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Preferential Release of Aspartic Acid by Dilute Acid Treatment of Tryptic Peptides*

Chun Ming Tsung† and H. Fraenkel-Conrat

ABSTRACT: The kinetics, mechanism, and degree of specificity of cleavage and release of aspartic acid in 0.03 N HCl at *p*H 2.0 and at 105° was studied with several tryptic peptides obtained from the protein of tobacco mosaic virus. It was observed that aspartyl or asparaginyl peptide bonds were cleaved at both sides and free aspartic acid was liberated. Aspartic acid was released according to first-order kinetics, and much more slowly from asparaginyl than from aspartyl peptide bonds. This suggests that asparaginyl peptide bonds must be deamidated prior to the release of the free amino acid. Traces of other amino acids were also released with no particular predominance of serine and threonine, but with predominance of alanine when this amino acid occurred adjacent to aspartyl but not to asparaginyl peptide bonds. If the dilute acid hydrolysis of peptides was carried out for relatively short times, more than half the aspartic acid residues were liberated as free amino acids, but the nonspecific cleavages at sites other than aspartic acid residues were minimal. Thus, dilute acid hydrolysis may be successfully applied to the structural analysis of peptides.

The remarkable selectivity of dilute acid at 100° in preferentially liberating free aspartic acid from proteins has been known since 1950 (Partridge and Davis, 1950). This effect is a function of the *p*H of the solution and not of the anion concerned (Leach, 1955). Although some studies suggested that the specificity of the reaction was insufficient for its useful application to the structural analysis of proteins (Thompson, 1960; Naughton *et al.*, 1960), the reaction has recently been reinvestigated with more success by Schultz *et al.* (1962). However, our understanding of this reaction is incomplete, and its usefulness has been obscured by the size of the proteins studied, and by the use of too extensive degradation conditions.

The present study represents a reinvestigation of the kinetics, mechanism, and specificity of this reaction, with particular reference to the action of 0.03 N hydrochloric acid on aspartic acid residues in several peptides obtained from the tryptic digestion of the protein of tobacco mosaic virus (for a preliminary report see Tsung and Fraenkel-Conrat, 1963). The kinetics and mechanisms of this reaction were studied extensively at early stages of this investigation. Subsequently, its specificity was investigated by recovering peptide fragments from partial hydrolysates using chromatographic separation techniques which have been developed in the course of these studies. The better knowledge of this reaction has extended its applicability to the structural analysis of tryptic peptides and has supplied confirmatory data concerning the amino acid sequence of tobacco mosaic virus (Tsung *et al.* 1964).

Materials and Methods

Materials. Peptides were obtained by tryptic digestion of the protein of tobacco mosaic virus (TMV pro-

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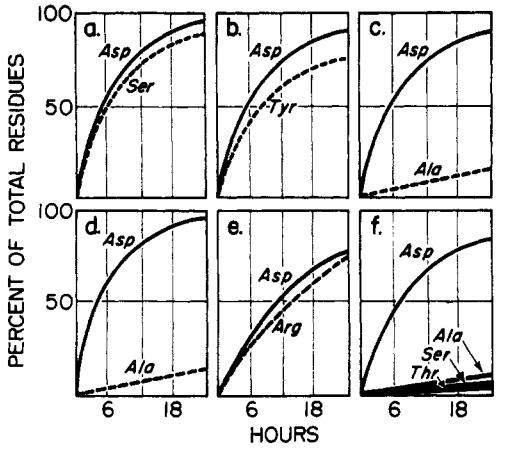

FIGURE 1: Liberation of amino acids from tryptic peptides upon partial hydrolysis in 0.03 N HCl. (a) Peptide 4, Phe-Pro-Asp-Ser-Asp-Phe-Lys. (b) Peptide 6, Tyr-AspNH_2-Ala-Val-Leu-Asp-Pro-Leu-Val-Thr-Ala-Leu-Leu-Gly-Ala-Phe-Asp-Thr-Arg. (c) Peptide 8, Ileu-Ileu-Glu-Val-Glu-AspNH_2-GluNH_2-Ala-AspNH_2-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg. (d) Peptide 9, Arg-Val-Asp-Asp-Ala-Thr-Val-Ala-Ileu-Arg. (e) Peptide 11, Gly-Thr-Gly-Ser-Tyr-AspNH_2-Arg. (f) TMV protein.

tein)[1] prepared according to the cold acetic acid method (Fraenkel-Conrat, 1957). They were separated chromatographically on Dowex 1-X2 columns and further purified by paper chromatography and paper electrophoresis (Tsung *et al.*, 1964). The number identifying the peptides refers to their sequential position on the polypeptide chain of TMV protein starting from the N terminus.

Dilute Acid Hydrolysis. The peptides to be studied were dissolved with 0.03 N HCl to a final concentration of 1 mg/ml, unless otherwise mentioned, and hydrolyzed in evacuated sealed tubes at 105° for the desired time.

Amino Acid Analysis. Amino acids were determined by a Beckman/Spinco automatic amino acid analyzer, Model 120, according to Spackman *et al.* (1958), after hydrolysis of peptides at 105° for 24 hours with glass-distilled constant-boiling HCl in evacuated sealed tubes.

End-Group Analysis. The dinitrophenylation (Sanger, 1945) and chromatographic separation for determination of N-terminal amino acids were standard (Fraenkel-Conrat *et al.*, 1955). DNP-aspartic acid and DNP-glutamic acid were further separated by paper chromatography using tertiary amyl alcohol–*p*H 5 phthalate (Blackburn and Lowther, 1951). Hydrazinolysis (Akabori *et al.*, 1952) as modified by Funatsu *et al.* (1964) was used to determine C-terminal amino acids.

Amide Content of Peptides. The amide content of peptides was determined by subtracting from the total ammonia content of the acid hydrolysate the adventitious ammonia detected in control aliquots of the peptide sample. To remove contaminating free ammonia, the peptides (0.1–0.5 μmole) were dissolved with 0.5 ml of 0.2 M sodium carbonate–bicarbonate buffer, *p*H 9.2, and dried over concentrated sulfuric acid *in vacuo* overnight. The dried material was then briefly hydrolyzed (6 hours with 1 ml of 6 N HCl at 105°) to minimize the decomposition of serine and threonine. The hydrolysates were dried by rotatory evaporation and the ammonia content was determined by means of the 15-cm column of the automatic amino acid analyzer. For the control analyses, the same amounts of reagents were carried through the entire procedure, excepting the hydrolysis with 6 N HCl, and analyzed for free ammonia present in the peptide sample or reagents.

Paper Chromatography and Paper Electrophoresis. Paper chromatography and paper electrophoresis were used for the purification of the peptides. The papers were Whatman No. 1 and/or Whatman 3MM which had been prewashed with 1% oxalic acid, water, and ethanol. The solvents used for paper chromatography were 1-butanol–acetic acid–water–pyridine (30:6:24:20) (Waley and Watson, 1953) and the organic phase of *t*-amyl alcohol–1 N ammonia (1:1) (Funatsu *et al.*, 1964). Paper electrophoresis was performed at 2000 v for 1–2 hours on Whatman 3MM paper using a pyridine acetate buffer of *p*H 6.5 (100 ml of pyridine and 4 ml of acetic acid diluted to 1 liter).

Ion-Exchange Chromatograph. Ion-exchange chromatography of the peptides or dilute acid hydrolysates of peptides was performed on Dowex 1-X2 columns (0.9 × 60 cm) (Tsung *et al.*, 1964). In some cases, a 15-cm column of Amberlite IR 120 was used with the automatic amino acid analyzer. For this purpose the peptides or dilute acid hydrolysates of peptides were adjusted to *p*H 2.0 with 0.1 N HCl and applied together with 2 drops of 1% phenol red solution to a 0.9 × 15-cm column of Amberlite IR 120 (Beckman resin Type 50A), which was preequilibrated with the starting buffer (0.05 M pyridine, 0.1 N acetic acid, *p*H 3.8). Chromatography was carried out at 52° with a flow rate of 30 ml/hour. The solvent system employed an Autograd (obtained from Technicon Chromatography Corp., Chauncey, N.Y.) consisting of 75 ml each of starting buffer in chambers 1 and 2, and a final buffer (2.5 M pyridine and 2.0 N acetic acid, *p*H 5.18) in chamber 3. The column was further developed with another 50 ml of final buffer. The details of setting up and operating the automatic peptide analyzer, devised by modifying the stream divider accessory for the Model 120 Beckman/Spinco amino acid analyzer (Beckman Technical Bulletin, 1962), will be reported elsewhere by C. M. Tsung.

Results

Hydrolysis of Tryptic Peptides of TMV Protein. The rate of release of amino acids from the tryptic peptides 4, 6, 8, 9, and 11 and from TMV protein are presented in Figure 1. It can be seen that aspartic acid was rapidly released from each peptide. The initial rate of release

[1] Abbreviation used in this work: TMV, tobacco mosaic virus.

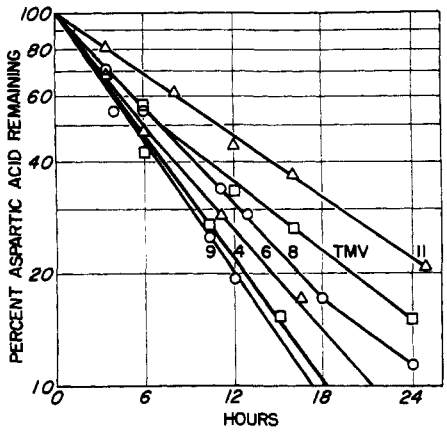

FIGURE 2: First-order plot of the liberation of aspartic acid from tryptic peptides (peptides 4, 6, 8, 9, and 11) and TMV protein during partial hydrolysis in 0.03 N HCl. The number along the line identifies the corresponding tryptic peptide number on the polypeptide chain.

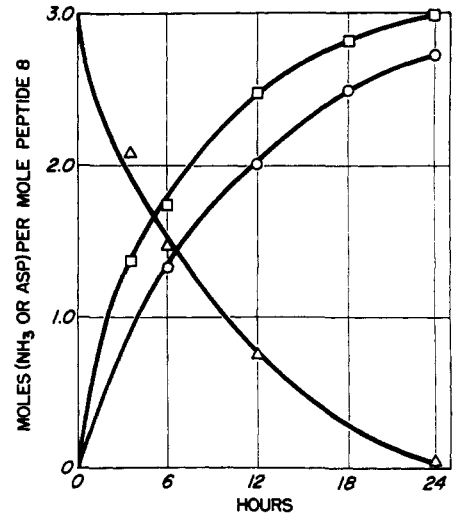

FIGURE 3: Rate of liberation of aspartic acid and ammonia from tryptic peptide 8 during partial hydrolysis in 0.03 N HCl. (-O-O-), free aspartic acid liberated; (-□-□-), free ammonia liberated; (-△-△-), remaining bound ammonia.

of aspartic acid was more than 100 times that of other amino acids, the latter appearing in significant amounts (but less than 10%) after the release of one-half of the total aspartic acid. An expected exception to this statement, however, was that certain amino acids were rapidly and quantitatively released from certain peptides because they were either N-terminal or C-terminal and followed by an aspartic acid residue, or because they were located between two aspartic acid residues (see legend for Figure 1). This was the case for the N-terminal tyrosine from peptide 6, the C-terminal arginine from peptide 11, and serine from peptide 4 (-Asp-Ser-Asp-).

Another exception is alanine which was released in small but significant amount from certain peptides. This was the case for Asp-Ala-Thr (in peptides 8 and 9), possibly because alanine is adjacent to aspartyl residues in these peptides. This, however, was not the case for $AspNH_2$-Ala-Val (in peptide 6), possibly because alanine is adjacent to an asparaginyl residue which is released much more slowly than aspartyl residues. In addition, Ala-Val might be more acid resistant than Ala-Thr.

The first-order plot of the aspartic acid released from each tryptic peptide and TMV protein indicates, as shown in Figure 2, that (1) free aspartic acid is released according to first-order kinetics, (2) the $t_{1/2}$ found for the cleavage of aspartyl peptide bonds (peptides 4 and 9) is 5.5 hours, while that of asparaginyl peptide bonds (peptide 11) is 11 hours, and that of the amide bond is on this basis calculated to be approximately 5.5 hours, (3) the first-order plot for peptides 6 and 8 (containing both aspartyl and asparaginyl residues) seems to be represented by two lines instead of one straight line. The point of intersection is near the $t_{1/2}$ point of the cleavage of aspartyl peptide bonds. This may be attributed to the difference in the rate of release of aspartic acid from aspartyl and from asparaginyl residues.

The half-lives for this reaction for various peptides and proteins are tabulated in Table I. The data indicate that the values calculated on the basis of $t_{1/2}$ for aspartyl

TABLE I: Half-Life Period ($t_{1/2}$) of the Cleavage of Aspartyl Peptide Bonds and of Asparaginyl Peptide Bonds.

Tryptic Peptides or Proteins Used	Moles of Aspartic Acid Residues per Mole Peptide or Protein Used		$t_{1/2}$ (hours)[a]	
	Asp	$AspNH_2$	Calculated	Found
11	0	1	11.0	11.0
10[b]	1	1	8.3	24.0
8	1	2	9.2	6.2
1[c]	1	3	9.6	12.5
4	2	0	5.5	5.5
9	2	0	5.5	5.0
6	2	1	7.3	5.8
TMV protein	9	9	8.3	7.5

[a] The $t_{1/2}$ was calculated by assuming $t_{1/2}$ of aspartyl peptide bond cleavage to be 5.5 hours and $t_{1/2}$ of asparaginyl peptide bond cleavage to be 11 hours. [b] Peptide 10 was not freely soluble in 0.03 N HCl. [c] Peptide 1 was treated with 0.03 N HCl in 40% acetic acid instead of 0.03 N HCl.

residues to be 5.5 hours and $t_{1/2}$ for asparaginyl residue 11 hours are in most cases very close to the observed $t_{1/2}$ values. Where they differ appreciably, this disparity may be attributed to the insolubility of the peptide in 0.03 N HCl and/or to the peculiar sequence adjacent to aspartic acid residues. When the release of free ammonia and free aspartic acid from peptide 8 was studied in detail, the $t_{1/2}$ of amide bond hydrolysis was found to be 5 hours, which is in agreement with the calculated value (Figure 3). The rate of hydrolysis of the amide groups from asparaginyl or from glutaminyl residues seems to be the same as judged from the foregoing result (peptide 8 contains 1 glutaminyl and 2 asparaginyl residues).

End-Group Analysis of Dilute Acid Hydrolysates of Peptide 8. End-group analyses were performed on dilute acid hydrolysates of peptide 8 in order to ascertain how specific the preferential hydrolysis was and whether a consecutive attack on one or the other side of the aspartic acid residues could be detected. Thus peptide 8 was treated with 0.03 N HCl at 105° for various times, and half of the hydrolysate was submitted to automatic amino acid analysis while the other half was used for end-group analysis. The N-terminal residues of peptides formed by dilute acid hydrolysis of peptide 8 were determined as the DNP derivatives while the free amino acids liberated were removed as DNP-amino acids in the organic phase. The analyses are summarized in Table II. The absorbance at 360 mμ of each N-terminal residue was compared to that of N-terminal isoleucine of peptide 8. Since the N-terminal Ileu-Ileu represents a rather acid-resistant peptide bond and was hydrolyzed to only 27–30% after 16 hours in 6 N HCl at 105°, 0.3 mole of DNP-Ileu is assumed to correspond to 1 mole N terminus per mole of the peptide 8 hydrolyzed. The absorbancy of the DNP-prolyl peptides was compared to that of peptide 8 (DNP-Ileu) at 360 mμ. The DNP-prolyl peptide was separated by paper chromatography as described in the next section. The recovery of the DNP derivative of glutamic acid, which would be one of the N-terminal residues after release of aspartic acid from peptide 8, was very low. This may be attributed to the conversion of the glutamic acid or glutamine residue to pyrrolidonecarboxylic acid.

The C-terminal amino acids in partial hydrolysates (0.03 N HCl, 105°) of peptide 8 were determined by hydrazinolysis (Table III). Since the amino acids liberated by the acid alone also appear as free amino acid in the hydrazinolysates, these were subtracted from the total in order to determine the abundance of each C-terminal amino acid. The aspartic acid liberated from aspartic acid residues in peptide 8 appeared somewhat higher (10–15%) upon direct amino acid analysis than after hydrazinolysis followed by automatic amino acid analysis.

The end-group analyses clearly indicated that aspartic acid did not appear in C-terminal or N-terminal position, but that all amino acids at either side of aspartic acid residues became new end groups with the exception of glutamic acid, which might have become cyclized (pyro-Glu-Ala). Thus it is concluded that free aspartic acid was preferentially liberated from the peptide by the double cleavage on both sides of aspartic acid residues rather than by consecutive attack on either side.

Isolation of Fragments after Dilute Acid Hydrolysis

TABLE II: N-Terminal Amino Acids of Peptide 8 after Hydrolysis in 0.03 N HCl at 105°.

DNP Derivative	Time of Hydrolysis[a] 8 hours	16 hours	24 hours
Isoleucine	0.30	0.27	0.31
Glutamic acid	0.02	0.03	0.03
Alanine	0.55	0.71	0.68
Proline[b]	0.17	0.29	0.23
Threonine	0.03	0.05	0.11
Total free aspartic acid	1.17	2.23	2.44

[a] The values given are in moles/mole peptide 8 (0.3 mole DNP-isoleucine). They are uncorrected for technical and hydrolytic losses. [b] DNP-proline was determined as DNP-prolyl peptide as separated by paper chromatography using the *t*-amyl alcohol–ammonia system (see text). Total free aspartic acid was determined as DNP-aspartic acid.

TABLE III: C-Terminal Amino Acids of Peptide 8 after Hydrolysis in 0.03 N HCl.

	Time of Hydrolysis[a] 4 hours	8 hours	16 hours	24 hours
Aspartic acid				
Alanine	Trace	0.03	0.09	0.17
Leucine	0.38	0.45	0.40	0.33
Threonine			Trace	Trace
Glutamic acid	Trace	0.16	0.28	0.23

[a] The values given are in moles/mole peptide used. The data for C-terminal amino acids represent the difference between the free amino acid found after and before hydrazinolysis, uncorrected for mechanical and technical losses during the hydrazinolysis and chromatographic separation on the automatic amino acid analyzer. The values for aspartic acid were negative.

TABLE IV: Amino Acid Composition of Fragments Obtained from Dilute Acid Hydrolysis of Peptide 8 (6-hour hydrolysis).

Peptide	Amino Acid Composition (mole ratios)	Remarks
S-1	DNP-$Asp_{0.77}Asp_{1.65}Thr_{1.93}Glu_{2.00}Pro_{0.61}$ $Ala_{1.59}Val_{0.53}Ileu_{0.64}Leu_{0.54}$	An impure peptide not characterized
S-2[a]	DNP-$ProThr_{2.62}Glu_{1.24}Ala_{1.02}Leu_{0.96}$	Peptide comprising residues 102–108
S-3	DNP-$Ala_{0.53}Thr_{0.89}Arg_{1.00}$	Peptide comprising residues 110–112
P-1	DNP-$Ileu_{0.27}Glu_{2.00}Val_{0.86}Ileu_{0.37}$	Peptide comprising residues 93–97
P-2	DNP-$Ileu_{0.31}Asp_{2.23}Thr_{2.18}Glu_{4.00}Pro_{0.73}$ $Ala_{1.98}Val_{1.02}Ileu_{0.35}Leu_{0.68}$	Probably comprising residues 93–108 $Leu_{0.71}$ found upon hydrazinolysis

[a] DNP proline was not determined.

of Peptide 8. Purified peptide 8 (2 μmoles) was hydrolyzed with 0.03 N HCl at 105° for 12 hours in an evacuated sealed tube. The acid hydrolysate was dinitrophenylated according to the standard procedure. After removal of DNP-amino acids by ether extraction after acidification, the acid-insoluble DNP peptides were centrifuged off. The soluble DNP peptides were separated into five yellow bands (S-1, S-2, S-3, S-4, and S-5 with R_F values of 0.01, 0.15, 0.3, and 0.4, respectively) and the insoluble DNP peptides yielded P-1 and P-2 with R_F values of 0 and 0.1 upon paper chromatography, using the *t*-amyl alcohol–ammonia system. Each DNP peptide was hydrolyzed with 6 N HCl for 16 hours and analyzed for N-terminal amino acid and for amino acid composition of the residual peptides. The results are summarized in Table IV. No further characterization was attempted for DNP peptides S-4 and S-5, which appeared to be minor products and probably were derived from cleavages at sites other than aspartic acid residues.

In another experiment, 2.8 μmoles of the peptide were hydrolyzed with 0.03 HCl at 105° for 12 hours and the partial hydrolysate was separated chromatographically on a 0.9 × 60-cm column of Dowex 1-X2. Figure 4 summarizes the results of ion-exchange column chromatography. Each major peak was pooled, lyophilized, and further purified by paper chromatography using the 1-butanol–acetic acid–water–pyridine system, or dinitrophenylated and subsequently separated by paper chromatography using the *t*-amyl alcohol–ammonia system. The amino acid composition of each major peak is summarized in Table V. The results indicate a reasonably good separation of the expected fragments from partial acid hydrolysis of peptide 8, excepting Glu-Ala which was not detected, a fact which might be attributed, as previously suggested, to its becoming cyclized to pyro Glu-Ala which might be overlooked during the chromatographic separation. The results indicate that the following sequence of events may take place during the hydrolysis of the peptide in 0.03 N HCl at 105° for 12 hours. The peptide fractions are designated according to Tables IV and V.

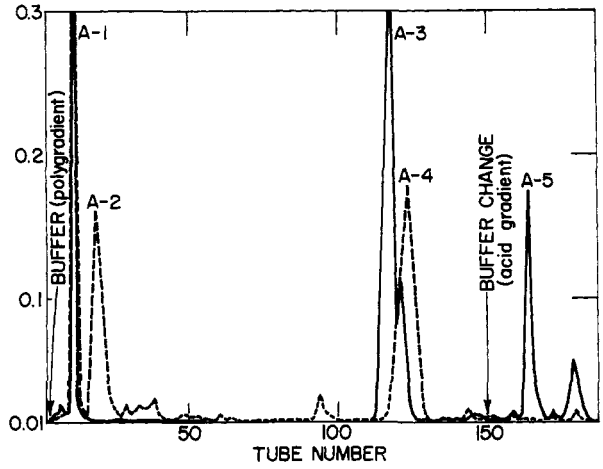

FIGURE 4: Chromatographic separation of fractions obtained from partial hydrolysate of tryptic peptide 8 (2.8 μmoles) in 0.03 N HCl. (———), Folin color (700 mμ); (– – – –), ninhydrin color (570 mμ). The 0.9 × 60-cm column of Dowex 1-X2 was used. Operation and elution of the column was described in the text. Left-hand ordinate represents ninhydrin (OD at 570 mμ) × 30, and folin color (OD at 700 mμ) × 30.

Peptide 8 (residues 93–112)[2] → peptide S-3 (93–108) + Asp (109) + peptide P-2 (110–112). Peptide S-3 (93–108) → peptide P-1 (93–97) + Asp (98 and 101) + peptide S-2 (102–108) + pyro-Glu-Ala (99–100). Also, peptide 8 (93–112) → peptide A-5a (93–108) + Asp (109) + peptide A-1 (110–112). Peptide A-5a (93–108) → peptide A-5b (93–97) + Asp (98) + pyro-Glu-Ala (99–100) + Asp (101) + peptide A-3 (102–108).

Dilute Acid Treatment of Peptide 12. It has been shown that in concentrated and moderate strength acid proteins undergo preferential cleavage of the peptide bonds

[2] The numbers identify the positions of residues in the complete amino acid sequence of TMV protein (Tsugita *et al.*, 1960).

TABLE V: Amino Acid Composition of Peaks Obtained from Dilute Acid Hydrolysis of Peptide 8 at 105°.[a]

Peak number	Amino Acid Compositions (mole ratios)	Remarks
A-1	$Thr_{1.37}Ala_{1.00}Arg_{1.12}$	Probably containing 2 peptides comprising residues 110–112 and 111–112
A-2	Ala	Free alanine released probably from Ala, Thr, Arg
A-3	$Asp_{0.6}$ and $Thr_{2.08}Glu_{1.02}$ $Pro_{0.92}Ala_{1.00}Leu_{0.85}$	Peptide comprising residues 102–108 contaminated with free aspartic acid
A-4	Asp	Free aspartic acid with minor contamination with unidentified peptides
A-5[b]	DNP $Ileu_{0.32}Glu_{2.00}Val_{0.67}$ $Ileu_{0.29}$ and DNP $Ileu_{0.26}Asp_{2.18}$ $Thr_{2.01}Glu_{4.00}Pro_{0.67}Ala_{1.79}$ $Val_{0.93}Ileu_{0.34}Leu_{0.71}$	A mixture of peptides comprising residues 93–97 and 93–108

[a] Automatic amino acid analyses indicate release of 53% of aspartic acid residues and 12.13% of alanine residues in peptide 8. [b] A-5 was purified by paper chromatography using the *t*-amyl alcohol–ammonia system which yields two bands similar to P-1 and P-2 from last experiment (Table IV).

involving the amino group of serine and threonine residues, with oxazoline ring formation and N→O acyl shift as intermediary steps (Desnuelle and Casal, 1948). To investigate the extent of N→O acyl migration of serine and threonine residues as well as the resistance of tryptophan residues under our experimental conditions, we chose peptide 12, which lacks aspartic acid residues but contains 6 serine, 2 threonine, and 1 tryptophan residues in the following sequence: ^{142}Ser-Ser-Phe-Glu-Ser-Ser-Ser-Gly-Leu-Val-Try-Thr-Ser-Gly-Pro-Ala-Thr.^{158}[2] If all serine and threonine residues were equally susceptible to hydrolysis owing to N→O acyl migration, it is estimated that about 3 moles of serine and 1 mole of threonine might be liberated as free amino acids because of their positions relative to each other. The automatic amino acid analyses of peptide 12 after 12-hour and 24-hour hydrolysis in 0.03 N HCl at 105° showed the release of only 0.05–0.1 mole of serine and 0.2–0.3 mole of threonine per mole of the peptide. Although the above estimates are no more than rough approximations, it is evident that the extent of cleavage of peptide bonds was very small for serine residues, though somewhat larger for threonine residues. The threonine residues in this peptide might be particularly labile owing to their locations (Try-Thr-Ser- and -Pro-Ala-Thr).

To search for a change in peptide 12 resulting from any N→O acyl migration or to detect fragmentation of the peptide occurring upon dilute acid treatment, the reaction mixture was applied to a 15-cm column of Amberlite IR 120 (automatic amino acid analyzer) using the pyridine acetate gradient system. The chromatographic pattern of peptide 12, treated with 0.03 N HCl at 105° for 12 hours (Figure 5A) and 24 hours (Figure 5B), showed only one major peak which was both ninhydrin and Folin reactive and appeared at the same early position. Figure 5C summarizes the chromatographic separation of the mixture of soluble tryptic peptides of TMV protein and demonstrates their good resolution by the Amberlite IR 120 on the automatic amino acid analyzer (Beckman Technical Bulletin, 1962). When the amino acid compositions of peptide 12 after treatment with 0.03 N HCl at 105° for 12 hours and 24 hours (Figure 5A,B) were compared with untreated peptide 12 (Figure 5C) they were found to be identical. The apparent yields of the acid-treated peptide were 51 and 72%, respectively, after 24 and 12 hours, uncorrected for mechanical losses. These results show that peptide 12, although containing many serine residues, remained largely unaffected by 0.03 N HCl at 105°. The treated peptide 12 (Figure 5B) yielded only one ninhydrin- and tryptophan-reactive spot of R_F 0.4 upon chromatographic purification by the 1-butanol–acetic acid–water–pyridine system. This result, as well as the fact that the ultraviolet spectrum of the acid-treated peptide 12 with its maximum about 275 mμ was superimposable on that of the untreated peptide, indicates that the tryptophan residue also remains undestroyed under the conditions of dilute acid treatment.

Dilute Acid Hydrolysis of Peptide 10. Peptide 10, with the sequence Ser-Ala-Ileu-AspNH_2-AspNH_2-Leu-Ileu-Val-Glu-Leu-Ileu-Arg, released only 50% of its total aspartic acid upon hydrolysis in 0.03 N HCl at 105° for 24 hours. The great discrepancy of the experimental and calculated value of $t_{1/2}$ (24 versus 11 hours) may be explained by the insolubility of this peptide in 0.03 N HCl. The following amino acids (moles/mole) were liberated from peptide 10 upon 90 hours of hydrolysis in 0.03 N HCl at 105°: $Asp_{(1.82)}$, $Ser_{(0.19)}$, $Ala_{(0.20)}$, $Ileu_{(0.39)}$, $Leu_{(0.25)}$, and "Tyr"$_{(0.33)}$ (a material which was shown to be the tripeptide Ser-Ala-Ileu which moves in the position of tyrosine on the auto-

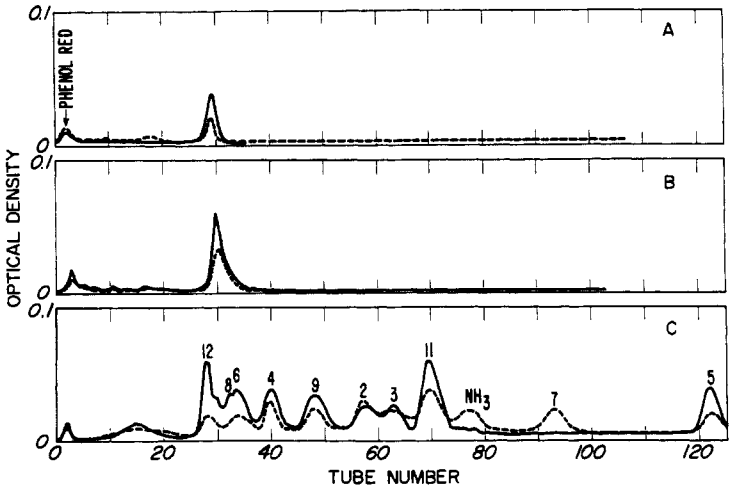

FIGURE 5: Chromatographic separation of peptides. Chromatographic separation of peptide 12 (2 μmoles) treated with 0.03 N HCl at 105° for 12 hours (A) and for 24 hours (B). (———), Folin color (700 mμ); (– – – –), ninhydrin color (570 mμ). (C) Chromatographic separation of soluble peptides (*p*H 4.6), approximately 12 μmoles, obtained by 2-hour tryptic digestion of TMV protein. (———), Folin color (700 mμ); (– – – –), ninhydrin color (570 mμ). The 0.9 × 15-cm column of Amberlite IR 120 on the amino acid analyzer was used. Flow rate 30 ml/hr, temperature 52°. The first peak with yellow color at 440 mμ was phenol red which served as index for solvent front. In (C) the number identifies the main component of each peak with the sequential peptide number on the polypeptide chain (peptide 1 was insoluble at *p*H 4.5 and was removed by centrifugation prior to chromatography).

matic amino acid analyzer). The hydrazinolysis of the peptide 10 hydrolysate yielded the following amino acids per mole of peptide 10: $Asp_{(1.79)}$, $Ala_{(0.18)}$, $Ileu_{(0.83)}$, and $Leu_{(0.35)}$ (ornithine from the C-terminal arginine was not determined). It is of interest to note that less than one-half mole of isoleucine was released by the acid, concomitant with the release of 2 moles of aspartic acid, and that isoleucine but not alanine was a new C-terminal amino acid upon the removal of aspartic acid residues. These data, therefore, suggested that the amino acid residues in position 125 and 126 were reversed from those earlier reported (Tsugita *et al.*, 1960) (Ileu-Asp-Asp instead of Asp-Ileu-Asp). The suggested sequence was further substantiated by the isolation of the above-mentioned tripeptide, Ser-Ala-Ileu, from the partial hydrolysate (Funatsu *et al.*, 1964).

Hydrolysis of Peptide 1. In general, 0.03 N HCl (Schultz *et al.*, 1962; Grannis, 1960), 0.25 N oxalic acid (Partridge and Davis, 1950), and 0.25 M acetic acid (Schroeder *et al.*, 1963) have been used for the preferential release of aspartic acid from peptides or from proteins. However, some proteins as well as some peptides are not soluble in these acid media and extensive liberation of aspartic acid appears to be prevented under these conditions. The hydrolysis of peptide 1 in 0.03 N HCl at 105° for 24 hours liberated only 33% of the total aspartic acid residues. The addition of various solubilizing agents to 0.03 N HCl (*p*H 2.0), such as 4.5 M guanidine HCl or 60% ethanol, did not increase the extent of liberation of aspartic acid. However, good results were obtained when peptide 1 was dissolved and hydrolyzed with 0.03 N HCl in 40% acetic acid (measured *p*H 1.4) though not with 40% acetic acid alone (measured *p*H 2.0). The hydrolysis of peptide 1 in these acids was studied, and the results are summarized in Figure 6. The results indicate that 22%, 33%, and 66% of the total aspartic acid residues were released as free amino acid when peptide 1 was hydrolyzed for 24 hours at 105° in 40% acetic acid, in 0.03 N HCl, or in a mixture of both 0.03 N HCl and 40% acetic acid, respectively. The liberation of amino acids from peptide 1 by the action of these acidic media is quantitatively similar. The fact that peptide 1, though soluble in 40% acetic acid, was hydrolyzed to a lesser extent in this medium than in 0.03 N HCl may be attributed to its high *pK*, and to the possible reaction of aspartic acid with acetic acid during the course of hydrolysis. The *p*H of 0.03 N HCl in 40% acetic acid is far below 2.0 but it seemed to exercise only slightly less specificity as compared with 0.03 N HCl alone. These results suggest that 0.03 N HCl in 40% acetic acid may be used for partial hydrolysis of acid-insoluble peptides.

Discussion

Automatic amino acid analysis and end-group analysis of the partial hydrolysates obtained from the tryptic peptides of TMV protein, heated in 0.03 N HCl at 105° for less than 16 hours, indicate that most of the aspartyl residues and more than half the asparaginyl residues in the peptides were liberated while other amino acids such as alanine, glycine, serine, threonine, and glutamic acid were liberated to a very small extent. These amino acids, however, were liberated to

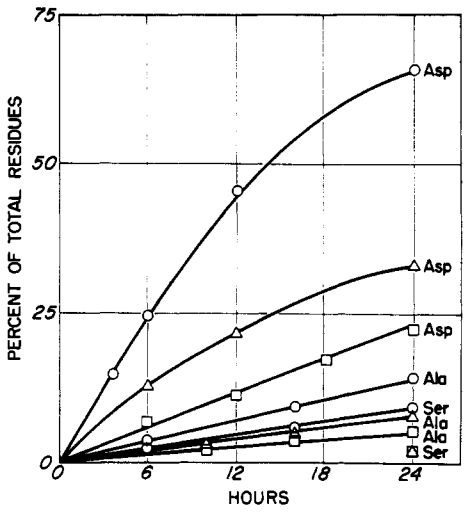

FIGURE 6: Liberation of amino acids from peptide 1 during partial hydrolysis in 0.03 N HCl, in 40% acetic acid, and in 0.03 N HCl in 40% acetic acid. (-O-O-), 0.03 N HCl in 40% acetic acid; (-□-□-), 40% acetic acid; (-△-△-), 0.03 N HCl. The complete amino acid sequence of peptide 1 is: *N*-acetyl-Ser-Tyr-Ser-Ileu-Thr-Thr-Pro-Ser-GluNH$_2$-Phe-Val-Phe-Leu-Ser-Ser-Ala-Tyr-Ala-Asp-Pro-Ileu-Glu-Leu-Ileu-AspNH$_2$-Leu-CySH-Thr-AspNH$_2$-Ala-Leu-Gly-AspNH$_2$-GluNH$_2$-Phe-GluNH$_2$-Thr-GluNH$_2$-GluNH$_2$-Ala-Arg.

a significant extent upon prolonged hydrolysis of the peptides, as is required and has usually been used for liberating essentially all the aspartic acid residues in the proteins and peptides. Asparagine appears to be more slowly liberated than aspartic acid, as shown by the kinetic study on the liberation of aspartic acid and amide-ammonia from peptide 8 in 0.03 N HCl at 105°. The data indicate that asparaginyl residues are deamidated prior to being liberated. This fact was also reflected in the broken line of the first-order plots of the peptides containing both aspartyl and asparaginyl residues. Thus it appears advisable to study the kinetics of this reaction at earlier stages, which may give a clue to aid in distinguishing aspartyl from asparaginyl residues in the original peptides.

The chromatographic separations of fragments obtained by the preferential hydrolysis of peptide 8 offer a much better insight into the specificity of this reaction than is possible by automatic amino acid analysis and end-group analysis alone. For example, alanine was shown to be liberated from Ala-Thr-Arg, a C-terminal peptide, after removal of the aspartyl residue in peptide 8. The data suggest that the dilute acid hydrolysis may be advantageously conducted in two stages. Peptides may first be hydrolyzed with 0.03 N HCl at 105° for 6 hours and high yields of both aspartyl-containing peptides (probably derived from deamidation of asparaginyl residues in the original peptides) and aspartyl-free peptides (derived from the removal of aspartyl residues and to a lesser extent of asparaginyl residues in the original peptides) may be obtainable, without appreciable amounts of fragments caused by nonspecific cleavage. Then the aspartyl-containing peptides may be isolated and again hydrolyzed with 0.03 N HCl at 105° for another 6 hours. The stepwise degradation of the peptides by dilute acid may be advantageous because (a) it can help to confirm whether the aspartic residues in the original peptides are amidated or not, (b) only a few peptides must be separated at each stage and this can be conveniently achieved by chromatographic procedures, (c) the aspartyl-free peptides may be degraded by other means, and (d) the secondary degradation of aspartyl-free peptides may be minimized.

The dilute-acid hydrolysis proved to be useful in confirming and in checking the neighboring sequences of aspartic acid residues. This can be easily achieved by the combined use of automatic amino acid analysis and end-group analysis of dilute-acid hydrolysates of the peptides. This was already demonstrated in correcting erroneously inverted sequences at residues 25–26 and residues 125–126 in the TMV protein (Tsung and Fraenkel-Conrat, 1963). Thus it is advisable to use this reaction for the confirmation of the sequences at both sides of aspartic acid residues, as ascertained by either the enzymatic or phenyl isothiocyanate degradation methods.

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Ultraviolet Rotatory Dispersion of Aspartic Aminotransferase*

Paolo Fasella† and Gordon G. Hammes

ABSTRACT: Rotatory dispersion measurements of the enzyme aspartic aminotransferase have been extended into the ultraviolet spectral region. All "native" forms of the enzyme, the pyridoxal, pyridoxamine, and oxime enzymes and the pyridoxal enzyme in the presence of excess substrates, have essentially the same ultraviolet dispersion curves which exhibit minima ($[\alpha] = -6600°$) at 231 mμ. The absolute rotation at the minimum is only slightly smaller for the apoenzyme ($[\alpha] = -5800°$). These results suggest the enzyme has a considerable amount of ordered structure. In 8 M urea the shape of the ultraviolet rotatory dispersion curve is considerably altered and the value of the rotation minimum is reduced to $[\alpha] = -4750°$. The visible portions of the rotatory dispersion curves (300–600 mμ) differ markedly for the various forms of the native enzyme owing to Cotton effects of the coenzyme. Although both the pyridoxal and pyridoxamine enzymes have marked Cotton effects, these Cotton effects disappear in the presence of high concentrations of the substrates. This suggests that rotatory dispersion measurements in the near ultraviolet and visible region of the spectrum may be useful as indicators of events occurring at the active site.

The rotatory dispersion properties of various forms of aspartic aminotransferase in the wavelength region 315–578 mμ have been previously reported (Fasella and Hammes, 1964a). Although pyridoxal and pyridoxamine phosphate are optically inactive, both the pyridoxal and pyridoxamine enzymes display Cotton effects centered about the wavelengths of the corresponding absorption maxima. On the other hand, the oxime enzyme, the apoenzyme, and the enzyme in urea appear to have plain dispersion curves. Similar results have been reported by Torchinsky and Koreneva (1963, 1964), who also found that other carbonyl reagents either abolished or inverted the coenzyme Cotton effect. The plain dispersion curves were analyzed according to the usual Moffit-Yang equation (Moffit and Yang, 1956). The resultant values of b_0 suggested that the apo- and denatured enzymes have very little ordered structure (e.g., α helices), while the oxime is highly ordered (Urnes and Doty, 1961); this indicates that the binding of coenzyme has an extraordinary effect on the protein structure. Unfortunately the theoretical basis for such an interpretation of the b_0 values is somewhat insecure; moreover b_0 values cannot be determined for other forms of the enzyme because of the Cotton effects occurring. In fact even in the case of the oxime enzyme a near-ultraviolet coenzyme Cotton effect might have given rise to an anomolous b_0 value.

An alternative measure of helical content or the ordered structure of proteins is the characteristic protein Cotton effect at about 232 mμ (Simmons *et al.*, 1961; Holzwarth *et al.*, 1962). Unfortunately a reliable theoretical analysis of this effect is also lacking, but in general this Cotton effect appears to be sensitive to changes in protein structure and should not be significantly influenced by the coenzyme Cotton effects occurring at longer wavelengths.

We present here the results of a study of the ultraviolet rotatory dispersion of various forms of aspartic aminotransferase. In addition the rotatory dispersion of the enzyme in the presence of excess substrates has been investigated.

Experimental

The preparation of materials was exactly as previously described (Fasella and Hammes, 1964a). Optical rotation was measured with a Cary 60 spectropolarimeter. All measurements were carried out at

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† Permanent address: Istituto di Chemica Biologica, Universita di Roma e. Centro di Enzimologia dell' C.N.R., Rome, Italy.