</td>
<td>(1)</td>
<td>(1)</td>
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<td>21</td>
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</table>

Data are presented as residues per peptide relative to lysine or arginine where present. When peptides from the AG50 chromatographic fractions were purified further, the purification methods, (paper electrophoresis and chromatography on Sephadex G25) are indicated as E and G25 respectively. The percentage yield of each peptide was calculated on the basis of the recovery after purification, assuming 100% hydrolysis of the appropriate cleavage points during tryptic digestion.

1 Residues per peptide relative to threonine.
2 Residues per peptide relative to phenylalanine.
3 Residues per peptide relative to isoleucine.
and it is remarkable that the only tryptic peptide that cowpea TMV and type TMV have in common is asparaginyl-arginine despite the fact that the former is very similar to type TYIV in a number of its properties. The cowpea TMV protein has the pH dependent reversible aggregation property of type TMV protein and forms tight helical aggregates similar to those obtained from type TMV protein (unpublished observations). This is in contrast to the nonfunctional protein synthesized by plants infected by the PM2 strain (Siegel et al., 1962; Siegel and Zaitlin, 1965), which is unable to form nucleoprotein virus particles in infected plants but aggregates to form elongated 2-stranded open helical structures (Zaitlin and Ferris, 1964). PM2 protein differs from common strain protein by only two amino acid replacements; an isoleucine replaces threonine at position 28, and a glutamate residue is replaced by an aspartate residue at position 95 (Zaitlin and McCaughey, 1965; Wittman, 1965).

Taking the simplest view of these data and those obtained from cowpea TMV, it appears that relatively few amino acid residues are critically involved in the formation of mature virus particles, a point that will be discussed in future publications.

ACKNOWLEDGMENTS

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REFERENCES


