OBSERVATIONS ON THE SPECIFICITY OF A THERMOSTABLE BACTERIAL PROTEASE "THERMOLYSIN"

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A proteolytic enzyme with the commercial name "Thermoase" was isolated by Endo (1962) from cultures of Bac. thermoproteolyticus Rokko, and was found to be comparatively stable at high temperatures ($\simeq 80^{o}$) in the presence of Ca^{++}. It was recently reported that the enzyme had a strong elastase-like activity (Morihara et al, 1965). We propose the trivial name thermolysin for this enzyme.

The substrate specificity of thermolysin was studied using beef heart cytochrome c as a substrate. The peptides produced were separated by two-dimensional paper chromatography and electrophoresis, and analysed. It was found that thermolysin hydrolysed 10 of the 12 peptide bonds involving the $-NH_2$ groups of isoleucine and leucine residues in cytochrome c under the conditions used. Three other bonds, involving the amino sites of phenylalanine and alanine residues, were also hydrolysed.

Materials and Methods: Chromatographically pure beef heart cytochrome c isolated according to Hagihara et al (1958) was dialysed against distilled and deionized water and lyophilised. Thermolysin was purified by crystallization x 4 times as described by Endo (1962). All other reagents used were analytical grade.

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About 10 mg of cytochrome c was dissolved in 2 ml of water and the pH was adjusted to 8.0 with 0.1 N NaOH. To this solution was added 0.5 ml of 0.01 % enzyme solution containing 0.1 M Tris (hydroxymethylamino methane) buffer, pH 8.0, and 0.002 M calcium chloride. The reaction was carried out for 4.5 hr. at 35°-37°C and during the reaction the pH was maintained at 8.0 with 0.1 N NaOH. At the end of the reaction, 1 drop of glacial acetic acid was added and the solution was lyophilised. The dried material was dissolved in 0.1 ml of water and 0.01-0.02 ml spotted on a sheet of Whatman No. 3MM paper. A peptide map was made first by paper chromatography and then by electrophoresis. A BPAW system (butanol:pyridine:acetic acid: water = 15:10: 3:12, v/v, Walley and Watson, 1953) was used for the chromatography and a pH 3.7 buffer (pyridine:acetic acid:water = 1:10:289, v/v, Katz et al, 1959) for the electrophoresis which was for 1.5 hr at 30 V/cm. Three sheets were made and one was sprayed with 0.1% ninhydrin solution in acetone to identify the location of the peptides and the others with 0.02% ninhydrin solution for the elution of the peptides with 30% acetic acid. After drying, each peptide was hydrolysed with 0.5 ml of twice-distilled 6 N HCl for 24 hr at 105°-106°C in a sealed and evacuated tube. The amino acid composition was analysed by a Spinco automatic analyser Model 120B with an accelerated system and sensitive cuvettes based on the method of Spackman et al, (1958). Minor components showed complicated compositions and mainly the clear spots were analysed. The composition of the peptides was sufficient to identify the fragments with respect to their positions in the sequence in cytochrome c (Yasunobu et al, 1963) except in a few cases in which a modified Edman's phenylisothiocyanate method (Light & Greenberg, 1965) or a carboxypeptidase method (Fraenkel-Conrat et al, 1956) was applied to identify either the amino or carboxyl terminal residue of the peptide. The tryptophan-containing peptide was isolated on a different sheet; it was separated only by BPAW and identified by Ehrlich reagent (Smith, 1953).

Results and Discussions: Fig. 1 shows the peptide map of the digest of beef

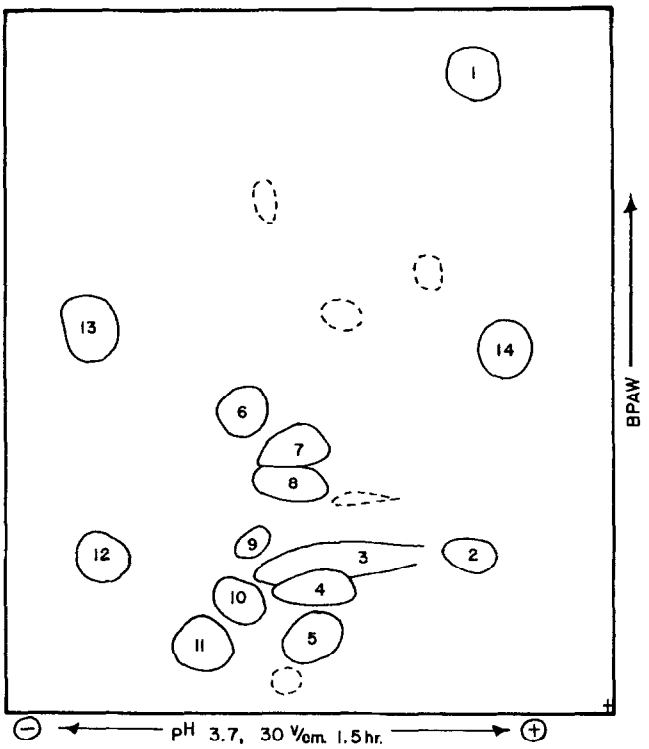

Fig. 1. Peptide map of thermolysin digest of beef cytochrome c. Ten mg of cytochrome c was digested with 0.05 mg of thermolysin for 3.5 hr. at 35°-37°C. The peptide map was made by two dimensional chromatography (first, BPAW), and electrophoresis (second, pH 3.7, 30 v/cm, 1.5 hr.). Spots were developed by spraying with 0.1% ninhydrin solution.

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cytochrome c with thermolysin. Fairly good separation of the peptides was obtained by two dimensional chromatography and electrophoresis. Peptide 3 showed the heme color of cytochrome c. Peptide 13 showed yellow color and was tris. Peptide 14 was the tryptophan peptide which was located separately (see above).

Table I shows the amino acid composition, relative yield, and the position in the sequence of each peptide separated as shown and numbered in Fig. 1. Peptides 2, 3, 5, 10, 12 and 14 were identified solely by their composition as corresponding to regions in the known amino-acid sequence of cytochrome c (Fig. 2). The yield of peptide 14, containing tryptophan and obtained sepa-

rately, was not calculated. Other peptides were examined for their amino or carboxyl terminal residues. Peptide 1 corresponded mainly to residues 64 to 67 or 65 to 68 with a minor component corresponding to residues 81 to 84 or 82 to 85. Since carboxy-peptidase released tyrosine as a main spot, peptide one must be the 64 to 67 sequence, and the minor component must be 81 to 84 since peptide eleven consisted of the sequence 85 to 94. Peptides 4, 6, 7, 8 and 11 were found by the Edman procedure to have amino-terminal residues of phenylalanine, leucine, leucine, leucine and isoleucine respectively. Peptide 9 was not examined for its terminal residue and its origin could not be distinguished between 36 to 45 or 37 to 46. However, in a separate experiment a small amount of peptide fragment corresponding to 32 to 35 was found, so that peptide 9 corresponds to 36 to 45. No peptide fragment corresponding to 95 to 97 was found. The results are in Fig. 2.

The following peptide bonds were hydrolysed: gly-ileu(2), lys-ileu, met-ileu, leu-ileu, tyr-leu(2), aspNH_2-leu, gly-leu, thr-leu, gly-phe, leu-phe, and lys-ala.

Ten out of 12 (83%) of the isoleucine and leucine residues in beef cytochrome c were hydrolysed at the amino site of their peptide linkages. Calculated

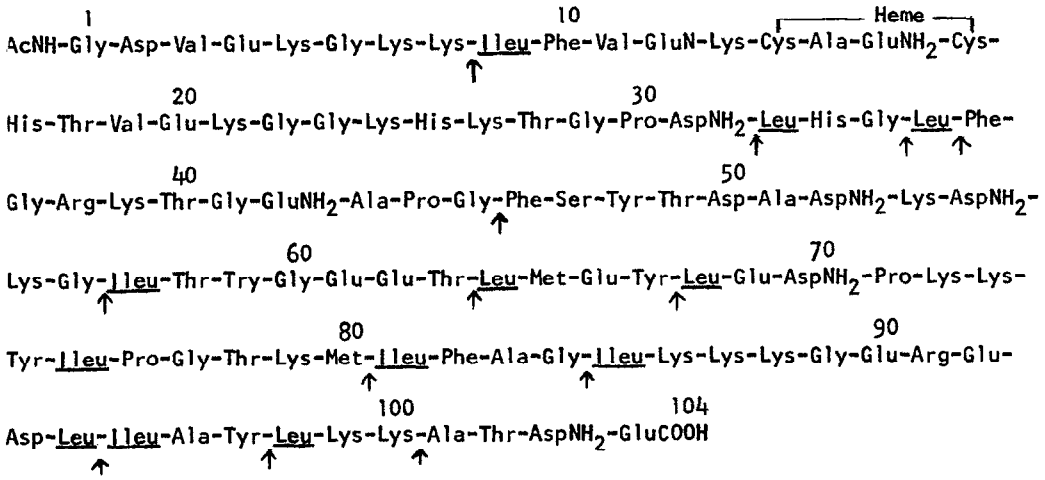

Fig. 2. Amino acid sequence of cytochrome c, showing peptide bonds hydrolysed by thermolysin (arrows). The isoleucine and leucine residues in cytochrome c are underlined.

TABLE 1

AMINO ACID COMPOSITION OF BEEF CYTOCHROME C PEPTIDES
PRODUCED BY HYDROLYSIS WITH THERMOLYSIN

The analytical values for each peptide are followed by the assumed number of residues, underlined, unless the analytical value represents an impurity. Glutamic and aspartic acid values include glutamine and asparagine respectively.

Peptide:	1	2	3	4	5	6	7	8	9	10	11	12	14
Amino Acid													
Lysine		0.18	4.0, 4	1.9, 2	2.8, 3		0.84, 1	2.9, 3	1.36, 1	1.9, 2	3.0, 3	2.2, 2	
Histidine			1.8, 2			0.98, 1							
Arginine							0.92, 1		0.70, 1		0.88, 1		
Aspartic acid	0.04	1.1, 1	1.1, 1	3.0, 3	1.1, 1			1.1, 1	0.25	1.1, 1	1.0, 1		
Threonine	0.05	0.78, 1	1.8, 2	0.86, 1			0.88, 1	0.96, 1	0.92, 1	0.73, 1			1.7, 2
Serine	0.08			0.78, 1									
Glutamic acid	1.35, 1	1.0, 1	3.3, 3		0.96, 1		1.0, 1	1.2, 1	1.10, 1	1.2, 1	2.1, 2		2.1, 2
Proline			0.8, 1				0.76, 1	1.9, 2	1.07, 1				
Glycine	0.22	0.32	3.1, 3	1.1, 1	1.90, 2	1.1, 1	3.4, 3	1.3, 1	2.75, 3		1.1, 1		1.0, 1
Alanine	0.31	0.94, 1	1.1, 1	1.1, 1			1.2, 1		0.93 1	1.0, 1			
1/2 Cysteine			+, 2										
Valine			2.1, 2		0.90, 1				0.17				
Methionine	1.17, 1							0.76, 1					
Isoleucine	0.23		0.9, 1					0.76, 1			0.94, 1		0.8, 1
Leucine	0.51, 1					0.96, 1	0.92, 1	0.96, 1		1.1, 1	1.0, 1	0.95, 1	
Tyrosine	0.96, 1			0.24, 1				0.50, 1					
Phenylalanine	0.14		0.9, 1	0.86, 1			1.0, 1		0.55, 1				
Tryptophan													+, 1
Yield %	50	62	16	50	100	42	50	29	18	30	70	38	7
Residues *	64-67	101-104	9-31	46-56	1-8	32-34	35-45	68-80	36-45	98-104	85-94	98-100	57-63

on the basis of the total number of bonds split by the enzyme, about 77% hydrolysis occurred at isoleucine and leucine residues. In contrast, the residues involved in the carboxyl sites of the bonds hydrolysed varied between hydrophilic and hydrophobic, and between basic and neutral.

An elastase isolated from Pseudomonas aeruginosa was recently found to hydrolyse peptide bonds involving the amino sites of leucine, valine, tyrosine, and phenylalanine residues in the B chain of insulin (Morihara et al, 1965). The fact that thermolysin has a high elastase activity (Morihara et al, 1965) suggests a similarity between it and the Pseudomonas enzyme. A proteolytic enzyme from the venom of the snake Crotalus atrox was reported to hydrolyse insulin and glucagon at peptide bonds involving the amino sites of leucine and phenylalanine residues (Pfleiderer & Krauss, 1965).

The property of thermolysin in preferentially attacking leucine and isoleucine residues in peptide linkage is of potential usefulness in studies of the primary structure of proteins. The fact that hydrolysis takes place at the $-NH_2$ group is of further interest in that it enables the DNP reaction to be used as a rapid test for the number of residues so liberated.

Further studies are in progress, using insulin and tobacco mosaic virus protein. Prolonging the time of hydrolysis or increasing the enzyme: substrate ratio seem to lead to the hydrolysis of other bonds.

References:
Endo, S., J. Fermentation Tech., 40, 346 (1962).
Fraenkel-Conrat, H., Harris, J. I. and Levy, A. L., in D. Glick (ed). Methods of Biochemical Analysis, Vol. II, Interscience Publishers, Inc., New York, (1956) p. 397.
Hagihara, B., Tagawa, K., Morikawa, I., Shin, M., and Okunuki, K., J. Biochem., Tokyo, 45, 725 (1958).
Katz, A. M., Dreyer, W. J. and Anfinsen, C. B., J. Biol. Chem., 234, 2897 (1959).
Light, A. and Greenberg, J., J. Biol. Chem., 240, 258 (1965).
Morihara, K., Tsuzuki, H., and Oka, T., The 17th Symposium on Enzyme Chemistry, (1965) p. 293.
Pfleiderer, G., and Krauss, A., Biochem. Z., 342, 85 (1965).
Smith, I., Nature, 171, 43 (1953).
Spackman, D. H., Stein, W. H. and Moore, S., Anal. Chem. 30, 1190 (1958).
Walley, S. G. and Watson, J., Biochem. J., 55, 328 (1953)
Yasunobu, K., Nakashima, T., Higa, H., Matsubara, H. and Benson, A., Biochim. Biophys. Acta, 78, 791 (1963).