Separation of Tryptic Peptides of Tobacco Mosaic Virus and Strain Proteins by an Improved Method of Column Chromatography*

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A chromatographic-elution schedule for the separation of peptides is described which gives reproducible patterns for a given mixture. The location of each peptide is affected in predictable manner by its net charge, content of tyrosine and phenylalanine residues, and chain length. The exact location of amino acid exchanges in mutants is facilitated by this procedure.

Studies of the amino acid exchanges in chemically evoked mutants of tobacco mosaic virus (TMV)[1] have been carried out in our laboratory and at the Max Planck Institut für Biologie at Tübingen for about 5 years. The best experimental approach to the localization of such exchanges has proved to be that starting from tryptic digests according to the general procedure of protein structure analysis.

When TMV protein is digested with trypsin, twelve peptides are obtained. The acetyl N-terminal peptide 1,[2] consisting of forty-one amino acids, is precipitated at *p*H 4.5–4.7 together with small amounts of other peptides, but it can be purified by three cycles of isoelectric precipitation. The other eleven peptides which remain in solution at *p*H 4.6 can be separated by Dowex 1 × 2 column chromatography using pyridine–collidine–acetic acid, as reported originally by Wittmann and Braunitzer (1959), and later by Tsugita (1962a). This chromatographic system, however, was not completely adequate for the location of all amino acid exchanges in mutants, because the separation of each peptide, especially peptide 10, was incomplete and the chromatographic pattern was not readily reproducible.

In the present paper, improvements in this chromatographic system will be described. The reproducible pattern yields a chromatographic map on which known and modified peptides can be identified by their location, which is in a predictable manner determined by their chemical nature.

Materials and Methods

The strains of TMV known as yellow tomato atypical mosaic virus (Y-TAMV), green tomato atypical mosaic virus (G-TAMV) and Holmes rib-grass virus (Knight, 1963), and chemically induced mutants (Tsugita and Fraenkel-Conrat, 1962) were used, as well as common TMV.

The virus proteins were isolated by the acetic acid method (Fraenkel-Conrat, 1957). Iodinated TMV protein was prepared by Dr. Fraenkel-Conrat either by treating the intact virus or the native or denatured protein with KI_3 at *p*H 7.

Trypsin was obtained from the Worthington Biochemical Corp. Three-times-crystallized trypsin (sterile) was used preferentially since it showed less chymotryptic activity than the twice-crystallized material.

The protein (50–100 mg) was digested with 1% of trypsin at 37° for 2 hours using an autotitrator to maintain the *p*H at 8.0. The reaction mixtures were adjusted to *p*H 4.5 with 1 N acetic acid and allowed to stand for 30 minutes in an ice bath. The precipitate was dissolved at *p*H 7 and purified by repeated isoelectric precipitation. The resulting supernatants were combined and lyophilized.

TABLE I

COMPOSITION OF PYRIDINE COLLIDINE–ACETIC ACID USED TO SEPARATE TRYPTIC PEPTIDES OF TMV PROTEIN ON DOWEX 1 × 2 COLUMN[a] (see text and Fig. 1)

Procedure:	(Present study)	(Tsugita and Fraenkel-Conrat, 1962)
(1)	1% collidine–1% pyridine–6.25 × 10^{-3} N acetic acid (250 ml, *p*H 8.2)	
(2)	Gradient (by Autograd, Technicon Chromatography Corp., N. Y.) (chambers 1–9)	
1	1% collidine, 1% pyridine, 0.03 N acetic acid (*p*H 7.3)	1% collidine, 1% pyridine, 0.03 N acetic acid (*p*H 7.3)
2	Same	Same
3	Same	0.1 N acetic acid
4	0.02 N acetic acid	0.5 N
5	Same	0.5 N
6	0.2 N	1 N
7	0.35 N	2 N
8	0.50 N	Glacial AcOH
9	0.65 N (each chamber contains 135 ml)	Glacial AcOH (each chamber contains 120 ml)
(3)	Acid gradient (chambers 1–3)	
1	0.65 N acetic acid	
2	1.7 N (10%)	
3	5 N (30%)	

[a] See text and Fig. 1. Anderer and Handschuh (1962), employ a three-step gradient system composed of pyridine acetate buffer (*p*H 8.2) with increasing acetic acid concentration to 1.8 M.

The mixture of soluble peptides was applied to a Dowex 1 × 2 (200–400 mesh; acetate form) column (0.9 × 150 cm) which had been equilibrated with a *p*H 8.8 pyridine–collidine–acetic acid buffer (10, 10, and 0.06 ml, respectively, in 1000 ml) and the column was developed at 35° with a *p*H 8.2 pyridine–collidine–acetic acid buffer (10, 10, and 0.2 ml, respectively, in 1000 ml). After 250 ml of this buffer had been added gradient elution was started. The standard elution schedule using the 9-chamber Autograde (Technicon Chromatography Corp.) is given in Table I, in comparison with previously used elution methods. It is evident that the neutral and acidic peptides are eluted at lower acetic acid concentration than those used in the other methods. Finally the column is washed with higher acetic acid concentrations. The flow rate was about 40 ml/hr, and 3.3 ml of eluate was collected per tube. One-tenth ml of each fraction was dried at about 50° by an air

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[1] Abbreviations used in this work: TMV, tobacco mosaic virus; Y-TAMV, yellow tomato atypical mosaic virus; G-TAMV, green tomato atypical mosaic virus.

[2] All peptides will be referred to by their sequential numbers, starting with the acetyl N-terminal peptide.

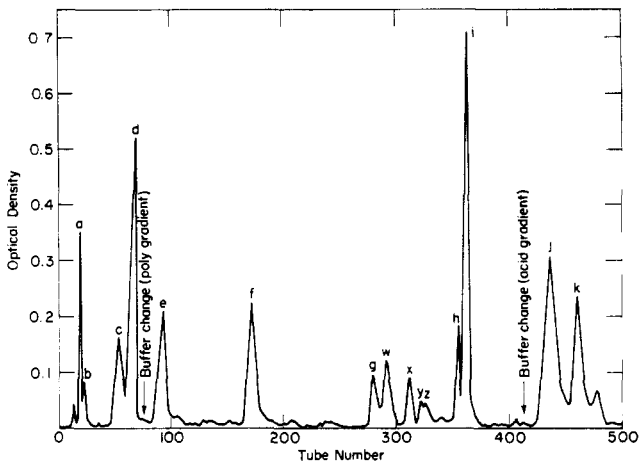

FIG. 1.—Chromatography on Dowex 1 × 2 column (0.9 × 150 cm) of the *p*H 4.5 soluble tryptic peptides of TMV protein. The tryptic peptide mixture was applied on the Dowex 1 × 2 column which was equilibrated with pyridine-collidine-AcOH buffer (*p*H 8.2) and then eluted by means of a polygradient elution system (Table I). Flow rate, 40 ml (= 12 tubes)/hr. Optical density stands for the absorbance at 700 mμ of the Folin-Lowry color obtained with 0.1 ml (of 3.3) eluate per hour. Temperature, 35°. Tubes containing peptide 10 (peak g) and peptide 8 (peak i) contained precipitates. Table II lists the amino acid composition of the peptides.

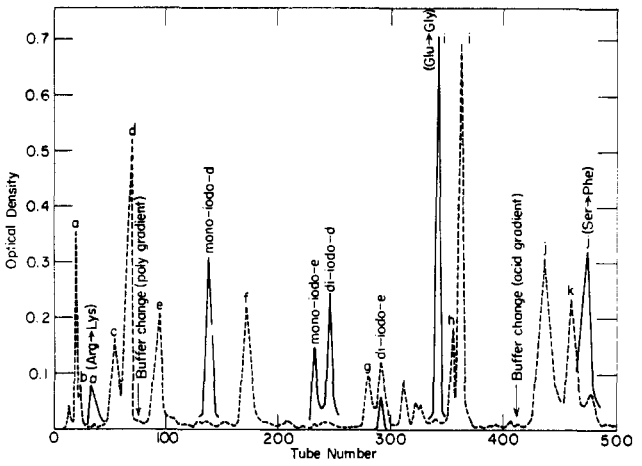

FIG. 2.—Comparison of the position of tryptic peptides of common TMV with those found displaced in chemically induced mutants and in the iodinated protein on Dowex 1 × 2 column (0.9 × 150 cm) chromatography. The position of the modified peptides, the composition of which was ascertained by analysis (Funatsu and Fraenkel-Conrat, 1964), is superimposed on the pattern for common TMV (dotted line). Iodinated peptides were distinguished from other peptides by their radioactivities, and mono- and diiodotyrosines were identified on paper chromatography after alkaline hydrolysis of the peptides (Fraenkel-Conrat, 1962).

stream and analyzed by the Folin-Lowry method (Lowry *et al.*, 1951). To determine the amino acid composition of each peptide, and detect exchanges, analyses were performed on individual tubes selecting those most likely to contain pure peptide. One- to 2-ml samples were evaporated to dryness and then hydrolyzed with 6 N HCl at 108° in evacuated sealed tubes for 24 hours, again evaporated, and applied to the Spinco amino acid analyzer. For preparative purposes, all tubes under a peak were pooled and either tested for purity, or purified by paper chromatography using *n*-BuOH–AcOH–H_2O–pyridine (30:6:24:20).

Results and Discussion

The chromatographic pattern and amino acid compositions of the tryptic peptides of common TMV-protein are shown in Figure 1 and Table II. Almost all peptides can be separated, but the yields of peak g (peptide 10) and peak k (peptide 6) were usually low, due to partial adsorption or precipitation of these peptides on the top of the column (Funatsu *et al.*, 1964). However, the present elution schedule gives an improved resolution in the midsection of the chromatogram resulting in a clear separation of peptide 10 as a discrete

TABLE II
AMINO ACID COMPOSITION OF EACH TRYPTIC PEPTIDE PEAK FROM TMV[a]

Amino Acids	Peaks										
	a	b	c	d	e	f	g	h	i	j	k
Asp		1		1		2	2	2	3		3
Thr	1		1	1		1			4	2	2
Ser			2	1			1	1		6	
Glu	1		3				1		4	1	
Pro			2					1	1	1	1
Gly				2						2	1
Ala						2	1		3	1	3
Val	2		3		1	2	1		1	1	2
Met											
Ileu						1	3		2		
Leu							2		1	1	4
Tyr				1	1						1
Phe			1					2		1	1
(Try)			(1)							(1)	
Lys			1					1			
His											
Arg	1	1	1	1	1	2	1		1		1
Total	5	2	15	7	3	10	12	7	20	17	19
Peptide[b]	2	7	3	11	5	9	10	4	8	12	6

[a] Amino acid composition of each peak is shown as the nearest integer number of residues per mole of peptide. The analytical values were usually within the range of ±5% of this number. No analysis was made for tryptophane. [b] See text footnote 1.

TABLE III
NUMBERS OF ACIDIC, BASIC, AND AROMATIC AMINO ACIDS IN EACH TRYPTIC PEPTIDE OF TMV

Peaks	Peptide[a]	Total Amino Acids	Acidic Amino Acids	Basic Amino Acids	Net Charges (at *p*H 7)	Tyrosine and Phenylalanine[b]
a	2	5	0	1	+1	0
b	7	2	0	1	+1	0
c	3	15	0	2 (1 Lys)	+2	2
d	11	7	0	1	+1	1
e	5	3	0	1	+1	1
f	9	10	2	2	0	0
g	10	12	1	1	0	0
h	4	7	2	1 (Lys)	−1	2
i	8	20	4	1	−3	0
j	12	17	1	0	−1	2
k	6	19	2	1	−1	2

[a] See text footnote 1. [b] Peptides 3 and 12 (peaks c and j) contain one tryptophan each.

TABLE IV
AMINO ACID COMPOSITION OF EACH TRYPTIC PEPTIDE PEAK FROM Y-TAMV[a]

Amino Acids	Peaks									
	a	b	c	d	e	f	g	h	i	Peptide 1
Asp	1			2	3	1	2	3	2	4
Thr		3		1			4	3	3	3
Ser		2			1		1		4	7
Glu		5			1		5		2	6
Pro		2				1	1	1	1	2
Gly						1		1	3	1
Ala				2	1		2	2	2	2
Val		3	1	2	2	1	1	1	1	3
Met									1	
Ileu				1	1		2	1		2
Leu					2		1	4	2	4
Tyr			1			1		1	1	1
Phe		2				1		1	1	3
Try		1							1	1
Lys		1				1				
His										
Arg	1	1	1	2	1		1	1		1
CySH										1
Total	2	20	3	10	12	7	20	19	24	41

[a] Single-peak tubes were hydrolyzed and analyzed for amino acid compositions. Tryptophane was determined spectrophotometrically. [b] See text footnote 1.

peak. This peptide as well as peptide 8 was partly insoluble upon elution. The small peaks w and y between peaks g and i resulted from incomplete digestion of the bond between peptides 3 and 4, and 7 and 8, respectively. Peak z was peptide 9 lacking the N-terminal arginine and peak x analyzed like peptide 8, possibly with an extra arginine. Other minor peaks showed indefinite amino acid composition and probably represented decomposition products.

As evident from the data summarized in Table III, the elution of peptides in this chromatographic system depends mainly on their number of acidic, basic, and certain aromatic amino acids, as well as somewhat on their chain length. As expected, arginine and to a lesser extent lysine residues accelerate the elution of peptides, while aspartic and glutamic acid, as well as tyrosine and phenylalanine, retard it. These facts are well illustrated by a comparison of the positions of peptide-elution peaks in chemically induced mutants. Note, for example, in Figure 2 the effect on peak a of the change arginine to lysine, the effect on peak i of the change glutamic acid to glycine, and on peak j of the change serine to phenylalanine. However, the exact order of the position of each peptide is quite sensitive to changes in acetic acid concentration. Thus peaks h and i in Figure 1 are interchanged at higher concentrations of acetic acid. At lower acetic acid concen-

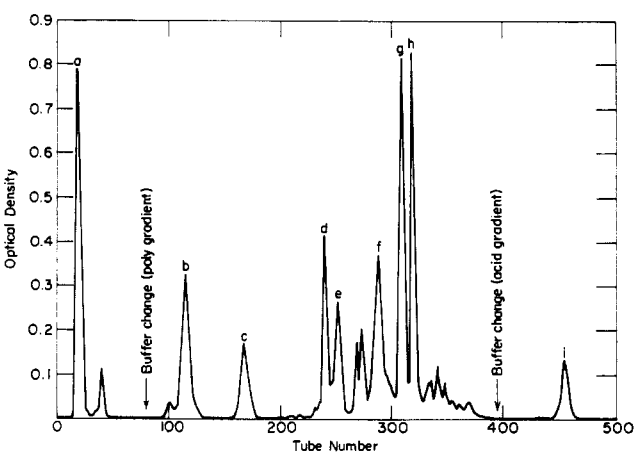

FIG. 3.—Chromatography of the *p*H 4.5 soluble tryptic peptides of Holmes rib-grass virus protein on Dowex 1 × 2 (0.9 × 150 cm) column. Chromatographic conditions were the same as in Fig. 1. Peak a was resolved by *n*-BuOH-AcOH-H_2O-pyridine (30:6:24:20) chromatography into three peptides, one of which (1a) precipitated. The yield of peak f is variable, owing to partial precipitation at *p*H 4.5. Table IV lists the amino acid composition of the peptides after purification by paper chromatography using *n*-BuOH-AcOH-H_2O-pyridine (30:6:24:20).

TABLE V
AMINO ACID COMPOSITIONS OF EACH TRYPTIC PEPTIDE PEAK FROM HOLMES RIB-GRASS VIRUS[a]

Amino Acids	Peaks											
	a-1	a-2	a-3	b	c	d	e	f	g-1	g-2	h	i
Asp	2		1	1	1	1	1	3	2	1	3	
Thr	1	1			1	1	1		1	2	3	3
Ser	1			1		1		2		3	3	
Glu	3	2		1				3		6	6	2
Pro	1			1			1			1	1	2
Gly		1					1	2				
Ala	2	1		2					2	3	5	4
Val	1			3	1				2	1	3	
Met				1				1				
Ileu	1			1				1	1	2	3	1
Leu	2			2				4				1
Tyr				2				1				
Phe	1					1	2					1
Lys				2								
His								1				
Arg	1	1	1		1	1	1	1	1	1	2	
Total	16	6	2	17	4	5	7	19	9	20	29	14

[a] Each peak was purified by paper chromatography using *n*-BuOH-AcOH-H_2O-pyridine (30:6:24:20), and analyzed for amino acid compositions. No analysis was made for cysteine and tryptophane.

TABLE VI
AMINO ACID COMPOSITIONS OF EACH TRYPTIC PEPTIDE PEAK FROM G-TAMV[a]

Amino Acids	Peaks								
	a	b	c	d	e	f	h	i	m
Asp		1	1		3	1	2	3	1
Thr	1		3			1	1	2	
Ser			1		1	2			1
Glu	3		3		1	1		2	
Pro			2			2		1	1
Gly									
Ala	1		2		2		2	2	1
Val			3	1	1		2	1	
Met			1						
Leu					1	1	1	2	
Tyr				1	2	1			1
Phe			1			1			2
Lys			1						
His									
Arg	1	1	1	1	1		1	1	
Total	6	2	19	3	12	10	9	14	7

[a] Each peak was purified by paper chromatography using *n*-BuOH-AcOH-H_2O-pyridine (30:6:24:20) and analyzed for amino acid compositions. Amino acid compositions of peaks g, j, k and l were not close to integer, suggesting incomplete separation. No analysis was made for cysteine and tryptophane.

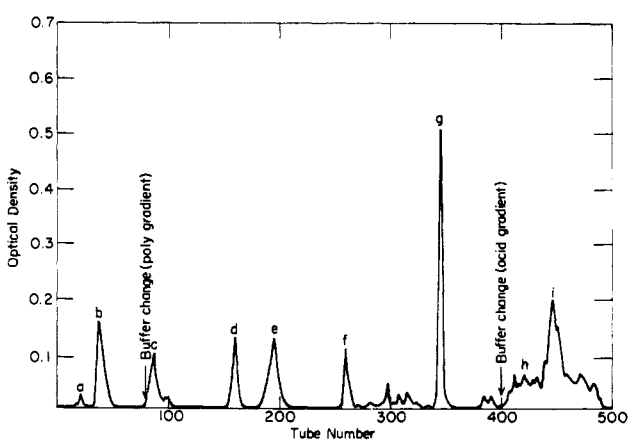

FIG. 4.—Chromatography of the *p*H 4.5 soluble tryptic peptides of Y-TAMV protein on a Dowex 1 × 2 column (0.9 × 150 cm). The chromatographic conditions were the same as used for Fig. 1. Contents of single-peak tubes were hydrolyzed and analyzed for the amino acid composition (Table V).

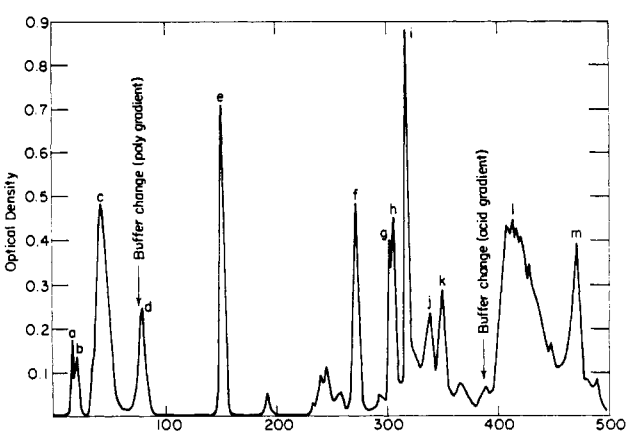

FIG. 5.—Chromatography of the *p*H 4.5 soluble tryptic (twice-crystallized trypsin) peptides of G-TAMV protein. Chromatographic conditions were the same as in Fig. 1. The peptides were purified by *n*-BuOH-AcOH-H_2O-pyridine (30:6:24:20) chromatography, and analyzed for amino acid composition (Table VI).

tration, in the case of the Holmes rib-grass virus strain (Fig. 3), peak g was separated into two peaks, while peak d (Asp_1, Phe_1) and peak e (Asp_1, Phe_2) were eluted together. It appears that the effect of acid groups is stronger at higher *p*H and weaker at lower *p*H. On the other hand, the effect of tyrosine and phenylalanine residues is the reverse. The iodination of tyrosine in peptides probably owes its marked effect on the peak position to the resultant change to a lower *pK* (Fig. 2). With consideration of these facts, the chromatographic elution schedule can be adjusted to suit each particular purpose.

The reproducible location of peptides under standard elution conditions permits the pinpointing of mutational exchanges in a manner similar to that used in paper chromatograph mapping ("fingerprints"). However, the possibility to verify the composition and structure of each peptide makes the column method a far more decisive and powerful tool.

The tryptic peptides of Y-TAMV could be clearly separated by the same elution schedule as those of TMV (Fig. 4). The amino acid compositions of each peak (Table IV) were identical to those reported for the Dahlemense strain (Wittmann, 1960). The single difference (Ser → Asp) was found in peptide 1.

The chromatographic patterns and amino acid compositions of some peaks from the Holmes rib-grass virus and G-TAMV strains are shown in Figs. 3 and 5 and Tables V and VI. In spite of the marked difference in amino acid composition, it is noteworthy that there are two peptides, 7 and 9, which have the same amino acid compositions as those of common TMV, except for one less arginine in peptide 9. This suggests, as pointed out before (Tsugita, 1962b; Wittmann, 1963), that these peptides play an important role in assuring the conformation of the protein necessary for aggregation to virus particles. The amino acid composition of peak h in Holmes rib-grass virus (Fig. 3), the protein of which is unusually resistant to dissociation by 67% acetic acid (Fraenkel-Conrat, 1957), was the same as that of peak g. Peak g separated into two peptides upon paper chromatography, but peak h was a single peptide. The two components of peak g (g 1, g 2) corresponded to peptides 8 and 9 of common TMV. Therefore peak h is formed by incomplete digestion of these two peptides. This peak h was obtained even after longer tryptic digestion. It is not clear whether this partial resistance to trypsin is due to steric hindrance or to some unusual type of masking of this arginine.

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