THE BASIC TRYPSIN INHIBITOR OF BOVINE PANCREAS. III. A PROGRESS REPORT ON THE TRYPTIC PEPTIDES*

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A derivative of the basic trypsin inhibitor of Kunitz and Northrop (1936) has been digested with trypsin, and the peptides isolated and analyzed. Nine peptides, all present in high yield in the digest, account for the 58 amino acids of the protein. The N-terminal peptide is a pentadecapeptide, which contains all 4 proline residues of the protein. The sequence of this peptide, as well as of some of the smaller ones, has been determined.

The preparation of trypsin inhibitor and trypsin were described previously (Kassell et al., 1963). The S-S linkages of the inhibitor were reduced and the carboxymethyl (RCM) derivative prepared according to Crestfield et al. (1963). It was digested with trypsin and the peptides separated on Dowex 50-X2 (Margoliash and Smith, 1962). Amino acid analysis was carried out as previously described (Kassell and Laskowski, 1961) using 24 or 72 hour hydrolysis. Three methods were used for N-terminal analysis: a) the Sanger fluorodinitrobenzene method (Fraenkel-Conrat et al., 1955), b) the subtractive Edman method of Konigsberg et al. (1962), and c) following (b) the phenylthiohydantoin (PTH) derivatives were hydrolyzed to free amino acids (Fraenkel-Conrat et al., 1955), which were

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determined on the automatic amino acid analyzer. C-terminal amino acids were identified by hydrazinolysis (Niu and Fraenkel-Conrat, 1955).

(1) Arg-Pro-Asp-Phe-Cys-Leu-Glu-Pro-Pro-Tyr-Thr-Gly-Pro-Cys-Lys
(2) Ala-Lys-Arg (also some Ala-Lys + Arg)
(3) Ileu-Ileu-Arg
(4) Ala-Arg
(5) Ala,Asp,Phe,Tyr_2,Lys
(6) Ala,Asp,Cys,Glu,Meth,Ser,Arg
(7) Ala,Cys_2,Glu,Gly_3,Leu,Phe,Thr,Tyr,Val,Arg
(8) Asp_2,Phe,Lys
(9) (Cys,Gly_2,Thr) Ala

Figure 1. The 9 main peptides of the tryptic digest.

The three amino acids at the N-terminus of the RCM-inhibitor (determined by method c above) are Arg-Pro-Asp. The C-terminal amino acid is alanine. Fig. 1 shows the main tryptic peptides. Small quantities of additional peptides, consisting of parts of these peptides, were also obtained. The sequences of peptides (3) and (4) are assumed from the specificity of trypsin. The sequence determination of peptides (1) and (2) will be described in detail.

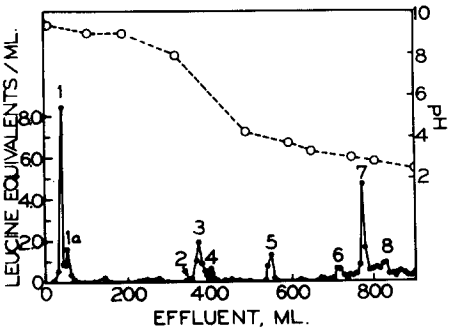

Figure 2. Chromatography of the products of partial acid hydrolysis of peptide (1) on Dowex 1-X2. Column 95 x 1 cm, 38°, load 78 mg, fractions 2 ml. The starting buffer was 250 ml of pyridine-N-ethylmorpholine-acetic acid, pH 9.3, in a constant volume gradient bottle; the gradient was 0.1-2 M acetic acid (Schroeder et al., 1962). The peaks are identified in Fig. 3.

In peptide (1) arginine is the N-terminal found by method (a). Lysine is the C-terminal. Partial acid hydrolysis (Schultz et al.,

1962) was used to obtain smaller pieces. Peptide (1), 78 mg, was heated in 78 ml of 0.03 M HCl, previously deaerated with nitrogen, in 4 sealed, evacuated tubes, at 105° for 10 hours. The digest was lyophilized and chromatographed as indicated in Fig. 2. The peptides isolated are given in Fig. 3. In addition to the 3 components expected, i.e., peak 1 (Arg-Pro), peak 5 (aspartic acid) and peak 7 (the remaining 12-membered peptide), further breakdown of peptide 7 occurred, yielding the peptides below #7 in Fig. 3. These provide confirmation of the sequence. Peptide

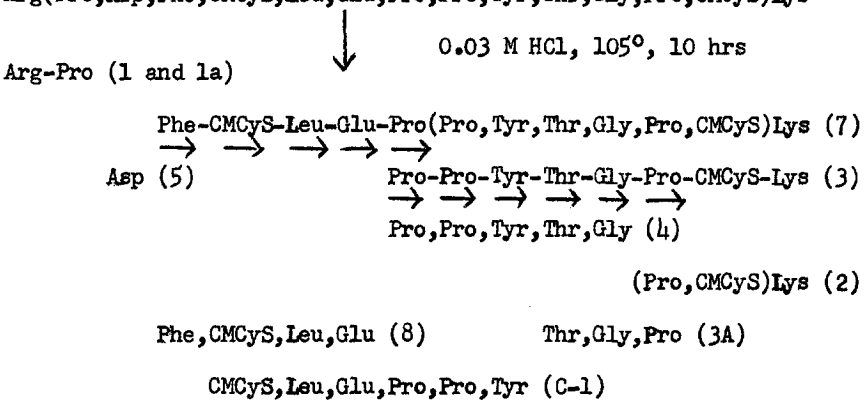

Figure 3. Protocol for sequence determination. Horizontal arrows indicate stepwise degradation by Edman's method summarized in Tables I (peak 3) and II (peak 7).

3A was derived by further acid hydrolysis of peptide 3 (heating in a boiling water bath in 5.7 N HCl for 40 minutes) and was the only peptide isolated after this procedure in pure form by paper electrophoresis (Katz et al., 1959). Peptide C-1 was isolated from a chymotryptic digest to be described elsewhere. The small peaks not identified in Fig. 2 proved on analysis to be either free amino acids or mixtures present in quantities too small to separate. Peak 6 is impure starting peptide.

Peptides 3 and 7 were used for the stepwise Edman degradation, indicated by the arrows of Fig. 3. The results are shown in Tables I and II respectively. In each case, identification of the PTH compound split off (last column) agreed with the subtractive method. As expected,

PTH-threonine and PTH-carboxymethylcysteine were not recovered as free amino acids. The composite results establish the sequence given in Fig. 1 for peptide 1.

TABLE I. Edman Degradation of Peak 3

Step	Molar Ratio of Amino Acids Remaining						Free Amino Acid from PTH
	CM-Cys	Gly	Lys	Pro	Thr	Tyr	
0	0.90	0.93	1.00	2.60	0.80	0.60	---
1	1.05	0.89	1.00	[1.93]	0.68	0.66	Pro
2	0.79	0.65	1.00	[0.87]	0.61	0.54	Pro
3	0.98	0.97	1.00	1.12	0.82	[0]	Tyr
4	1.31	1.75	1.00	1.16	[tr]		None
5	1.17	[0.31]	1.00	1.43			Gly
6	0.86		1.00	[tr]			Pro

TABLE II. Edman Degradation of Peak 7

Step	Molar Ratio of Amino Acids Remaining									Free Amino Acid from PTH
	CM-Cys	Glu	Gly	Leu	Lys	Phe	Pro	Thr	Tyr	
0	1.40	0.91	1.13	1.14	1.00	1.19	2.69	1.09	0.97	---
1	1.81	0.98	1.00	0.98	0.82	[tr]	2.82	0.89	0.89	Phe
2	[1.01]	0.96	1.00	0.93	0.82		2.92	0.90	0.82	None
3	0.92	0.97	1.00	[0.09]	0.80		2.80	0.87	0.89	Leu
4	0.94	[0.12]	1.00		0.58		2.78	0.78	0.80	Glu
5	0.51*		1.00		0.40		[2.15]	0.80	0.70	Pro

* Includes some cysteic acid.

The sequence of peptide 2 of Fig. 1 was established by the subtractive Edman method with the results in Table III.

TABLE III. Subtractive Edman Degradation of Peptide 2

Step	Molar Ratio of Amino Acids Remaining		
	Ala	Lys	Arg
0	1.00	1.00	1.01
1	[0.19]	1.00	1.11
2	0.09	[0.07]	1.00

Discussion — In contrast to the resistance of native inhibitor to tryptic digestion (Kassell and Laskowski, 1956), the RCM-derivative was digested at all the linkages of the basic amino acids, except the N-terminal Arg-Pro linkage, which is known to be resistant to trypsin (Bell, 1954, Hirs et al., 1956, Jones et al., 1963). It seems rather doubtful that the sequence Arg-Pro can by itself account for the blocking of the active center of trypsin, since oxidized and RCM-inhibitors are inactive and since Arg-Pro and Lys-Pro sequences occur in many proteins, e.g. ACTH (Bell, 1954), ribonuclease (Hirs et al., 1956), clupeine (Ando et al., 1962) and melanocyte stimulating hormone (Lee et al., 1961). However, participation in a more complex "inhibiting center" is not excluded.

The concentration of proline residues found in the N-terminal fourth of the inhibitor, while uncommon in proteins other than the collagens, is not unique, e.g. cytochrome C-551 (Gray and Hartley, 1963) and catalase (Schroeder et al., 1964) contain regions even higher in proline.

REFERENCES

Ando, T., Iwai, K., Ishii, S.-I., Azegami, M. and Nakahara, C., Biochem. Biophys. Acta 56, 628 (1962).
Crestfield, A. M., Moore, S. and Stein, W. H., J. Biol. Chem. 238, 622 (1963).
Fraenkel-Conrat, H., Harris, J. I. and Levy, A. L., in D. Glick (Editor), Methods of Biochemical Analysis, Vol. II, Interscience Publishers, Inc., New York, 1955, p. 359.
Gray, W. R. and Hartley, B. S., Biochem. J., 89, 379 (1963).
Kassell, B. and Laskowski, M., Sr., J. Biol. Chem. 219, 203 (1956).
Kassell, B. and Laskowski, M., Sr., J. Biol. Chem. 236, 1996 (1961).

Kassell, B., Radicevic, M., Berlow, S., Peanasky, R. J. and Laskowski, M., Sr., J. Biol. Chem. 238, 3274 (1963).
Katz, A. M., Dreyer, W. J. and Anfinsen, C. B., J. Biol. Chem. 234, 2897 (1959).
Konigsberg, W. and Hill, R. J., J. Biol. Chem. 237, 2547 (1962).
Kunitz, M. and Northrop, J. H., J. Gen. Physiol. 19, 991 (1936).
Lee, T. H., Lerner, A. B. and Buettner-Janusch, V., J. Biol. Chem. 236, 1390 (1961).
Margoliash, E. and Smith, E.L., J. Biol. Chem. 237, 2151 (1962).
Niu, C.-I. and Fraenkel-Conrat, H., J. Am. Chem. Soc. 77, 5882 (1955).
Schroeder, W. A., Jones, R. T., Cormick, J. and McCalla, K., Anal. Chem. 34, 1570 (1962).
Schroeder, W. A., Shelton, R. J., Shelton, J. B. and Olson, B. M., Biochim. Biophys. Acta 89, 47 (1964).
Schultz, J., Allison, H. and Grice, M., Biochemistry 1, 694 (1962).