NASA CR70649

Specific Nature of Hydrolysis of Insulin and Tobacco Mosaic Virus Protein by Thermolysin[1]

H. Matsubara, R. Sasaki, A. Singer and T. H. Jukes

Space Sciences Laboratory, University of California, Berkeley, California 94720

(Received

[redacted]rs

ABSTRACT

Oxidized bovine insulin, and tobacco mosaic virus protein were used as substrates in an investigation of the specificity of thermolysin, a thermostable protease. Thermolysin hydrolysed the peptide bonds involving the amino-groups of leucine and phenylalanine residues of insulin under the conditions used, and preferentially those of leucine, isoleucine, valine and phenylalanine residues of tobacco mosaic virus protein. It was concluded that thermolysin hydrolyses preferentially the peptide bonds involving the amino-groups of hydrophobic amino acid residues with bulky side chains.

Author

[redacted]

A previous publication from this laboratory (1) described the selective hydrolysis of peptide bonds on the amino sides of isoleucine, leucine and phenylalanine residues in bovine cytochrome c by thermolysin, a heat stable protease from Bacillus thermoproteolyticus Rokko (2). The study has now been extended to the hydrolysis of oxidized bovine insulin (3), the A and B chains of which are commonly used for examining the substrate specificity of proteases, and also to tobacco mosaic virus protein (4), the sequence of which differs markedly in its amino acid distribution from proteins such as insulin and cytochrome c.

The specificity of thermolysin in the hydrolysis of oxidized insulin and tobacco mosaic virus protein was in good agreement with that found for cytochrome c. The enzyme promises to be useful in the structural study of proteins, and is of interest in a comparison of the mechanism of action of various proteases.

MATERIALS AND METHODS

Thermolysin: This was crystallized four times by means of pH change of the concentrated enzyme solution as described by Endo (2), and lyophilized.[2] It was dissolved in 0.1 M tris-(hydroxymethyl) amino methane buffer, pH 8.0, containing 0.003M $CaCl_2$ to give a 0.01 % solution which was stable for several months. Thermolysin was found to hydrolyse carbobenzoxy-glycyl-L-phenylalaninamide (New England Nuclear Corp., Boston, Mass.) at a rate of 3×10^{-1} μmole/min./mg protein at pH 8.0 and 40° at 2×10^{-3} M substrate concentration[3].

Insulin: Crystalline bovine insulin was purchased from Calbiochem.,

Los Angeles, Calif., and oxidized at 0° for 4 hours with performic acid (5). The product was lyophilized and used without further purification.

Tobacco mosaic virus protein: Tobacco mosaic virus protein (strain vulgare) (4) was kindly supplied by Dr. C. A. Knight, Department of Molecular Biology, University of California, Berkeley, California, and stored as a lyophilized powder.

Hydrolysis of the substrate by thermolysin: (a) Insulin. About 30 mg of oxidized insulin was dissolved in 2 ml of distilled and deionized water, the pH was adjusted to about 8.0 with 0.1 N NaOH, and 0.25 ml of thermolysin solution (0.01%) was added to give an enzyme:substrate ratio of 1:1200 by weight. The mixture was incubated for 30 minutes at 36°, following which the reaction was terminated by adding one drop of glacial acetic acid, and the solution was lyophilized. (b) Tobacco mosaic virus (TMV) protein: This was digested with thermolysin as described for insulin except that the substrate concentration was 0.75% and the enzyme:substrate ratio 1:200. The reaction was terminated in separate experiments at 15 minutes and 2 hours respectively by adding one drop of glacial acetic acid. A portion of the 2 hour digest was lyophilized; the remainder was held for about 30 minutes at 40°, and 0.15 ml of 25% trimethylamine was added to the mixture (2 ml), producing a pH of 8.5 for the dinitrophenylation.

Analysis of peptides: The lyophilized products were dissolved in about 0.2 ml of 1% trimethylamine, and an aliquot of about 0.05 ml was placed on Whatman No. 3MM paper. Separation of peptides was by

two-dimensional paper chromatography and electrophoresis as described previously (1). For chromatography, a BPAW system (butanol:pyridine: acetic acid:water=15:10:3:12 by volume) was used, this was followed by electrophoresis in pH 3.7 buffer (pyridine:acetic acid:water=1:10: 289 by volume) for 1.5 hours at 35 V/cm for the insulin digest and at 30 V/cm for the TMV protein digest. Only the 2-hour digest of TMV protein was used for the separation of peptides.

Three sheets were usually prepared. One was sprayed with 0.1% ninhydrin solution in acetone to mark the location of the peptides and the others with 0.02% ninhydrin solution to locate peptides for elution with 30% acetic acid. The eluates were then dried. They were hydrolysed with 0.5 ml of twice-distilled 6N HCl for 24 hours at 105°-106° in sealed and evacuated tubes. The hydrolysates were dried in a rotary evaporator at 80° within 30 minutes and the amino-acid composition was determined by a Spinco automatic analyser Model 120B, operated by a variant of the method of Spackman, et al. (6). The susceptibility of various peptide bonds to thermolysin was gauged by calculating the relative yields of each peptide, assuming that all peptides underwent similar losses during isolation and that the yield of the peptide recovered in the highest quantity was 100%.

The amino-terminal residues of some of the peptides were identified by a modification of Edman's phenylisothiocyanate method (7), when this was necessary for their characterization.

Amino-terminal analysis of peptides: Sanger's dinitrofluorobenzene method (8) was applied to the reaction mixtures obtained from TMV

protein after 15 minute and 2 hour digestion periods, and to a zero time sample, to identify the amino-terminal residues.

The solution was adjusted to pH 8.5 with trimethylamine, mixed with 0.05 ml of dinitrofluorobenzene dissolved in 4 ml of ethanol, and the reaction was carried out for 2 hours at room temperature. Ethanol was removed by vacuum distillation and the excess dinitrofluorobenzene was extracted with ether several times in the presence of 1-2 drops of trimethylamine. The aqueous phase was dried in a rotary evaporator at 45° and the residue was hydrolysed with 4 ml of distilled 6N HCl for 16 hours at 105°-106°. The dinitrophenyl (DNP)-amino acids were separated into ether-soluble and water-soluble fractions and identified by paper chromatography (8,9). DNP-arginine was identified by spraying with Sakaguchi reagent (10) following paper chromatography of the water-soluble fraction. For quantitative estimation of the DNP-amino acids recovered from the digest, the spots on paper were eluted with 1% $NaHCO_3$ for 15 minutes at 55° and the absorbancies of the solutions were measured as usual (8). One portion of the untreated digest was used for determination of the basic amino acids with the analyser for estimation of the amount of digest taken in the DNP-amino acid analysis. No corrections were made for losses of DNP-amino acids. DNP-isoleucine and DNP-leucine did not become separated under the conditions used and the combined values for these were taken. The ratio of isoleucine:leucine terminal groups was estimated in the 2-hour digest with the analyser after regeneration of the leucines with concentrated ammonia (8). The recovery factors were assumed to be equal.

RESULTS AND DISCUSSION

Hydrolysis of oxidized insulin: The conditions used for the digestion of oxidized insulin by thermolysin, described above, produced a partial hydrolysate and various overlapping peptides were obtained. Fig. 1 shows a peptide map of the digest of oxidized insulin.

Fig. 1

Clear-cut separation was obtained by two-dimensional paper chromatography and electrophoresis. Each spot was from a peptide with a unique composition except for spot 7 which was due to two components. Table 1 shows the analytical results of peptides recovered from the spots shown in Fig. 1. From the results it was possible to locate

Table 1 and Fig. 2

each peptide in the primary structure of insulin (3). Only peptide 9 was subjected to amino-terminal group analysis and leucine was found to be its terminal residue. Peptides 7b and 16 were not subjected to terminal analysis. Therefore, it remains possible that peptide 7b was derived from residues 7 to 15 in the B chain and peptide 16 from residues 7 to 11 (see Fig. 2). However, this is unlikely, because no peptides were found corresponding to the sequence from residues 1 to 6, or to sequences beginning at residues 12 or 16 in the B chain.

A peptide derived from residues 1 to 12 in the A chain was not

obtained; it may have run off the paper to the anode side during electrophoresis.

The results are summarized in Table 1 and Fig. 2. Only peptide bonds involving the amino-groups of leucine and phenylalanine residues in oxidized insulin were selectively cleaved. The specificity agrees well with that observed in the digestion of cytochrome c (1). The sole isoleucine residue in insulin is adjacent to the amino-terminal glycine residue of the A chain and the bond on the amino side of this isoleucine residue was not attacked. A glycyl-isoleucine bond in cytochrome c was easily cleaved by thermolysin (1). It is possible that the presence of a free α amino group on the adjacent residue prevents hydrolysis by thermolysin of a peptide bond which would otherwise be sensitive.

In Table 1 the relative yield of each peptide is shown. The results suggest, for instance, that the glutaminyl-leucine (residues 15-16, A chain) bond was more susceptible than the seryl-leucine (residue 12-13, A chain) bond to thermolysin. However, no decisive conclusion can be drawn from these results about the relative susceptibility of peptide bonds and more reliable procedures are required to obtain such information.

Hydrolysis of tobacco mosaic virus protein: (a) Amino-terminal analysis of peptides produced. Tobacco mosaic virus protein was digested with thermolysin for 15 minutes and 2 hours at a substrate: enzyme ratio of 200:1 by weight to compare the hydrolysis rates at different peptide bonds. Since thermolysin attacks peptide bonds on

the amino sides of certain residues, amino-terminal group analysis of the peptides produced by the DNP method should provide information on hydrolysis rates.

The protein itself did not yield any detectable DNP-amino acids as was expected from the presence of an acetylated amino-terminal residue (11). Table 11 shows the results for the digests. It is evident that isoleucine+leucine, valine, and phenylalanine residues

Table 11

predominated as the amino-terminal residues of the peptides produced. In addition to leucine, isoleucine and phenylalanine cleavages which were found in a cytochrome c digest (1) under the same conditions, it is noteworthy that valine bonds were also attacked by thermolysin. This suggests that peptide linkages involving the amino-groups of certain valine residues in TMV protein may be present in a state that is susceptible to this enzyme.

To facilitate a comparison, each value in column (a) of Table 11 was divided by the total number of residues of the respective amino acids in the protein, namely 21 isoleucine+leucine, 14 valine, 8 phenylalanine and 14 alanine residues (4), and these ratios were compared with the ratio for isoleucine+leucine, taken as 100. More isoleucine+leucine bonds than valine bonds were cleaved as hydrolysis was prolonged (see column (b)).

The DNP-isoleucine+leucine mixture was cleaved with ammonia and

it was found that the ratio of isoleucine:leucine was about 1.5:1. This suggests that isoleucine bonds may be more susceptible than leucine bonds in the virus protein to splitting with thermolysin.

(b) Analysis of peptides produced: Several peptide maps were made and analysed as described earlier. Only the 2-hour digest was subjected to analysis; a peptide map is shown in Fig. 3.

Fig. 3

No peptide core was obtained under the conditions used. Fairly good separation was obtained, but several spots gave complicated analyses which could not be related to the sequence of amino acids in the protein. Further studies are needed to establish the composition of all the peptides produced by thermolysin; therefore, only those peptides which could be identified by reference to the sequence are listed in Table III and are shown by arrows in Fig. 4.

Table III and Fig. 4

Peptide 1, 3 and 10 were subjected to amino-terminal group analysis. Leucine, valine and isoleucine were found to be the respective terminal residues, thus, serving to locate these peptides in the sequence. The other peptides were located by their unique composition. Several other spots were analyzed, and satisfactory ratios were obtained from peptides that corresponded to spots 5, 8, 13, 17, 18, 21 and 23. However, end-group analyses did not provide

convincing evidence and the peptides could not be located in the sequence. The following summarizes these analyses and the possible origins (residue numbers) of the peptides. Peptide 5a, (Asp_2, Thr_3, Glu_4, Pro_1, Ala_2, Val_1, $Ileu_2$=93-107); peptide 5b (Ser_3, Glu_1, Gly_1, Phe_1=144-149); peptide 8a (Lys_2, Arg_1, Asp_2, Thr_1, Ser_2, Glu_1, Pro_3, Val_2, Phe_2=52 or 53-68); peptide 13 (Ser_1, Glu_1, Phe_1=8-10, 47-49 or 48-50), peptide 17 (Ala_1, Val_1=74-75 or 119-120); peptide 18 (Gly_1, Ala_1, Leu_1=30-32 or 84-86); peptide 21 (Asp_1, Thr_1, Pro_1, Ala_{1-2}, Val_2, Leu_2=74-81 or 75-82); peptide 23a (Glu_1, Val_1, $Ileu_1$, Leu_1=128-131, 129-132, or 130-133); and peptide 23b (Val_1, Leu_1=10-11 or 11-12).

Spots 5 and 8 contained at least two components; spot 5 could be separated into two fractions, 5a and 5b, in approximately equal quantity, and spot 8 gave a main component, 8a. Peptide 13 was more probably derived from residues 48 to 50 than from 8 to 10; in the latter case, a prolylserine bond would have to be split and this appears unlikely. Moreover, peptide 3 (Table III) revealed that hydrolysis of the glutaminyl-phenylalanine bond at 47-48 had taken place. Though incomplete, these results agree with those obtained by end-group analysis.

General discussion: Table IV shows the types of peptide bond cleaved by thermolysin in cytochrome c (1), insulin and tobacco mosaic virus protein.

Table IV

It is evident that thermolysin is specific for peptide bonds involving

the amino-groups of hydrophobic amino acids with bulky side chains, such as isoleucine, leucine, valine and phenylalanine. So far, the amino acid residues that contribute carboxyl-groups to the peptide bonds attacked have varied between hydrophilic and hydrophobic, and between basic and neutral, but have not involved the acidic amino-acid residues.

This specificity may well be useful in studies of the chemistry of proteins. It is possible that thermolysin may be useful for the study of proteins which produce "cores" on digestion by trypsin.

The specificity shown by thermolysin is more distinctive than that of other well-known proteases (12), which usually attack the carbonyl bonds of certain amino acid residues. It will be of interest to study more precisely the mode of action of thermolysin and the structure of the protein in terms of structure-function relationship.

Recently a similar specificity has been reported in a snake venom protease (13) and in Pseudomonas aeruginosa elastase (14). The latter has properties similar to those of thermolysin with respect to metal requirement, inhibition by ethylenediaminetetraacetate, and insensitivity to diisopropylfluorophosphate (15, 16). A number of amino acid esters are not attacked by either enzyme (15, 16). The amino acid compositions of these enzymes showed differences.

A more detailed study of the specificity of thermolysin with various synthetic substrates is in progress. After these experiments were completed, Dr. K. Morihara (Shionogi Research Institute, Osaka, Japan) informed us that he had obtained similar results with thermolysin in the hydrolysis of the B chain of insulin (17).

REFERENCES

1. Matsubara, H., Singer, A., Sasaki, R. and Jukes, T.H., Biochem. Biophys. Res. Comm., 21, 242 (1965).

2. Endo, S., J. Fermentation Tech., 40, 346 (1962); Chem. Abstract., 62, 5504 (1965).

3. Sanger, F., in D. E. Green, Currents in Biochemical Research, Wiley (Interscience), New York (1956) p. 434.

4. Funatsu, G. and Fraenkel-Conrat, H., Biochemistry, 3, 1356 (1964).

5. Moore, S., J. Biol. Chem., 238, 235 (1963).

6. Spackman, D. H., Stein, W. H. and Moore, S., Anal. Chem., 30, 1190 (1958).

7. Light, A. and Greenberg, J., J. Biol. Chem., 240, 258 (1965).

8. Fraenkel-Conrat, H., Harris, J. I. and Levy, A. L., in D. Glick, Methods of Biochemical Analysis, Vol. 2 (Interscience Publishers, Inc.), New York (1956) p. 397.

9. Braunitzer, G., Chem. Ber., 88, 2025 (1955).

10. Acher, R. and Crocker, C., Biochim. Biophys. Acta, 9, 704 (1952).

11. Narita, K., Biochim. Biophys. Acta, 28, 184 (1958).

12. Smith, E. L., in Boyer et al., The Enzymes, Vol. 4, (Academic Press) New York (1960) p. 1.

13. Pfleiderer, G. and Krauss, A., Biochem. Z., 342, 85 (1965).

14. Morihara, K., Tsuzuki, H. and Oka, T., The 17th Symposium on Enzyme Chemistry (Tokushima) (1965) p. 293.

15. Morihara, K., Tsuzuki, H., Oka, T., Inoue, H. and Ebata, M., J. Biol. Chem., 240, 3295 (1965).

16. Matsubara, H., 132nd Meeting of the American Association for the Advancement of Science, Molecular Mechanisms of Temperature Adaptation, Berkeley, Calif., Dec. 27-29, (1965), (to be published).

17. Morihara, K. and Tsuzuki, H., Seikagaku, 37, 538 (1965).

FOOTNOTES

1. Supported by Grant NsG 479 by the National Aeronautics and Space Administration to the University of California, Berkeley, Calif..

2. We are indebted to Dr. S. Endo, Daiwa Kasei Co., Osaka, Japan for the supply of the commercial enzyme, Thermoase, which is available from this company.

3. Unpublished results.

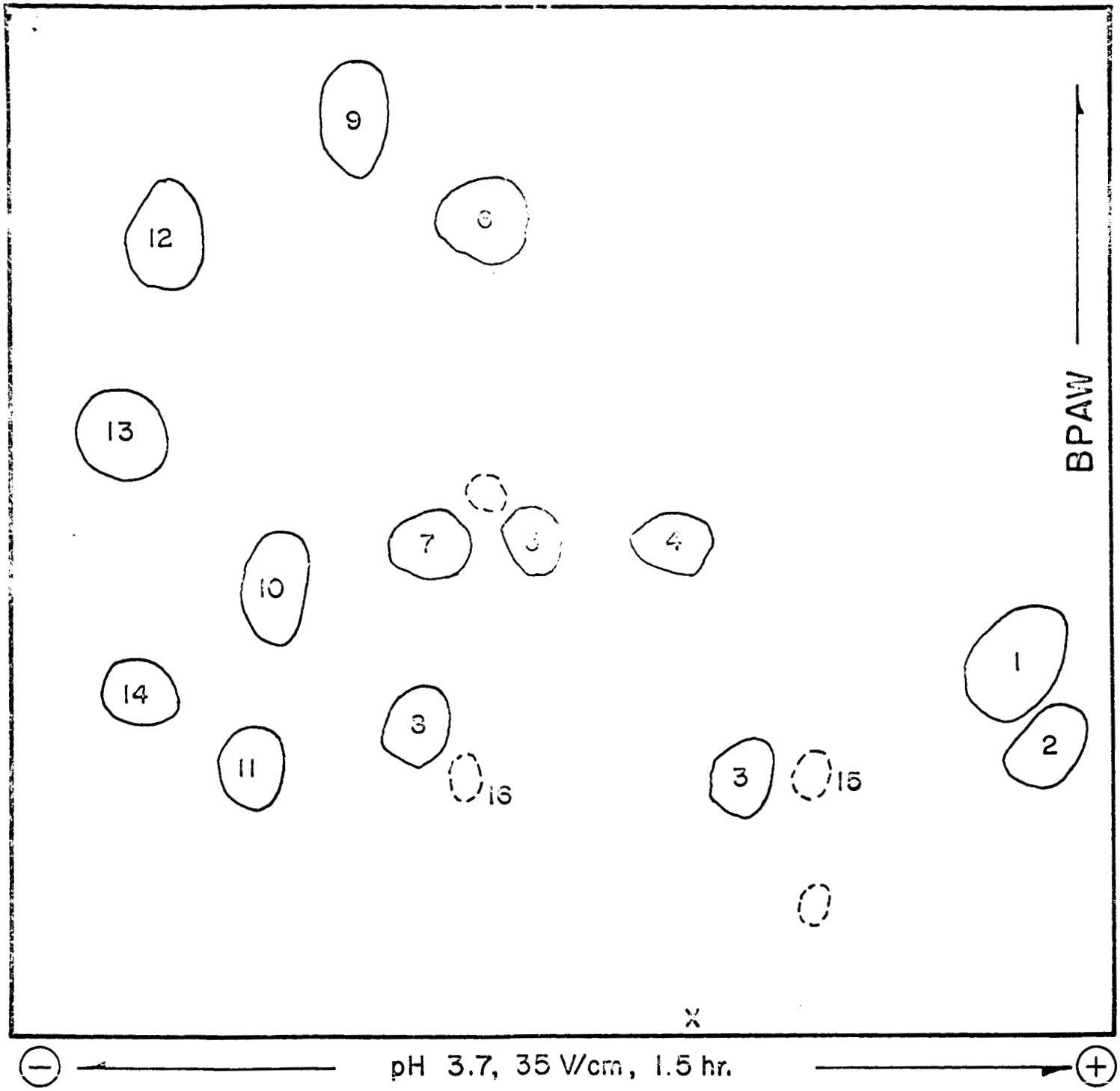
LEGENDS TO FIGURES

Fig. 1. Peptides produced by thermolysin digestion of oxidized insulin (see text). The peptide map was made by two dimensional paper chromatography (as described in the text). Spots were located by spraying with 0.1% ninhydrin solution.

Fig. 2. Amino acid sequence of oxidized insulin A and B chains, showing the peptide bonds attacked by thermolysin (arrows).

Fig. 3. Peptides produced by thermolysin digestion of tobacco mosaic virus protein. Thirty mg of the virus protein were treated with 0.15 mg of thermolysin for 2 hours at 36° and pH 8.0. The peptide separation was obtained as described in Fig. 1, except that electrophoresis was at 30 V/cm.

Fig. 4. Amino acid sequence of tobacco mosaic virus protein, strain vulgare, showing the probable location of peptide bonds attacked by thermolysin (arrows).


9
12
6
13
7
5
4
10
1
14
3
2
11
3
16
3
15
BPAW
pH 3.7, 35 V/cm, 1.5 hr.

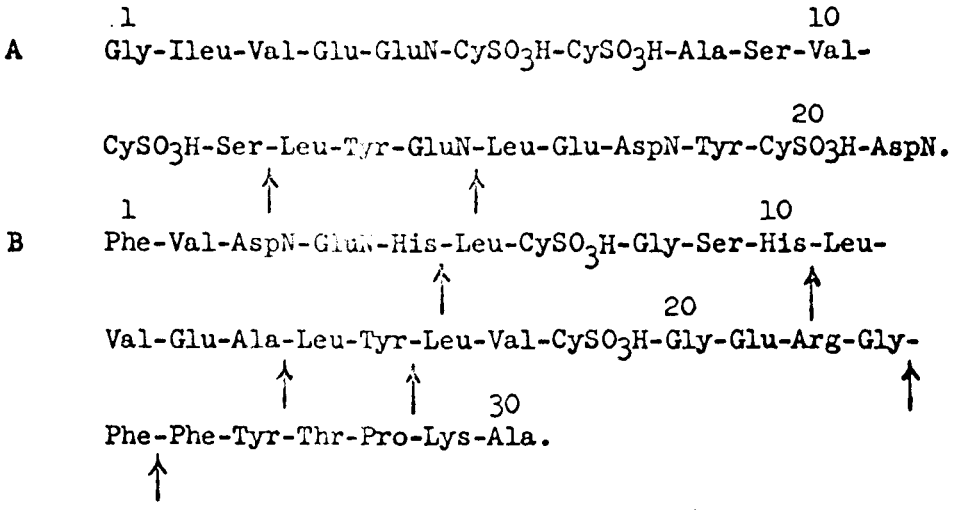

1
10
A
Gly-Ileu-Val-Glu-GluN-CySO3H-CySO3H-Ala-Ser-Val-
20
CySO3H-Ser-Leu-Tyr-GluN-Leu-Glu-AspN-Tyr-CySO3H-AspN.
1
10
B
Phe-Val-AspN-GluN-His-Leu-CySO3H-Gly-Ser-His-Leu-
20
Val-Glu-Ala-Leu-Tyr-Leu-Val-CySO3H-Gly-Glu-Arg-Gly-
30
Phe-Phe-Tyr-Thr-Pro-Lys-Ala.

Fig. 2.

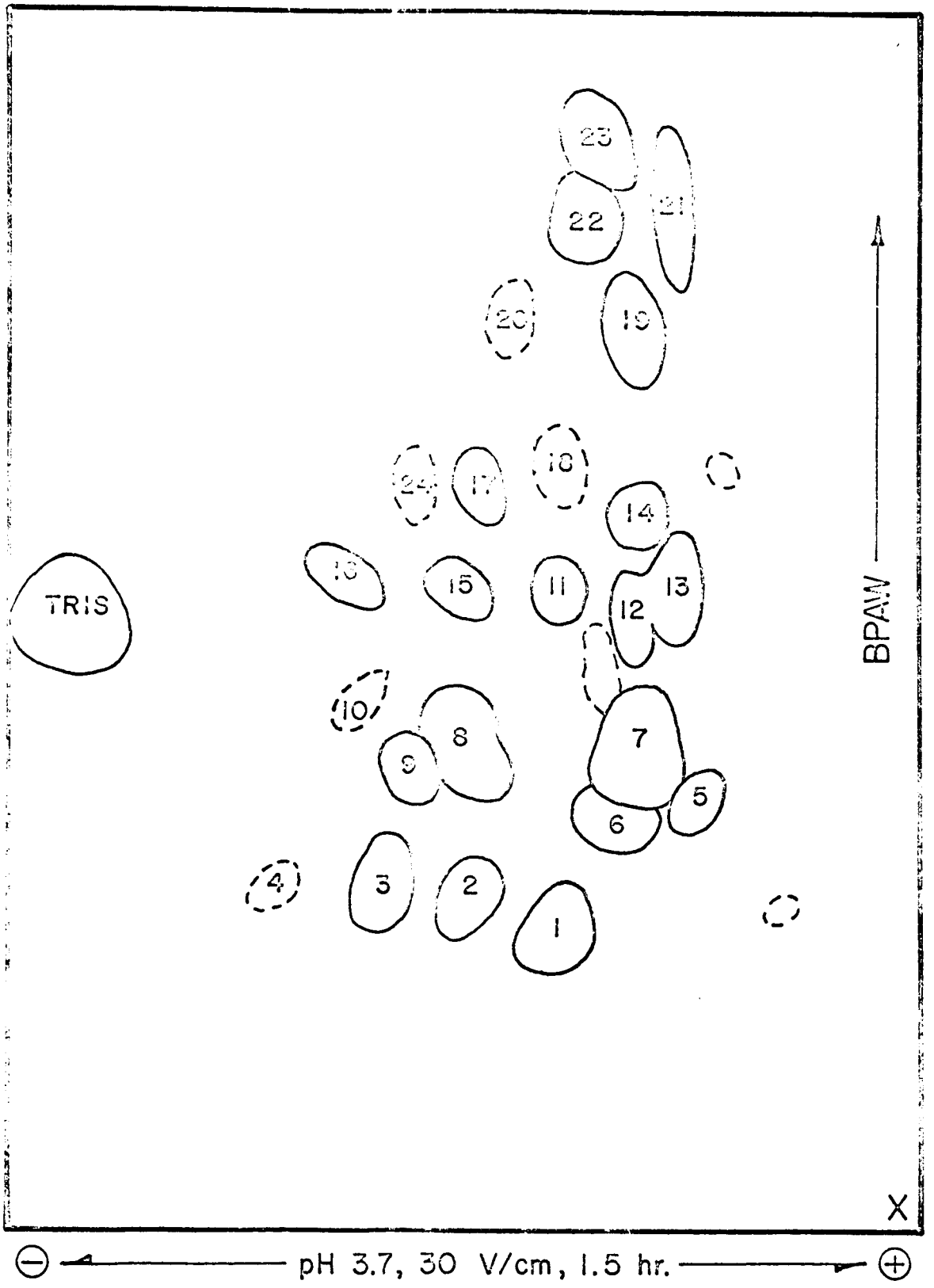

23
22
21
20
19
18
24
17
14
TRIS
15
11
13
12
10
8
9
7
5
6
4
3
2
1
BPAW
X
pH 3.7, 30 V/cm, 1.5 hr.

Fig 3

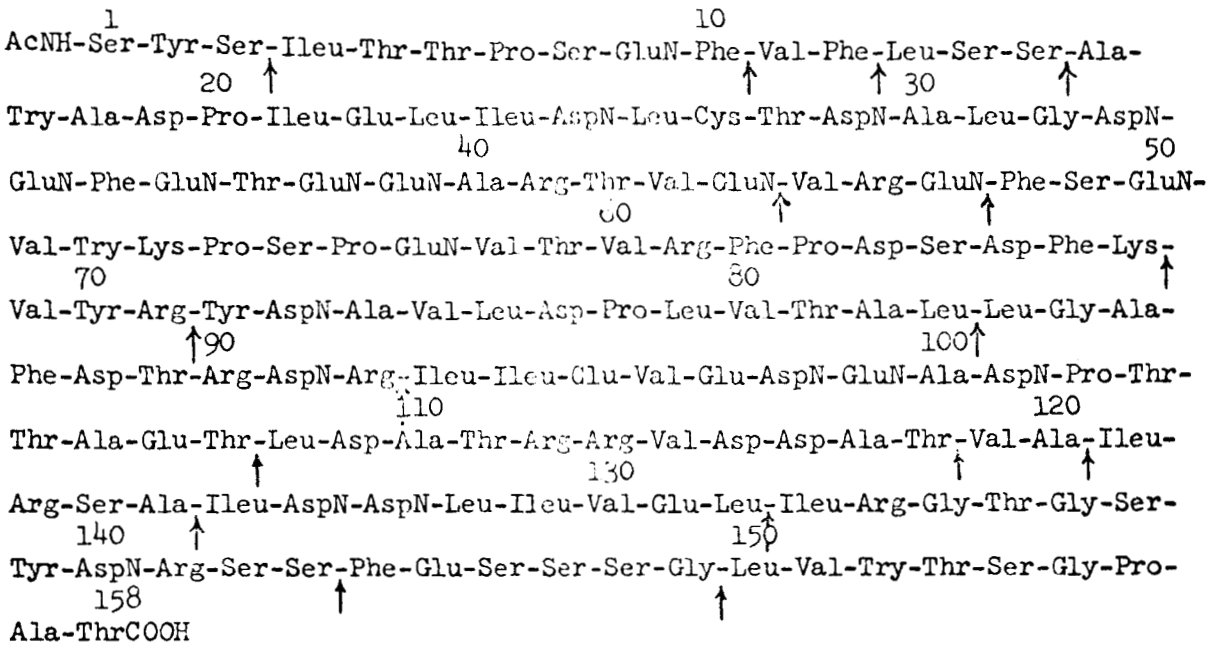

1
10
AcNH-Ser-Tyr-Ser-Ileu-Thr-Thr-Pro-Ser-GluN-Phe-Val-Phe-Leu-Ser-Ser-Ala-
20
30
Try-Ala-Asp-Pro-Ileu-Glu-Leu-Ileu-AspN-Leu-Cys-Thr-AspN-Ala-Leu-Gly-AspN-
40
50
GluN-Phe-GluN-Thr-GluN-GluN-Ala-Arg-Thr-Val-GluN-Val-Arg-GluN-Phe-Ser-GluN-
60
Val-Try-Lys-Pro-Ser-Pro-GluN-Val-Thr-Val-Arg-Phe-Pro-Asp-Ser-Asp-Phe-Lys-
70
80
Val-Tyr-Arg-Tyr-AspN-Ala-Val-Leu-Asp-Pro-Leu-Val-Thr-Ala-Leu-Leu-Gly-Ala-
90
100
Phe-Asp-Thr-Arg-AspN-Arg-Ileu-Ileu-Glu-Val-Glu-AspN-GluN-Ala-AspN-Pro-Thr-
110
120
Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg-Arg-Val-Asp-Asp-Ala-Thr-Val-Ala-Ileu-
130
Arg-Ser-Ala-Ileu-AspN-AspN-Leu-Ileu-Val-Glu-Leu-Ileu-Arg-Gly-Thr-Gly-Ser-
140
150
Tyr-AspN-Arg-Ser-Ser-Phe-Glu-Ser-Ser-Ser-Gly-Leu-Val-Try-Thr-Ser-Gly-Pro-
158
Ala-ThrCOOH

Table I

AMINO ACID COMPOSITION OF PEPTIDES PRODUCED BY THERMOLYSIN FROM OXIDIZED BOVINE INSULIN

The analytical values for each peptide are followed by the assumed number of residues, underlined. Glutamic and aspartic acid values include their amides.

Peptide:	1	2	3	4	5	6	7a	7b	8	9	10	11	12	13	14	15	16
Amino Acid																	
Lysine					0.20		0.92, 1						0.98, 1	1.0 , 1			0.32
Histidine					0.34		2.0 , 2	0.89, 1			1.9 , 2	1.7 , 2			0.91, 1		0.88, 1
Arginine					0.85, 1	0.70, 1	1.2 , 1		0.80, 1								
Cysteic acid	3.1 , 3	4.0 , 4	1.0 , 1	1.2 , 1	0.94, 1	0.92, 1	2.0 , 2	1.1 , 1	0.96, 1		1.0 , 1	0.89, 1				0.98, 1	1.0 , 1
Aspartic acid		1.7 , 2	2.0 , 2	1.8 , 2			1.1 , 1				1.0 , 1	0.94, 1			0.98, 1	1.8 , 2	
Threonine							0.81, 1						1.1 , 1	1.0 , 1			
Serine	1.7 , 2	1.8 , 2		0.26			1.0 , 1	0.75, 1			0.87, 1	0.84, 1					0.88, 1
Glutamic acid	3.2 , 3	4.1 , 4	1.0 , 1	2.2 , 2	1.3 , 1	1.1 , 1	3.0 , 3	1.2 , 1	1.0 , 1	1.4 , 1	2.1 , 2	1.1 , 1			1.0 , 1	1.0 , 1	0.42
Proline							0.72, 1			0.20				0.99, 1			
Glycine	0.65, 1	0.54, 1		0.46	1.9 , 2	1.9 , 2	3.0 , 3	1.2 , 1	1.9 , 2	0.43	1.1 , 1	1.0 , 1					1.3 , 1
Alanine	0.90, 1	0.90, 1		0.33	0.32		2.0 , 2	0.70, 1		0.99, 1	1.0 , 1		1.2 , 1	1.2 , 1			
Valine	1.8 , 2	1.9 , 2		0.47	1.1 , 1	1.1 , 1	3.0 , 3	1.4 , 1	1.2 , 1	1.0 , 1	2.0 , 2	1.1 , 1			1.1 , 1		0.37
Isoleucine	0.94, 1	0.94, 1															
Leucine	1.1 , 1	1.9 , 2	0.90, 1	1.8 , 2	1.2 , 1	1.6 , 2	4.0 , 4	1.6 , 2	0.71, 1	1.5 , 2	2.0 , 2	0.83, 1				0.57, 1	0.84, 1
Tyrosine	0.93, 1	1.6 , 2	0.85, 1	1.5 , 2	0.18	0.18	1.6 , 2			1.0 , 1			0.98, 1	0.98, 1		0.72, 1	
Phenylalanine				0.33	0.94, 1	1.0 , 1	2.2 , 2			0.37	0.64, 1	0.48, 1	1.64, 2	0.76, 1	0.48, 1		
Yield %*	55	24	100	21	36	18	7	11	25	31	58	11	27	72	40	16	13
Residues	A,1-15	A,1-21	A,16-21	A,13-21	B,17-24	B,15-24	B,1-30	B,6-14	B,17-23	B,11-16	B,1-14	B,1-10	B,24-30	B,25-30	B,1-5	A,16-21	B,6-10

*Assuming 100% for peptide 3.

Table II

DNP-AMINO ACIDS IN THERMOLYSIN DIGESTS

OF TOBACCO MOSAIC VIRUS PROTEIN

DNP-amino acid	Recovery from digests			
	(a) mole/mole protein		(b) ratio of content of amino acid end groups to content of Ileu + Leu terminal groups	
	15 min	2 hr	15 min	2 hr
Isoleucine + Leucine	0.99	2.4	100	100*
Valine	0.66	1.0	101	63
Phenylalanine	0.25	0.55	66	61
Alanine	trace	0.13	-	8
Tyrosine	0.0	trace	0	-
Aspartic and Glutamic acid	trace	?	-	-

After dinitrophenylation the peptides were heated with 6N HCl for 16 hours at 105°-106°; the ether and water-soluble fractions were separately analysed (see text). No corrections were made for losses of DNP-amino acids. Under (a) are the recoveries of DNP-amino acids on a molar basis, and under (b) the ratios (as percent) of the fraction of an amino acid in a terminal position to the fraction of terminal isoleucine + leucine.

* The ratio of isoleucine:leucine is about 1.5:1 by an analysis after regeneration from the DNP-isoleucine + -leucine mixture with ammonia.

Table III

AMINO ACID COMPOSITION OF PEPTIDES PRODUCED BY THERMOLYSIN FROM TOBACCO MOSAIC VIRUS PROTEIN

The analytical values for each peptide are followed by the assumed number of residues, underlined. Glutami and aspartic acid values include their amides.

Peptide / Amino Acid	1	3	9	10	12	14	15	16	19	24
Lysine			0.2				0.23			
Arginine	1.8, 2	1.2, 1	1.8, 2	1.2 , 1			1.6, 2	1.0, 1		0.71, 1
Aspartic acid	3.3, 3	0.62	1.4, 1		0.14	0.38	2.2, 2		0.3	
Threonine	1.7, 2	0.37	0.95,1			1.7, 2	1.0, 1		1.8, 2	
Serine		0.22	2.4 ,3	0.80, 1	2.2 , 2	0.86,1	0.52		0.88,1	
Glutamic acid		1.0, 1	0.17			1.1 ,1	0.30		0.23	
Proline						0.98,1			0.88,1	
Glycine		0.23	2.0 ,2			0.23	1.1, 1		1.0 ,1	
Alanine	2.2, 2	0.17	0.16	0.84, 1	0.10	0.17	1.2, 1		1.1 ,1	
Valine	0.91,1	0.88,1	0.24			0.10	0.2	1.1, 1	1.4 ,1	1.1 , 1
Isoleucine			0.95,1	0.99, 1		0.79,1	0.2		0.17	
Leucine	0.72,1		0.38		0.90, 1	0.21	1.3, 1		0.93,1	
Tyrosine			0.78,1					0.90,1		1.2 , 1
Phenylalanine			0.30			0.93,1	0.8, 1			
Residues	108-118	45-47	133-143	121-124	13-15	4 - 10	84 - 92	69-71	150-158	69-71

Table IV

SUMMARY OF THE PEPTIDE BONDS ATTACKED BY THERMOLYSIN

R_1	R_2
Ileu	Lys, Arg, Ser, Gly, Ala, Met, Leu
Leu	His, AspN, Thr, Ser, GluN, Gly, Ala
	Leu, Tyr, Phe
Val	Lys, GluN, Thr, Phe
Phe	GluN, Ser, Gly, Leu, Phe
Ala	Lys, Ser
Tyr	Arg