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Immunochemical Studies on the Tobacco Mosaic Virus Protein. III. The Degradation of an Immunologically Active Tryptic Peptide of Tobacco Mosaic Virus Protein and the Reactivity of the Degradation Products with Antibodies to the Whole Protein*

Janis Dillaha Young, E. Benjamini, M. Shimizu, and Cherry Y. Leung

ABSTRACT: Tobacco mosaic virus protein (TMVP) tryptic peptide 8 representing residues 93–112 of the protein subunit and having the amino acid sequence Ile-Ile-Glu-Val-Glu-Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg has previously been shown to exhibit immunological activity related to the whole tobacco mosaic virus protein. TMVP tryptic peptide 8 has been degraded by enzymes and by N-terminal stepwise cleavage, yielding desarginated peptide 8 (Ile-Ile-Glu-Val-Glu-Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr), peptide 8 (−5) (Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg), and desarginated peptide 8 (−5) (Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr). The latter two peptides were further degraded by digestion with carboxypeptidase A. The immunological activities of the isolated peptides were tested by their capacity to inhibit the fixation of complement by TMVP and anti-TMVP, by their ability to compete with radioactive peptide 8 for antibodies to TMVP, or by their direct combination with anti-TMVP.

Such tests showed that the removal of five amino acids from the N-terminus of peptide 8 and the removal of at least two amino acids from the C-terminus of the peptide yielded an immunologically active peptide having the sequence Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala. The binding to anti-TMVP was somewhat reduced by the removal of five amino acids from the N-terminus of peptide 8 and was reduced to a greater extent by the removal of amino acids from the C-terminus of the peptide. Both pepsin and subtilisin digestion of the peptide destroyed its immunological activity.

Studies on the immunological activity of tryptic peptides of TMVP[1] showed that tryptic peptide 8 having the amino acid sequence: Ile-Ile-Glu-Val-Glu-Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg (Gish, 1961; Funatsu *et al.*, 1964) specifically inhibited the fixation of complement by TMVP and anti-TMVP (Benjamini *et al.*, 1964). Acetylation of peptide 8 with 2-[^{14}C]acetic anhydride to yield [^{14}C]-acetyl peptide 8 ([^{14}C]peptide 8) resulted in no loss of its inhibitory activity as judged by inhibition of complement fixation. Furthermore [^{14}C]peptide 8 has been shown to bind specifically to anti-TMVP (Benjamini *et al.*, 1965).

In an effort to elucidate the antigenic determinant area contained in peptide 8, studies on the degradation of the peptide have been undertaken. The present paper reports the degradation of peptide 8 by digestion with

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[1] Abbreviations used in this work: TMVP, tobacco mosaic virus protein; [^{14}C]peptide 8, 2-[^{14}C]acetyl TMVP tryptic peptide 8; peptide 8 (−4), peptide 8 after four stepwise cleavages from the N-terminal end; peptide 8 (−5), peptide 8 after five stepwise cleavages from the N-terminal end; desarginated peptide 8, peptide 8 after removal of the C-terminal arginine; desarginated peptide 8 (−5), peptide 8 (−5) after removal of the C-terminal arginine; [^{14}C]desarginated peptide 8, 2-[^{14}C]acetyl peptide 8 after the removal of the C-terminal arginine.

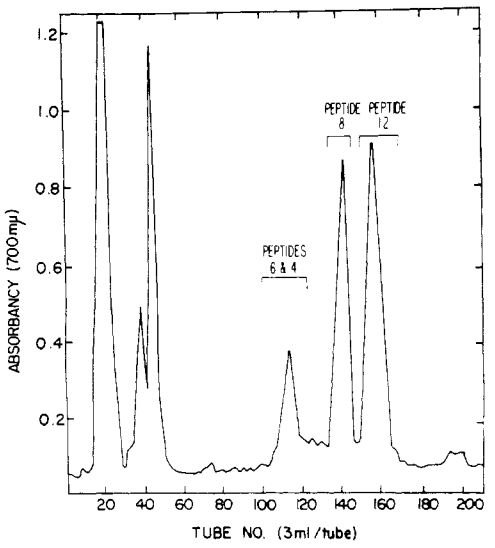

FIGURE 1: Chromatography on Dowex 1 X 2 column (0.9 × 150 cm) of the pH 4.5 soluble tryptic peptides obtained from 100 mg of TMVP. The peptide mixture was applied to the column which was equilibrated with a pH 8.8 collidine–pyridine–acetic acid buffer and eluted by means of an acetic acid gradient elution system (see text); flow rate, 42 ml/hr; temperature, 40°. Folin–Lowry color in 2.2 ml was obtained from 0.1-ml aliquots which were evaporated to dryness prior to analysis.

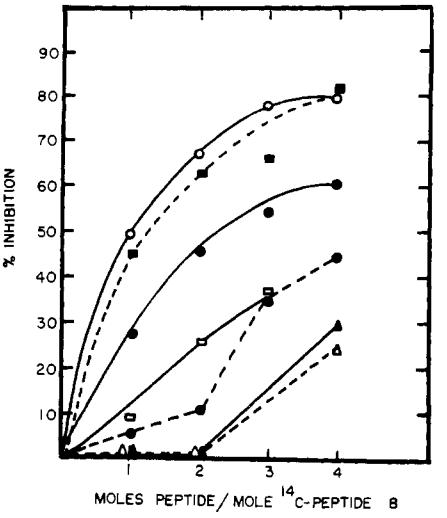

FIGURE 2: Inhibition of [^{14}C]peptide 8-anti-TMVP complex by TMVP tryptic peptide 8, and its degradation products. Globulins derived from 0.5 or 0.7 ml of anti-TMVP were mixed with unlabeled peptides and 3.65 mμM [^{14}C]peptide 8; the complex was precipitated at 50% saturation of ammonium sulfate, the precipitate was washed, dissolved in saline, and radioactivity determined on an aliquot. O———O, expected inhibition of [^{14}C]peptide 8–anti-TMVP complex by peptides having the same binding as [^{14}C]peptide 8 (for explaination see text); ■- - -■, inhibition by tryptic peptide 8; ●———●, inhibition by peptide 8 (−5); □———□, inhibition by desarginated peptide 8; ●- - - -●, inhibition by desarginated peptide 8 (−5); ▲———▲, inhibition by carboxypeptidase A digest of desarginated peptide 8; Δ- - -Δ, inhibition by carboxypeptidase A digest of desarginated peptide 8 (−5). In no case did [^{14}C]peptide 8 bind to control globulins.

pepsin, subtilisin, carboxypeptidases B and A, and stepwise degradation of the peptide from the amino-terminal end, using the Edman phenyl isothiocyanate method. The immunological activities of the resulting peptides were measured by their ability to inhibit the fixation of complement by TMVP and anti-TMVP, by their capacity to inhibit the specific combination of [^{14}C]peptide 8 with anti-TMVP, or by their specific binding to anti-TMVP.

Materials and Methods

Tobacco mosaic virus protein (TMVP) was prepared from tobacco mosaic virus[2] by 67% acetic acid treatment (Fraenkel-Conrat, 1957).

TMVP tryptic peptide 8[3] was obtained from tryptic digestion of TMVP and ion exchange chromatography of the pH 4.5 soluble peptides on a Dowex 1 X 2 column as described by Funatsu (1964), except that for a rapid isolation of peptide 8, the column, after equilibration with the pH 8.8 collidine–pyridine–acetic acid buffer (40:40:0.25 ml, in 4 l.), was developed with increasing concentrations of acetic acid pumped from an autograd (Technicon Chromatography Corp.), with each cylinder loaded with 150 ml of acetic acid at the following concentrations: (1) 0.02 N, (2) 0.02 N, (3) 0.2 N, (4) 0.35 N, (5) 0.5 N, (6) 0.65 N, (7) 0.65 N, (8) 30% (v/v), (9) 50% (v/v). The elution pattern obtained from a tryptic digest of TMVP is given in Figure 1. When amino acid analysis of peptide 8 indicated impurities, the peptide was further purified by precipitation at pH 3.8 at 0° as described by Funatsu *et al.* (1964). Some of the tryptic peptide 8 used in these experiments was obtained from Dr. Duane Gish (now of the Upjohn Co., Kalamazoo, Mich.).

Amino acid analyses were performed on hydrolysates (20 hr, 6 N HCl, 110°, in sealed tubes) using the Spinco Model 120B amino acid analyzer. The columns contained the resin AA-27 for separation of the basic amino acids and AA-15 for the separation of the neutral and acidic amino acids (Spinco Division, Beckman Instruments, Palo Alto, Calif.).

Acetylation of peptides with 2-[^{14}C]acetic anhydride was performed as previously described (Benjamini

[2] The tobacco mosaic virus was generously supplied by Dr C. A. Knight of the Virus Laboratory, University of California Berkeley, Calif.

[3] According to the nomenclature of Tsugita *et al.* (1960).

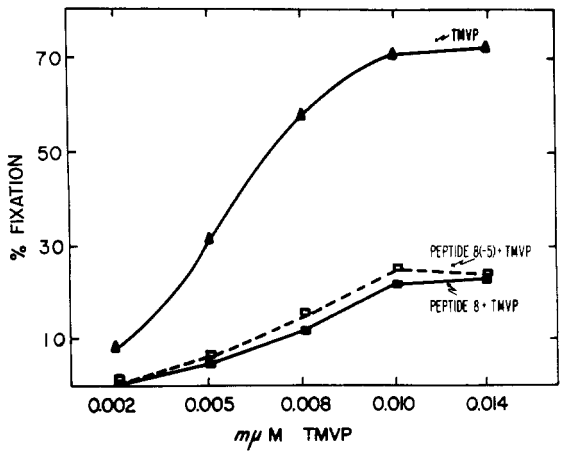

FIGURE 3: Complement fixation by TMVP and 5 μl of anti-TMVP and its inhibition by TMVP tryptic peptide 8 and peptide 8 (−5). ▲———▲, TMVP alone; ■———■, inhibition by TMVP tryptic peptide 8 (5.5 mμM); □- - -□, inhibition by peptide 8 (−5) (5.5 mμM).

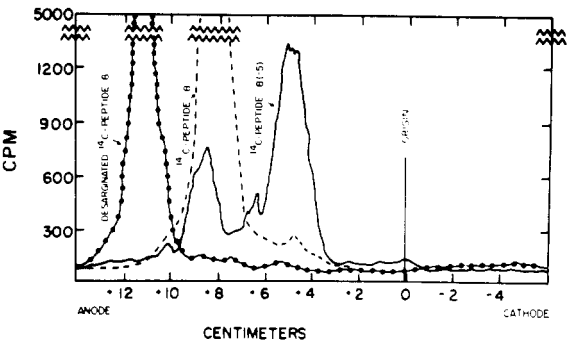

FIGURE 4: Electrophoretic pattern of [^{14}C]acetyl peptide 8 (−5) (———), [^{14}C]acetyl peptide 8 (- - -), and [^{14}C]acetyl desarginated peptide 8 (●-●-●-●). The electrophoresis was performed using a pH 6.4 buffer (pyridine–acetic acid–water (100:4:90)) for approximately 4 hr at 30 v/cm on Whatman 3MM. Radioactivity was scanned using a Nuclear Chicago Model C 100B Actigraph II.

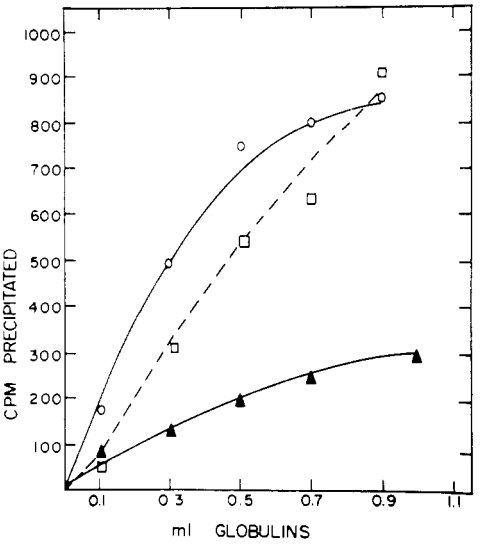

FIGURE 5: The binding of [^{14}C]acetyl tryptic peptide 8 and its [^{14}C]-acetylated degradation products with immune globulins to TMVP. Anti-TMVP globulins were mixed with 3.65 mμM of ^{14}C-acetylated peptides and the complexes precipitated at 50% saturation of ammonium sulfate. The term, ml globulins, represents the volume of serum from which they were obtained. O———O, [^{14}C]peptide 8; □- - -□, [^{14}C]peptide 8 (−5); ▲———▲, [^{14}C] desarginated peptide 8. None of the peptides were found to bind with control globulins.

et al., 1965) except that the [^{14}C]acetyl peptides were separated from [^{14}C]acetic acid by passage through a 2.3 × 37 cm column packed with Bio-Gel P-2 (50–100 mesh, Bio-Rad Laboratories, Richmond, Calif.) equilibrated and eluted with a pH 8 pyridine–collidine–acetic acid buffer (20 ml of pyridine, 20 ml of collidine, and 0.75 ml of acetic acid diluted to 1 l.).

Paper electrophoresis at pH 6.4 and radioactive scanning of electrophoretograms were performed as previously described (Benjamini *et al.*, 1965).

Anti-TMVP serum, anti-TMVP globulins, control serum, control globulins (both antiacetylcholinesterase), and complement fixation inhibition assays were obtained and performed as previously described (Benjamini *et al.*, 1964; 1965).

Assays of specific binding between [^{14}C]acetylated peptides and anti-TMVP globulins were performed as described by Benjamini *et al.* (1965).

The activities of the peptides were tested for their capacity to inhibit the specific reaction between [^{14}C]-peptide 8 and anti-TMVP. The peptides (at molar ratios of 1:4 to [^{14}C]peptide 8) were added to 0.7–1.0 ml of anti-TMVP globulins prior to the addition of 3.65 mμM [^{14}C]peptide 8. The antibody–peptide complexes were precipitated at 50% saturation of ammonium sulfate, and the precipitates were washed twice with 50% saturated ammonium sulfate. The washed precipitates were dissolved in saline and their radioactivity was measured.

Experimental Section and Results

Stepwise N-Terminal Cleavage of Peptide 8. Stepwise cleavage from the N-terminal end of peptide 8 was performed as described by Crestfield *et al.* (1963), except that instead of drying the samples under vacuum, they were air dried in a 40–50° water bath. The amino acid analysis of the residual peptide after five stepwise cleavages of peptide 8, which is designated as peptide 8 (−5), is recorded in Table I. The high molar ratio for glutamic acid, 2.29, rather than 2, indicates the presence of peptide 8 (−4). This preparation was found to be

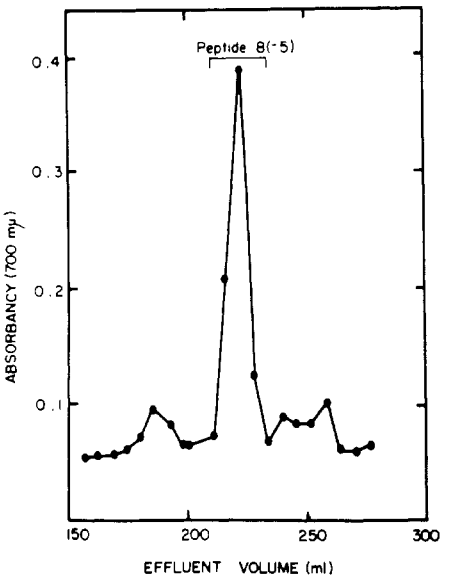

FIGURE 6: Chromatography on Dowex 1 X 2 column (0.9 × 150 cm) of 0.5 μM peptide 8 (−5) which was obtained by five N-terminal stepwise degradations of TMVP tryptic peptide 8. Conditions for chromatography were the same as those described for Figure 1. Folin–Lowry color in 2.2 ml was obtained from 0.5-ml aliquots which were evaporated to dryness prior to analysis.

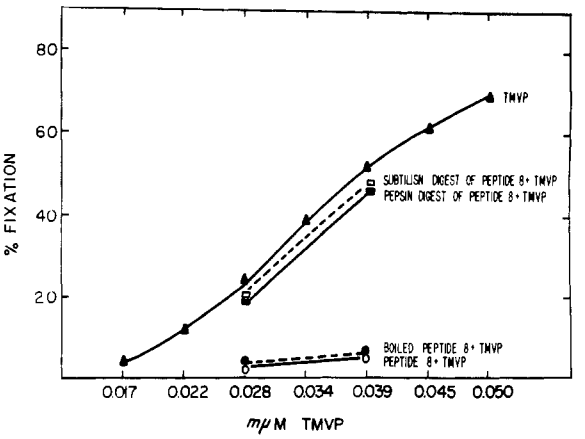

FIGURE 7: Complement fixation by TMVP and 5 μl of anti-TMVP and its inhibition by TMVP tryptic peptide 8 and by the enzymatic digests of the peptide. The peptides were assayed at a concentration of 1.40 mμM. ▲———▲, TMVP alone; O———O, inhibition by TMVP tryptic peptide 8; ●- - -●, inhibition by boiled TMVP tryptic peptide 8; □- - -□, inhibition by subtilisin digest of TMVP tryptic peptide 8; ■———■, inhibition by pepsin digest of TMVP tryptic peptide 8. No complement was fixed by the peptides and anti-TMVP.

immunologically active as evident by its successful competition with [^{14}C]peptide 8 for anti-TMVP (Figure 2), and by its capacity to inhibit the fixation of complement by TMVP and anti-TMVP (Figure 3).

Peptide 8 (−5) was acetylated with [^{14}C]acetic anhydride and subjected to electrophoresis at pH 6.4. Scanning the electrophoretogram for radioactivity revealed two radioactive areas, the major area probably peptide 8 (−5), and a smaller area which migrated more toward the anode, probably corresponding to peptide 8 (−4) which has an additional negatively charged glutamic acid residue (Figure 4). The immunological activity of this [^{14}C]peptide 8 (−5) preparation is evident by its direct binding with anti-TMVP as is shown in Figure 5. No binding occurred between [^{14}C]-peptide 8 (−5) and control globulins.

Ion exchange chromatography of the peptide 8 (−5) preparation gave the elution pattern as shown in Figure 6. The area designated peptide 8 (−5) on the figure was pooled and, as recorded in Table I, gave the theoretically expected amino acid analysis for peptide 8 (−5). The immunological activity of this peptide was tested by measuring its competition with [^{14}C]peptide 8 to anti-TMVP. The peptide exhibited the same activity as peptide 8 (−5) prior to ion-exchange chromatography (Figure 2).

Pepsin and Subtilisin Digestions. Peptide 8 was digested with pepsin using the conditions of Gish (1961). Peptide 8 (0.2 μM) was digested with 0.1 mg of pepsin (Worthington Lot 635, two times crystallized from EtOH) in 1 ml of 0.01 N HCl, pH 1.95, at 37° for 17 hr, at which time the digest was adjusted with ammonia to pH 8.2 and was boiled for 15 min to destroy the enzymatic activity. Assay for activity of the digest re-

TABLE I: Amino Acid Composition of Peptide 8 after Five N-Terminal Cleavages.

	Mole Ratio			
Amino Acid	Peptide 8 (−5) (Exptl)	Peptide 8 (−5) after Ion Exchange Chromatography (Exptl)	Peptide 8 (−5) (Expected)	Peptide 8 (Expected)
Arg	1.02	...	1	1
Asp	2.92	3.00	3	3
Thr	3.75	3.78	4	4
Glu[a]	2.29	2.08	2	*4*
Pro	1.06	1.02	1	1
Ala	2.87	3.00	3	3
Val[a]	Trace	0.0	0	*1*
Ile[a]	Trace	0.0	0	*2*
Leu	1.02	1.00	1	1

[a] Amino acids present in the first five residues of peptide 8.

vealed that it was immunologically inactive since it did not inhibit the fixation of complement by TMVP and anti-TMVP (Figure 7). Peptide 8 which was subjected to a similar treatment but in the absence of the enzyme was active (Figure 7).

Subtilisin digestion was performed using the conditions of Funatsu *et al.* (1964). Peptide 8 (0.2 μM) was digested with 0.1 mg of subtilisin (Nagarse bacterial Al-proteinase, 20 $\times$ 10^4 P.U.N., obtained from Nagase, Osaka, Japan) at room temperature, in a total volume of 1 ml. The pH was adjusted to 8 with 0.2 N NH_3. After 20 min the digest was adjusted to pH 4 with 1 N acetic acid and boiled for 15 min to destroy the enzymatic activity. Assay of this digest by complement fixation inhibition failed to reveal immunological activity (Figure 7).

Desarginated Peptides. Carboxypeptidase B (Worthington, Lot 6052) was treated with diisopropyl flurophosphate (20 μl/6 mg of enzyme in 1 ml) for 1 hr and then dialyzed at 3° *vs.* water for several days. The dialyzed enzyme was diluted to *ca.* 0.6 mg/ml with 10% LiCl.

Peptide 8 was digested at 40° for 1 hr with carboxypeptidase B in 2% $NaHCO_3$ at a concentration of 0.125 μM peptide and 0.02 mg of enzyme/ml. The digest was boiled for 15 min. Complement fixation inhibition assay of the digest revealed that it was immunologically active (Figure 8).

In another series of experiments, peptide 8, [^{14}C]-peptide 8, and peptide 8, (−5) (the peptide 8 (−5) used was the preparation prior to ion exchange chromatography) were each digested in the same manner as described above. The arginine released was determined on aliquots of the digests using the amino acid analyzer (AA-27 column). The residual peptides from the carboxypeptidase B digestion were obtained by passing the digests through 0.9 $\times$ 99 cm or 2.1 $\times$ 42 cm, G-50 Sephadex columns, equilibrated, and eluted with a pH 8, pyridine–collidine–acetic acid buffer (20:20:0.75 ml, respectively, diluted to 1 l.). The location in the eluates of the residual peptides was revealed by passage of a mixture of blue dextran 2000 (Pharmacia, Uppsala, Sweden), desarginated [^{14}C]peptide 8, and arginine through the column. The color of the blue dextran, the radioactivity of desarginated [^{14}C]peptide 8, and the ninhydrin color of the arginine were used to locate the materials. These materials eluted from the 2.1 $\times$ 42 cm G-50 Sephadex column at 40–60 ml for the blue dextran, 60–110 ml for the [^{14}C]desarginated peptide 8, and 130–160 ml for the arginine. The release of arginine and the recovery of the residual peptides from the G-50 Sephadex columns are recorded in Table II.

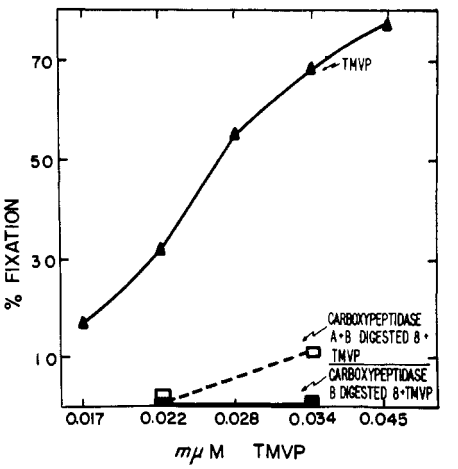

FIGURE 8: Complement fixation by TMVP and 5 μl of anti-TMVP and its inhibition by carboxypeptidase-digested TMVP tryptic peptide 8. ▲———▲, TMVP alone; ■———■, inhibition by carboxypeptidase B digest of peptide 8 (5.5 mμM); □- - -□, inhibition by carboxypeptidase A and B digested peptide 8 (5.5 mμM).

The radioactive areas of electrophoretograms of [^{14}C]peptide 8 and of desarginated [^{14}C]peptide 8 are shown in Figure 4. As expected, [^{14}C]desarginated peptide 8 moved farther toward the anode than did [^{14}C]-peptide 8; no [^{14}C]peptide 8 could be detected in the desarginated [^{14}C]peptide 8 preparation. As little as 2% of [^{14}C]peptide 8 could have been detected if it were present on the electrophoretogram. The immunological activity of [^{14}C]desarginated peptide 8 is shown in Figure 5; the peptide specifically binds with antibodies to TMVP, although its binding is reduced when

TABLE II: The Release of Arginine and Recovery of the Residual Peptide after Carboxypeptidase B Digestion of Peptide 8 and Its Derivatives.

Peptide Digested	Amount Digested (μM)	Arg Released by Carboxypeptidase B (μM)	Arg Released by Carboxypeptidase B % of Expected Release	G-50 Sephadex Column Amount Chromatographed (μM)	G-50 Sephadex Column Amount Recovered (μM)	G-50 Sephadex Column % Recovered
Peptide 8	0.518	0.362	70	0.319	0.278	87
[^{14}C]Peptide 8	0.400	0.392	98	0.350	0.280	80
Peptide 8 (−5)	0.434	0.403	93	0.397	0.336	85

compared to that of [^{14}C]peptide 8. Desarginated peptide 8 and desarginated peptide 8 (−5) exhibited activity, as judged by the finding that both competed, although weakly, with [^{14}C]peptide 8 for anti-TMVP as is seen in Figure 2.

Carboxypeptidase A Digestion Desarginated Peptides. Peptides 8 and 8 (−5) (0.25 μM each) were each digested as previously described with carboxypeptidase B. Following a 45-min digestion, 0.1 mg of carboxypeptidase A (Calbiochem Co., Lot 503162) was added. After 18 hr the preparations were boiled and assayed for immunological activity by complement fixation inhibition. The carboxypeptidase A and B digests of both peptide 8 (Figure 8) and peptide 8 (−5) (not shown) were immunologically active.

In another series of experiments desarginated peptide 8 and desarginated peptide 8 (−5) (both isolated by G-50 Sephadex chromatography from the carboxypeptidase B digest of peptide 8 and peptide 8 (−5) as described in the previous section) were digested at 37° for 19 hr in 2% $NaHCO_3$ with 0.1 mg/ml of carboxypeptidase A; *ca.* 0.1 μM/ml of peptides were digested. The enzyme was added in three aliquots: at 0 time, 1 hr, and 15 hr. The neutral and acidic amino acids released were determined on aliquots of each digest on the amino acid analyzer (AA-15 column). The rest of each digest was passed through a G-50 Sephadex column in the same manner as that described for the recovery of peptides after carboxypeptidase B digestion.

Carboxypeptidase A digestion of desarginated peptide 8 released over 1 mole of threonine, almost 1 mole of alanine, and less than 1 mole of both aspartic acid and leucine/mole of peptide 8. Carboxypeptidase A digestion of desarginated peptide 8 (−5) yielded a residual peptide which on analysis revealed that only the carboxyl threonine residue was completely cleaved. Partial cleavage of the aspartic acid, leucine, and threonine residues on the amino side of the carboxyl threonine was obtained. The immunological activity of both peptides was tested by their capacity to compete with [^{14}C]peptide 8 for antibodies to TMVP. Results presented in Figure 2 show that the preparations were active, since they competed, although weakly, with [^{14}C]peptide 8 for anti-TMVP.

Discussion

Isolation and Identification of Peptides. The peptide 8 (−5) preparation which was obtained by five stepwise degradations from the N-terminal end of peptide 8 gave the expected amino acid analysis as seen in Table I except that the glutamic acid content was high. This indicates that there was some peptide 8 (−4) present. The absence of isoleucine and valine indicated the loss of the first three N-terminal residues. Electrophoresis of this peptide 8 (−5), after acetylation with acetic 2-[^{14}C]acetic anhydride (Figure 4), revealed in addition to the major component another peptide, possibly peptide 8 (−4), since it migrated as a more negatively charged material. Ion exchange chromatography on Dowex 1X2 effected purification of the peptide 8 (−5) as judged from its amino acid analysis (Table I).

The quantitative removal of arginine from [^{14}C]peptide 8 by carboxypeptidase B is shown in Table II. This is corroborated by electrophoresis of desarginated [^{14}C]peptide 8 which shows the absence of [^{14}C]peptide 8 (Figure 4). As little as 2% [^{14}C]peptide 8 in the desarginated [^{14}C]peptide 8 preparation could have been easily detected.

Assays for Immunological Activity. The immunological activities of the peptides which are reported in this communication were ascertained using the technique of the inhibition of the complement fixation by TMVP and anti-TMVP, by direct binding of the [^{14}C]-acetyl peptide with anti-TMVP, or by the competition between the peptide and [^{14}C]acetyl peptide 8 to anti-TMVP. In most cases the activity of each peptide was ascertained by all three methods with comparable results. The assay for immunological activity by complement fixation inhibition and by direct binding with anti-TMVP has been discussed in previous communications (Benjamini *et al.*, 1964, 1965). The assay for immunological activity by the competition between a peptide and [^{14}C]acetyl peptide 8 for anti-TMVP shows not only whether or not a given peptide combines with anti-TMVP but also gives an indication of the binding of the peptide with anti-TMVP in comparison to the binding of [^{14}C]peptide 8. Using this method, data presented in Figure 2 show that the presence of the acetyl group on peptide 8 does not appreciably affect its binding with anti-TMVP. It would be expected that if [^{14}C]acetyl peptide 8 were identical with peptide 8 in binding to anti-TMVP, these two peptides would compete with each other equally for the antibody. If, *e.g.*, instead of treating 0.7 ml of anti-TMVP with 3.65 mμM [^{14}C]acetyl peptide 8 (slight hapten excess), the same amount of antibodies were treated with 3.65 mμM peptide 8 and 3.65 mμM [^{14}C]acetyl peptide 8, only 50% of the total label would be expected to be associated with the antibody. Similarily, if twice the amount of peptide 8 were used, only 33% of the label should be associated with antibody. Thus, a theoretical line for the inhibition of label bound with antibodies by unlabeled peptide can be calculated as shown in Figure 2. That the binding between [^{14}C]acetyl peptide 8 and peptide 8 with antibodies is similar is also shown in Figure 2, where the competition between these two peptides for anti-TMVP follows the theoretical line.

The Immunological Activity of Peptides. Data presented in this report demonstrate that the removal of five of the N-terminal amino acids of peptide 8 does not appreciably affect the immunological activity of the peptide and that the resulting pentadecapeptide having the amino acid sequence: Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg is immunologically active. This was demonstrated by using all three methods which are described above. Data presented in Figure 3 show that the above pentadecapeptide inhibited the fixation of complement by TMVP and anti-TMVP to a degree similar in magnitude to the inhibition caused by the undegraded peptide 8. When peptide 8 (−5) was tested for immunological activity

by its competition with [^{14}C]peptide 8 to anti-TMVP (Figure 2), it was also found active, the competition being somewhat in favor of [^{14}C]peptide 8. The data (Figure 2) indicate that the binding of peptide 8 (−5) with anti-TMVP is approximately half of that of peptide 8. Data presented in Figure 5 demonstrate that the direct binding between anti-TMVP and [^{14}C]peptide 8 (−5) is similar in magnitude (although again somewhat lower) to the direct binding between anti-TMVP and [^{14}C]peptide 8. The somewhat lower binding of 8 (−5) with anti-TMVP than that of peptide 8 may perhaps be due to the smaller size of peptide 8 (−5) rather than due to the removal of an immunologically important amino acid residue or residues from peptide 8.

Experiments on the immunological activity of peptide 8 following the removal of the C-terminal arginine indicated that the desarginated peptide retained activity. A carboxypeptidase B digest of peptide 8 inhibited the fixation of complement by TMVP and anti-TMVP in a magnitude similar to that exhibited by the complete peptide 8, as can be realized from Figure 8. Further experiments dealing with the immunological activity of desarginated peptide 8 following its isolation and characterization by amino acid analysis clearly demonstrate its activity. This was shown by the successful competition of desarginated peptide 8 with [^{14}C]peptide 8 for anti-TMVP (Figure 2) and by the direct binding of [^{14}C]desarginated peptide 8 with anti-TMVP (Figure 5). When desarginated peptide 8 was tested by its competition with [^{14}C]peptide 8 for anti-TMVP (Figure 2) the immunological activity of the desarginated peptide was apparent, although the competition was in favor of [^{14}C]peptide 8. The data presented in Figure 2 indicate that the binding of desarginated peptide 8 with anti-TMVP is approximately one-sixth of that of [^{14}C]peptide 8, since it required approximately six times (on a molar basis) more desarginated peptide 8 than peptide 8 for the equal competition with [^{14}C]peptide 8 for anti-TMVP. Data presented in Figure 5 show that anti-TMVP combines specifically with [^{14}C]desarginated peptide 8 although here again the binding between the [^{14}C]desarginated peptide and anti-TMVP is lower than that between [^{14}C]peptide 8, or between [^{14}C]peptide 8 (−5) and anti-TMVP. However, in view of the high binding of peptide 8 with anti-TMVP [$>10^9$ l./mole, Benjamini *et al.* (1965)] the binding of desarginated peptide 8 with anti-TMVP remains substantial. It is premature to speculate the reason for the reduction in binding of peptide 8 by the removal of arginine; it probably cannot be attributed merely to the reduction in the size of the peptide by one amino acid residue, since the binding of the desarginated peptide with anti-TMVP was similar in magnitude to the binding of desarginated peptide 8 (−5) (Figure 2).

Having ascertained that the removal of the C-terminal arginine from peptide 8 yields an immunologically active peptide, attempts were made to ascertain the role of other C-terminal amino acid residues of peptide 8 in the immunological activity of the peptide. Data

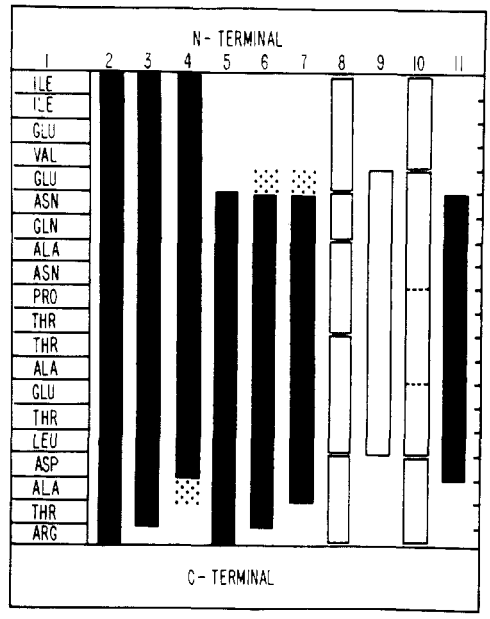

FIGURE 9: Peptides obtained from TMVP tryptic peptide 8 and their immunological activity. Black bars, immunologically active peptides; dotted areas, incomplete removal of these amino acids; open bars, immunologically inactive peptides (not isolated); (1), TMVP tryptic peptide 8 amino acid sequence; (2), peptide 8; (3), desarginated peptide 8; (4), carboxypeptidase A digested desarginated peptide 8; (5), peptide 8 (−5); (6), desarginated peptide 8 (−5); (7), carboxypeptidase A digested desarginated peptide 8 (−5); (8), peptide obtained from subtilisin digestion of peptide 8 by Gish (1961) and Funatsu *et al.* (1964); (9), peptides obtained by pepsin digestion of peptide 8 by Gish (1961); (10), peptides obtained by pepsin digestion of peptide 8 by Anderer and Handschuh (1962) (peptides indicated by the dotted lines were also isolated); (11), smallest immunologically active peptide deduced in this report.

presented in Figure 2 on the competition between [^{14}C]peptide 8 and peptide 8 from which two of the C-terminal residues were removed by treatment with carboxypeptidase B and A show that this peptide having the amino acid sequence: Ile-Ile-Glu-Val-Glu-Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala is still immunologically active. The activity of this peptide was also demonstrated by its capacity to inhibit the fixation of complement by TMVP and anti-TMVP (Figure 8).

From the foregoing it would be expected that the removal of five N-terminal and at least two C-terminal amino acids from peptide 8 should yield a peptide which is immunologically active. That this is so may be realized from data presented in Figure 2 on the successful competition between such a peptide and [^{14}C]peptide 8 for anti-TMVP. Although the competition is greatly in favor of [^{14}C]peptide 8, the immuno-

logical activity of the peptide having the amino acid sequence: Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala is apparent, and is close in magnitude to that of peptide 8 lacking only the two C-terminal amino acids.

That the peptide having the amino acid sequence given above or this peptide lacking the C-terminal alanine contains the sequence responsible for much of the immunological activity of peptide 8 is further indicated from experiments on the destruction of immunological activity of peptide 8 following subtilisin and pepsin digestion. It has been shown by Gish (1961), Anderer and Handschuh (1962), and Funatsu *et al.* (1964) that the digestion of peptide 8 by pepsin or subtilisin results in cleavages in the above proposed active peptide (see Figure 9). Since pepsin and subtilisin digestion of peptide 8 reported in the present communication were done using the conditions of Gish (1961) and Funatsu *et al.* (1964), respectively, cleavage or cleavages in the proposed active peptide are expected. Testing the immunological activity of the subtilisin and pepsin digests of peptide 8 by the complement fixation inhibition method revealed that it was not active (Figure 7). Therefore, sequences important for activity must be present in the peptide: Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala.

An attempt is made in Figure 9 to summarize the results presented in this paper. The black bars represent the part of peptide 8 which has immunological activity. The open bars represent fragments of the peptide which do not have immunological activity. The black bar in column 11 represents the smallest peptide sequence which is deduced from this work to be immunologically active.

The immunologically important characteristics of the active peptide cannot be presently delineated but the size (12 residues), the one proline residue, the total charge of -2, and the hydrophobic groups are possible aspects of the peptide which give it immunological activity.

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