ISOLATION OF ACETYLSERYLTYROSINE FROM THE CHYMOTRYPTIC DIGESTS OF PROTEINS OF FIVE STRAINS OF TOBACCO MOSAIC VIRUS*

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The first experimental approach to the problem of the comparative chemistry of the proteins of several strains of tobacco mosaic virus (TMV) was the amino acid analyses performed by KNIGHT[1,2]. These showed significant differences in many cases. In contrast, C-terminal amino acid analyses of thirteen strains by HARRIS AND KNIGHT[3,4] showed remarkable similarity, since from all strains equal amounts of threonine were split off by carboxypeptidase. NIU AND FRAENKEL-CONRAT[5] employing hydrazinolysis extended this study to the C-terminal sequence of the protein and found the same C-terminal tripeptide in every strain studied. A subsequent isolation of the C-terminal hexapeptide from several strains revealed the first difference in structure between HR on the one hand and common TMV, M and YA, on the other[6]. As previously demonstrated, no N-terminal group could be found in common TMV[7]. The N-acetylseryltyrosine recently isolated from both the chymotryptic and the peptic digests of the protein[8] probably occupies the N-terminal end of the peptide chain. A comparison of five strains of TMV (common TMV, M, J14D1, HR and YA), namely a search for N-acylpeptides, represents the subject of the present investigation.

The present contribution also includes the results of amino acid analyses of the N-acetylpeptide from common TMV protein by Dowex-50 X4 column chromatography[9], and the comparison of the natural and the corresponding synthetic acetylpeptides.

MATERIALS AND METHODS

TMV protein sample

The protein samples were prepared by either the detergent (sodium dodecylsulphate) method of degradation of the viruses into proteins and nucleic acids[7,10], or by the heat-denaturation method[7,11]. The latter method was used to isolate the acetylpeptide from common TMV for amino acid analysis by ion exchange chromatography[9]. 35 ml of the virus solution (32.5 mg/ml) were added to 60 ml of 0.1 *M* NaCl solution at 100° with stirring, and heating was continued for 10 min. After the solution was cooled, the denatured protein was centrifuged and washed with NaCl solution and water. The precipitated protein was suspended in water and digested with chymotrypsin.

The proteins from some strains prepared by the detergent method were kindly supplied by Dr. C. A. KNIGHT of this laboratory.

Isolation of acetylpeptide

About 50 mg of the detergent-treated protein was dissolved in 5 ml of water at pH 10, then the pH was adjusted to 8 and crystalline chymotrypsin (Worthington Biochemical Corp.)

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was added (enzyme to substrate ratio = 1:100). After 24 h at room temperature (pH was maintained at 7.8 ~ 8.0 during digestion), the precipitates resulting at pH 3.8 were centrifuged off. The supernatant was applied to a Dowex-50 X2 column (hydrogen form, 1 × 5 cm) which was then eluted with water. Of each 5 ml, collected automatically, a 0.5-ml aliquot was analyzed by the modified Folin reagent[12]. Folin-positive fractions, which contained the acetylpeptide and were eluted immediately after the hold-up volume, were combined, lyophilized and weighed. The yields of the acidic peptide fraction from the various strains are listed in Table I.

These fractions contained several minor components besides a main component as revealed by paper chromatograms developed with *n*-butanol–acetic acid–water (4:1:1, vol.) and detected with the chlorine–starch–iodide reagent[13]. The R_F values of the spots are listed in Table II. Each peptide was eluted with water from the paper, the eluate was purified by paper chromatography using the same solvent as above and finally lyophilized.

The acid-insoluble precipitate in the chymotryptic digest of the heat-denatured TMV protein was 13.9% of the original virus. The acidic peptide fraction of this digest, separated as usual on a Dowex-50 column (2 × 15 cm), was further purified by a Dowex-2 X10 (chloride form) column, 2 × 15 cm, as previously described[8]. The weight of the acetylpeptide fraction was 1.75% of that of the virus sample used.

TABLE I

YIELDS OF ACIDIC PEPTIDE FRACTION IN THE CHYMOTRYPTIC DIGESTS OF TMV PROTEINS

	Precipitate at pH 3.8 %	*Yield** by Folin color*** %	*Yield** by weight
Common TMV	40.3	1.1	1.7
M strain	41.9	1.2	2.0
J14D1 strain	42.3	1.1	1.5
HR strain	31.4	1.7	2.7
YA strain	48.6	1.6	3.2

* Average values of two experiments are listed. Yields were based on weight of protein used.
** Acetylseryltyrosine isolated from common TMV protein was used for the preparation of standard curve

TABLE II

R_F VALUES AND RELATIVE COLOR INTENSITIES OF PEPTIDES IN THE ACIDIC PEPTIDE FRACTION ON PAPER CHROMATOGRAM*

Spot No.	*1*	*2*	*3*	*4*	*5***	*6*
R_F	*0.00*	*0.19*	*0.30*	*0.57*	*0.69*	*0.74*
Common TMV	trace	trace	weak	trace	very strong	—
M strain	trace	trace	weak	trace	very strong	—
J14D1 strain	trace	trace	weak	trace	very strong	—
HR strain	trace	relatively strong***	weak	trace	very strong	weak
YA strain	trace	trace	weak	relatively strong	very strong	—

* Paper chromatograms were developed with *n*-butanol–acetic acid–water (4:1:1, vol). For detection of the peptides, the chlorine–starch–iodide reagent was used.
** The R_F value of spot No. 5 agreed with that of acetylseryltyrosine.
*** Spot No. 2 of HR was rather streaked.

Structural studies

Aliquots of the peptides were hydrolyzed with redistilled hydrochloric acid in sealed tubes at 100° for 8 h. The amino acids in hydrolysates were converted to dinitrophenyl (DNP) derivatives with 1-fluoro-2,4-dinitrobenzene (FDNB), separated by two-dimensional paper chromatography and estimated by the method of LEVY[14]. Amino acid analyses by ion-exchange chromatography[9] were kindly performed by Dr. L. K. RAMACHANDRAN and Mr. Y. KATO. Occasionally the amount of tyrosine was also estimated colorimetrically using 1-nitroso-2-naphthol[15].

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Hydrazinolysis of the peptides was done at 100° for 10 h in sealed tubes and the evaporated hydrazinolyzates were analyzed qualitatively by descending paper chromatography using collidine–water (10:2, vol.) as described in the previous paper[8]. Two paper chromatograms were prepared, one of which was sprayed with ninhydrin to characterize the C-terminal amino acid and the other with an alkaline silver nitrate reagent[16] to detect both the amino acid hydrazide and the acylhydrazide.

Carboxypeptidase digestion of the peptide was carried out at pH 7.8 with an enzyme to substrate ratio of 1:50.

Synthesis of acetyl-DL-seryl-L-tyrosine

N-acetyl-DL-serine. One g of DL-serine was dissolved in 5 ml of 2 *N* NaOH and 2.6 ml of acetic anhydride and 26 ml of 2 *N* NaOH were added alternatively at 0° with shaking. Then the reaction mixture was kept overnight at room temperature. The mixture was treated with a Dowex-50 X2 column (*ca.* 300 ml of acid form) and the column was washed with water until the effluent became neutral. Under these conditions sodium ions and unreacted amino acid were retained on the resin. It was easy to see whether the amount of resin was sufficient by watching the color change of the resin. The effluent was quickly frozen to avoid any acyl shift of the acetyl group from the N to the O atom, and then lyophilized. A partially crystalline sticky material was obtained. It was dissolved in absolute ethanol and crystallized by adding ether and recrystallized from the same solvent system. One g (68%) of N-acetyl-DL-serine was obtained melting at 129 ~ 130°. SYNGE[17] synthesized this compound but did not crystallize it. The R_F value on the paper chromatogram (*n*-butanol–acetic acid–water, 0.5% bromothymol blue solution neutralized to pH 7) was 0.62. Anal.: calcd. for $C_5H_9O_4N$: C, 40.89; H, 6.17; N, 9.52. Found, C, 40.93; H, 6.07; N, 9.49.

N-acetyl-DL-seryl-L-tyrosine methyl ester. 294 mg (2 mmole) acetyl DL-serine and 390 mg (2 mmole) L-tyrosine methyl ester (free form, prepared from its hydrochloride) were dissolved in 15 ml of tetrahydrofuran containing 1 ml of water. To this solution 425 mg (2.2 mmole) of dicyclohexylcarbodiimide[18] was added, the solution was kept for 4 h at room temperature and overnight in the refrigerator. One drop of acetic acid was added, the mixture kept 30 min at room temperature, and the precipitate of the urea derivative finally filtered. The filtrate was evaporated at room temperature, the residue was taken into ethyl acetate and a small amount of insoluble material was removed. The filtrate was washed consecutively with dilute $NaHCO_3$ (saturated with NaCl) and dilute HCl (saturated with NaCl) and dried over sodium sulfate. On evaporation a syrupy material was obtained, which failed to crystallize and was dried over P_2O_5. Yield was 260 mg (40%).

Anal.: Calcd. for $C_{15}H_{20}O_6N_2$: N, 8.64. Found, N, 8.72.

N-acetyl-DL-seryl-L-tyrosine. N-acetyl-DL-seryl-L-tyrosine methyl ester (240 mg) was dissolved in methanol (5 ml) and *N* NaOH (2 ml) was added. The mixture was kept at room temperature for 2 h and was acidified to pH 1.7 with HCl. The organic solvent was removed at room temperature, the solution was saturated with NaCl and shaken with ethylacetate. The peptide derivative was extracted with $NaHCO_3$, the solution was acidified, saturated with NaCl and extracted with ethylacetate. The solution was dried over Na_2SO_4 and evaporated. Only 42 mg (18%) of the compound was obtained in amorphous form. It was found by paper chromatography that the material still contained a minor impurity and it was purified on a Dowex-2 column as described in the previous paper[8]. The yield in this step was 60%.

Anal.: Calcd. for $C_{14}H_{18}O_6N_2$: N, 9.03. Found, N, 9.28.

N, O-diacetyl-DL-serine. 1 g of DL-serine was dissolved in 5 ml of 2 *N* NaOH and 1.5 ml of acetic anhydride and 15 ml of 2 *N* NaOH were added alternatively with shaking at room temperature. Then 1.5 ml more of acetic anhydride was added and shaken for 4 h at room temperature to acetylate also the hydroxyl group. The mixture was acidified with HCl to pH 1.7, saturated with NaCl and extracted 10 times with 10 ml of ethylacetate. After drying the solution over Na_2SO_4, the solvent was removed *in vacuo*. The oily material obtained was cooled, scratched and thus crystallized. After recrystallization from a small amount of water, 450 mg (25%) of the compound was isolated which melted at 136 ~ 7°. The R_F value on a paper chromatogram was 0.77 (butanol–acetic acid–water). Anal.: Calcd. for $C_7H_{11}O_5N$: C, 44.44; H, 5.86; N, 7.40. Found, C, 44.37; H, 5.82; N, 7.46.

N, O-diacetyl-DL-seryl-L-tyrosine. This compound was prepared by the same technique as N-acetyl-DL-seryl-L-tyrosine. The methyl ester of the diacetyldipeptide was not analyzed. The free peptide was obtained in 43% yield but it contained trace amounts of a contaminant and was purified by paper chromatography. The material isolated was insufficient for analysis but it was found that the peptide contained 1 mole each of serine and tyrosine by amino acid analysis after hydrolysis.

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RESULTS

Comparison of acidic peptides from five strains of TMV

The paper chromatographic patterns of the acidic peptides from the chymotryptic digests of the proteins from all strains were almost the same except for the spots No. 2 and No. 6 in HR and the spot No. 4 in YA strains (Table II). All of the peptides were negative to ninhydrin and were detected by the chlorine–starch–iodide reaction[13]. In the present paper, spot No. 5 and spot No. 3 were studied. The eluate of the main component of the chromatograms of all strains, spot No. 5 showed the typical ultraviolet absorption spectrum of tyrosine with an absorption maximum at 274 mμ in acid and 294 mμ in alkaline solutions. Amino acid analyses revealed only tyrosine and serine in the approximate molar ratio of 1:1 in every strain.

The paper chromatograms of the hydrazinolysates of the main components of every strain, obtained by developing with collidine–water, gave completely the same pattern as that for acetylseryltyrosine which was previously isolated from common TMV protein[8]. Alkaline silver nitrate revealed spots corresponding to hydrazine (R_F 0.00), serine hydrazide (R_F 0.054), acetyl hydrazide (R_F 0.32) and an artifact (R_F 0.27) which might be derived from either serine or serine hydrazide[8]. Ninhydrin revealed spots corresponding to tyrosine (R_F 0.02) and to serine hydrazide. The free tyrosine must be liberated from the C-terminal position of the peptide by the action of hydrazine. Therefore the structure of this peptide should be acetylseryltyrosine.

Amino acid analysis of spot No. 3 in every strain showed that it consisted of 2 moles of glutamic acid and 1 mole of threonine. Consequently this peptide seems to have no connection with the acetylpeptide. To investigate the structure of this interesting peptide, isolation of more of the material was attempted. The dipolar peptide fraction (677 mg) of the chymotryptic digest of common TMV protein, which was retained on a Dowex-50 column during fractionation of the acetyl peptide was eluted with ammonia. The eluate was then evaporated, the residue redissolved in water and heated at 100° at pH 6.6 for 1 h. The solution was again applied to the acid form of a Dowex-50 X2 column and washed with water. An acidic peptide fraction was again obtained, in a yield of 1.3% (by weight). It was observed that this fraction contained several ninhydrin-negative but chlorine–starch–iodide-positive components on the paper chromatogram developed with *n*-butanol–acetic acid–water. For the separation of these peptides a Dowex-2 X10 column (chloride form) was used in similar manner as used for the purification of the acetylseryltyrosine[8]. The elution curve is shown in Fig. 1. Peak A, from fractions 32 to 39, was lyophilized and 0.63 mg (9.3% of the acidic fraction) of an almost pure peptide was obtained. Judging from the R_F value on the paper chromatogram, it corresponded to spot No. 3. It was further purified by paper chromatography. The N-terminal analysis of this peptide gave a negative result by the FDNB method. A part of this peptide was incubated with carboxypeptidase and the 24-h digest was analyzed by one-dimensional paper chromatography using *n*-butanol–acetic acid–water as solvent. Strong glutamine (R_F 0.18) and weak threonine (R_F 0.29) spots were revealed by ninhydrin. Furthermore chlorine–starch–iodide revealed two additional spots, one of which was quite faint and corresponded to pyroglutamic acid (pyrrolidone carboxylic acid) (R_F 0.66) while the other strong spot (R_F 0.54) seemed to be a dipeptide which was produced from the original tripeptide by the action of carboxy-

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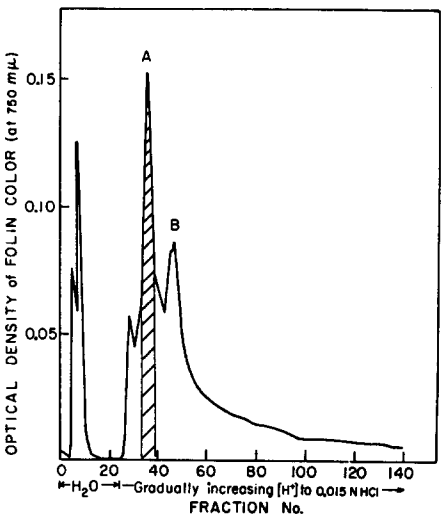
peptidase. From the above results this peptide was tentatively identified as pyroglutamylthreonylglutamine. To confirm this structure, the amino acids released from the peptide by carboxypeptidase were estimated as DNP-derivatives[19] after various times of incubation (Fig. 2). The small amount of DNP-glutamic acid, which was observed, might arise from glutamine during the reaction with FDNB and the sum of the DNP-glutamine and DNP-glutamic acid was therefore also plotted. The amount of peptide used was calculated from the estimation of DNP-threonine in the complete hydrolysate of an aliquot of the digest. The result of Fig. 2 confirmed that the amino acid sequence of this peptide was pyroglutamylthreonylglutamine. Peak B in Fig. 1 was also studied but clear-cut results could not be obtained.



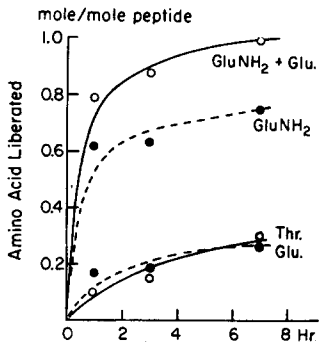


Fig. 2. Amino acids released from the pyroglutamyl peptide by carboxypeptidase; estimated as their DNP-derivatives.

Fig. 1. Fractionation of acidic peptide fraction prepared from heated dipolar peptide mixture of the chymotryptic digest (see text). 7.2 mg of the sample was chromatographed on a 1 × 23 cm column of chloride form of Dowex-2 X10. The effluent was collected in 2-ml fractions.

Amino acid analysis of acetylpeptide from common TMV protein by various methods

Previous preparations[8] of the acetylpeptide contained 41% of non-peptidic impurities after purification by a Dowex-2 column and the purity of the present preparation was 74%, as calculated from the UV absorption of the tyrosine residue. Further purification was attempted by paper chromatography using paper prewashed with the same solvent used as developer. The peptide was eluted from the paper with water, lyophilized, dissolved in absolute ethanol and precipitated with ether. This procedure, however, did not appreciably increase the purity of the peptide.

After hydrolysis of the peptide with twice-distilled constant-boiling hydrochloric acid at 110° C for 12 hours, the product, in both acid and alkaline solutions, showed marked UV absorption at and above 300 mμ. The peptide, before hydrolysis, did not absorb UV light at 325 mμ and showed little absorption at 300 mμ. The amount of tyrosine in the hydrolysate was measured by using the base line derived from extrapolation of the slope of absorption between 300 and 350 mμ. This value agreed with the colorimetric tyrosine estimation with the 1-nitroso-2-naphthol reagent[15]. In contrast, the tyrosine color value of the intact peptide corresponded to only 74%

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of the UV absorption value. This discrepancy may be due to the solubility of the colored peptide derivative in dichloroethylene which was used to extract the excess reagent after the reaction.

In previous amino acid analysis of the peptide[8] by the FDNB method, 0.81 and 0.67 moles of serine and tyrosine per mole of the peptide were found, respectively, as averages of four experiments. Column chromatography[9] of previous preparations[8] gave 1.14 and 0.85 moles for these two amino acids as average values of two analyses without correction for destruction during hydrolysis. The present preparation, when analysed by the latter method, gave 0.99 and 0.94 moles of serine and tyrosine (average of two analyses) respectively without correction, in support of the belief that this peptide is a dipeptide of serine and tyrosine.

Comparison of natural and synthetic acetylseryltyrosine

Neither the natural nor the synthetic acetylseryltyrosine could be obtained in crystalline form. These were therefore carefully compared by a variety of methods:

1. *R_F value.* R_F values of the natural and synthetic N-acetylseryltyrosine and N,O-diacetylseryltyrosine were 0.70, 0.70 and 0.82, respectively, on paper chromatograms developed with *n*-butanol–acetic acid–water (4:1:1).

2. *Position of DNP-derivative on paper chromatogram.* The peptides gave ethyl acetate soluble and colorless DNP-derivatives[8]. The natural and synthetic N-acetylpeptides were located at the same position as DNP-serine on the two-dimensional paper chromatogram prepared by the method of LEVY[14]. The DNP-N,O-diacetyl peptide was located just below the dinitrophenol spot. The UV-absorption spectra of these three DNP-peptides were the same as that of O-DNP-tyrosine[8,20].

3. *Mobility on a paper electrophoregram.* Both the natural and synthetic monoacetyl peptides migrated 4.7 cm from the starting line towards the anode, using Whatman No. 3 MM paper, collidine–acetic acid buffer of pH 6.7, and 8 V/cm and 6 h.

4. *Position of peak from Dowex-2 column.* The natural and synthetic N-acetylpeptides were applied to a Dowex-2 column (2 × 15 cm, chloride form) and eluted with 0.015 *N* hydrochloric acid as shown in Fig. 3. Both elution curves were the

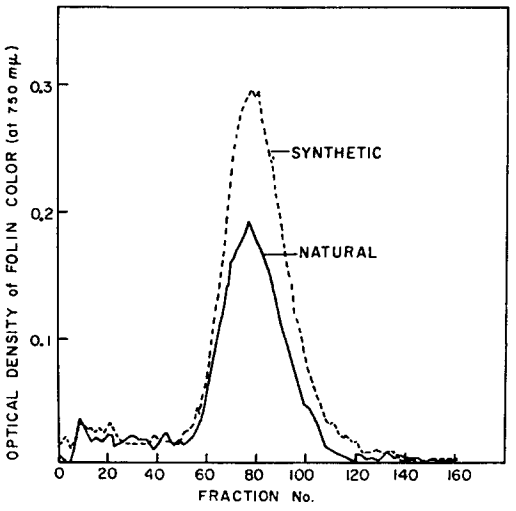

Fig. 3. Elution curves of the natural and synthetic N-acetylseryltyrosine from Dowex-2 X10 column (1 × 20 cm, chloride form). 0.41 mg of the natural and 0.65 mg of synthetic peptides were applied to the column separately, eluted with 0.015 *N* hydrochloric acid and the effluent was collected in 2-ml fractions. Recovery of the sample was about 90% in both cases.

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same and the elution curve upon chromatography of a mixture of the two *N*-acetylseryltyrosine samples also showed a similar pattern (not illustrated).

5. *Tyrosine color value with 1-nitroso-2-naphthol*[15]. As already described the acetylpeptide gave a tyrosine color value lower than expected. Both the natural and synthetic N-acetylpeptides gave the same amount of color, namely 73.9 and 74.1 % of the tyrosine content as estimated by UV absorption, respectively.

6. *Folin color*[12]. When the Folin color value of the natural peptide was taken as unity, that for the synthetic N-acetylpeptide was 0.96.

7. *Formic acid-treated product.* If the natural acetylpeptide was N-acetylseryltyrosine, an acyl shift should be possible under certain conditions, leading to O-acetylseryltyrosine *via* an assumed oxazoline compound as an intermediate[21-23]. Both natural and synthetic N-acetylpeptides were dissolved in anhydrous formic acid[23], the mixtures kept for 4 h at room temperature, lyophilized and applied to paper. A new spot appeared on the paper chromatogram developed with butanol–acetic acid–water, which had an R_F value of 0.77 in both cases. The acid-treated product was regenerated to the original peptide after incubation with 10 % ammonia for 4 h at room temperature. The N,O-diacetylpeptide did not change in chromatographic behavior under the above conditions.

The results described above, confirmed the previous conclusion that the natural acetylpeptide was N-acetylseryltyrosine.

DISCUSSION

Consideration of the results given in the present paper enable one to conclude that common TMV and four of its strains (M, YA, J14D1, and HR) contain the same acetylpeptide in their polypeptide chains, namely N-acetylseryltyrosine. This structure of the peptide suggested in the previous paper[8] was confirmed by the amino acid analysis with ion exchange chromatography and by the comparison with synthetic N-acetylseryltyrosine in various respects.

Differences of the proteins of various strains of TMV had been demonstrated by amino acid analyses[1,2], by the peptide pattern of the tryptic digests[24] (HR and YA), and by isolation of the C-terminal hexapeptide (HR)[6]. In the present studies, minor differences in the chromatographic patterns of the acidic peptide fractions from the chymotryptic digests were also observed for HR and YA (Table II) in accordance with the previous findings described above.

The pyroglutamylthreonylglutamine, demonstrated in the acidic fractions of the chymotryptic digests of five strains, might be derived from the corresponding glutaminyl peptide by cyclization during ion-exchange treatment. C-terminal glutamine peptides have not been previously found in chymotrypsin digests but would easily be missed in a hydrazinolysate. The present method to isolate N-acylpeptides seems to be applicable to all proteins which lack N-terminal groups. However, the present results have shown that not all acidic peptides need be acylpeptides, and in particular the presence of a glutamic acid residue in such a peptide suggests that it may be a pyroglutamyl peptide. As is well known, glutamine and glutaminyl peptide are quite labile even in neutral solution and they have the tendency to cyclize to pyroglutamyl residue[25-27]. Slight acid and a higher temperature accelerate this tendency. Actually more pyroglutamyl peptide was obtained from the dipolar

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peptide fraction, which was retained on the Dowex-50 column, after heating it at 100° and pH 6.6 for 1 h. Therefore these procedures are also available to isolate glutaminyl peptides from a peptide mixture as pyroglutamyl peptides. SANGER[28] also found both the glutaminyl and the corresponding pyroglutamyl peptides in a chymotryptic digest of oxidized A chain of insulin, which was heated to inactivate the enzyme.

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SUMMARY

Acetylseryltyrosine was isolated from the chymotryptic digests of the proteins of five tobacco mosaic virus strains: common TMV, M, J14D1, HR and YA. N-acetylseryltyrosine and N,O-diacetylseryltyrosine were synthesized. A comparison of these with the natural peptide in various respects confirmed that the natural acetylpeptide was N-acetylseryltyrosine. Small amounts of pyroglutamylthreonylglutamine were also detected in the chymotryptic digests of the TMV proteins in every strain. This peptide was probably derived from glutaminyl threonylglutamine by the catalytic action of the ion-exchange resin, which was employed during fractionation of the peptide.

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