UDC 547.965

A CHARACTERISTIC FRAGMENT OF THE POLYPEPTIDE CHAIN OF PEPSIN

R. A. Matveeva and V. M. Stepanov

Khimiya Prirodnykh Soedinenii, Vol. 3, No. 4, pp. 263-266, 1967

As a rule, the amino acids in the polypeptide chains of proteins are arranged irregularly. Accumulations of similar amino acid residues forming so-called "clusters" are found in individual sections. Information exists that indicates that such clusters may play an essential role in determining the specific spatial structure of a protein and, consequently, its functional properties. Thus, a comparison of the primary structure of cytochrome c of different origins has shown that in the evolutionary process of these proteins changes in the amino acids present in the clusters take place comparatively rarely. The evolutionary stability of these sections of the peptide chain can be explained on the assumption that a change in the amino acids forming a cluster interferes with the latter and leads to such a change in the specific spatial structure as to deprive the proteins of functionally important properties [1]. The tryptophan-tryptophan sequence (No. 62 and 63) found in the polypeptide chain of the lysozyme of hens' eggs participates in the structure of the hydrophobic "backbone" which is a characteristic element of the tertiary structure of this enzyme [2].

In a study of a tryptic hydrolyzate of porcine pepsin, we have isolated peptides rich in tyrosine corresponding to the following fragment of the primary structure: Gln-Tyr-Tyr-Tyr.*

As far as we know, this is the first time that such an accumulation of tyrosine residues forming a well-defined cluster has been observed. It would be premature to draw any conclusions concerning the possible role of this fragment in maintaining the spatial structure and, all the more, in the catalytic activity of pepsin. However, what is worthy of attention is the remarkable similarity of the sequence of amino acids found and the structure of the specific low-molecular-weight substrates for pepsin. For example, carbobenzoxyglutamyltyrosine has recently been used as one of the best synthetic pepsin substrates [3, 4], and the peptides carbobenzoxyglutamyltyrosyltyrosine and carbobenzoxyglutamyltyrosyltyrosyltyrosine are formed in a transpeptidation process catalyzed by pepsin [5]. The hydrolysis of the peptide bonds formed by tyrosine frequently takes place under the action of pepsin on protein substrates [6]. On the basis of these data, it may be assumed that the tyrosine-rich cluster plays some part in the specific binding of the substrate by pepsin. This assumption of course is of the nature of a working hypothesis.

We have previously carried out the trypsin hydrolysis of reduced carboxymethylated pepsin and the fractionation of the resulting mixture of peptides on Sephadex G-50 [7]. The experimental procedures used in this work on the separation of the peptides and the determination of their structures have been described in detail previously [8, 9].

The low-molecular-weight fraction of the trypsin hydrolyzate E was separated by preparative paper chromatography in the butanol-water-acetic acid (144:43:13) system. The treatment of control strips of the chromatogram with Pauly's diazo reagent showed up two zones: E-1, located close to the solvent front, and E-2. These peptides were eluted with water and were additionally purified by paper electrophoresis at 1000 V. At pH 5.6, peptides E-1 and E-2 bear negative charges and migrate towards the anode. No migration towards the cathode takes place at pH 2.2. Neither of the peptides was stained with ninhydrin. No N-terminal amino acid was detected by dinitrophenylation. Incubation of the peptides with leucine aminopeptidase was also unsuccessful. Consequently, in both peptides the N-terminal amino group is blocked.

The composition of peptide E-1 was determined by means of an automatic amino acid analyzer after hydrolysis with 5.7 N hydrochloric acid at 110° C for 24 hr. The same amount of peptide E-1 was hydrolyzed with carboxypeptidase A at pH 8.5 and 37° C for 20 hr. Peptide E-2 was hydrolyzed with acid and carboxypeptidase A similarly. The amino acid contents in the acid and carboxypeptidase hydrolyzates of peptides E-1 and E-2 (μmole) were as follows:

Amino acid	Peptide E-1 hydrolyzate		Peptide E-2 hydrolyzate	
	Acid	Carboxypeptidase	Acid	Carboxypeptidase
Tyrosine	0.42	0.37	0.53	0.51
Glutamic acid	0.20	0	0.16	0

Thus, the composition of peptide E-1 corresponds to the formula Glu_1, Tyr_2, and peptide E-2 has the composition Glu_1, Tyr_3. The incubation of both peptides with carboxypeptidase A leads to the practically complete splitting off of the tyrosine residues. These results agree well with the sequences Glu-Tyr-Tyr for E-1 and Glu-Tyr-Tyr-Tyr for E-2. Since neither of the peptides has a free α-amino group, it is possible that the N-terminal glutamic acid residues in them are cyclized to form pyroglutamic (pyrrolidone-α-carboxylic) acid. Such a conversion is found not infrequently in protein hydrolyzates.

*In accordance with established practice, we use the abbreviation Gln for glutamine and Asn for asparagine.

The presence of N-terminal pyroglutamic acid in the peptides E-1 and E-2 was confirmed in the following way. Under the conditions described above, 0.6 μmole of the peptide E-2 was incubated with carboxypeptidase A. The hydrolyzate was separated by paper electrophoresis at pH 2.2 and 1000 V for 2 hr. The treatment of a control strip showed the presence of free tyrosine and of a zone coinciding in mobility with the zone of an authentic sample of pyroglutamic acid. To detect this acid, the electrophoregram was kept in an atmosphere of chlorine and was then sprayed with a 1% solution of potassium iodide [10], after which the pyroglutamic acid appeared in the form of a brown spot. The zone corresponding to the acid was eluted with water, the eluate was evaporated, and the residue was hydrolyzed with 5.7 N hydrochloric acid at 110° C for 20 hr. Determinations of the composition on an amino acid analyzer gave the following results: glutamic acid 0.55 μmole, and tyrosine 0.16 μmole.

Thus, the carboxypeptidase hydrolyzate of peptide E-2 contained tyrosine and pyroglutamic acid. The presence of small amounts of tyrosine in the hydrolyzate of the zone corresponding to pyroglutamic acid is explained by the assumption that the degradation of the peptide with carboxypeptidase A did not take place completely. The same results were obtained in a study of the carboxypeptidase hydrolyzate of peptide E-1. These results permit the following structures to be ascribed to the peptides:

Pyroglu-Tyr-Tyr (E-1),

Pyroglu-Tyr-Tyr-Tyr (E-2).

The presence of N-terminal pyroglutamic acid explains the following peculiar behavior of the peptides: the absence of a N-terminal amino acid determinable by dinitrophenylation; the stability of the peptides to hydrolysis by leucine aminopeptidase; the negative reaction with ninhydrin; the absence of migration towards the cathode on electrophoresis in an acid medium; and the practically complete splitting off of tyrosine by carboxypeptidase A.

It is obvious that peptides E-1 and E-2 do not themselves represent any fragment of the polypeptide chain of pepsin and they are formed after the degradation of the protein with trypsin by the conversion of the N-terminal residue into pyroglutamic acid. It is known that such a conversion takes place particularly readily and, as a rule, completely, if a glutamine residue is present in the N-terminal position of the peptide [11, 12]. Consequently, it may be assumed that peptides E-1 and E-2 correspond to the following sequences of amino acids in the polypeptide chain of pepsin:

Gln-Tyr-Tyr (E-1),

Gln-Tyr-Tyr-Tyr (E-2),

the tripeptide sequence forming part of the tetrapeptide sequence.

The formation of peptides E-1 and E-2 is the result of the splitting of tyrosine peptide bonds, which is characteristic for the specificity of chymotrypsin but not of trypsin. As we have observed previously [7], porcine pepsin is extremely poor in arginine and lysine residues, at which trypsin hydrolysis takes place, and this apparently also explains the splitting of a fairly large number of bonds corresponding to the chymotrypsin type of specificity.

In addition to peptides E-1 and E-2, we isolated from the pepsin hydrolyzate a peptide owing its origin to the rupture of bonds characteristic for the specificity of trypsin. Fraction D of the trypsin hydrolyzate [7] was separated by preparative paper chromatography in the butanol-water-acetic acid (144:43:13) system. The zone D-31 located close to the solvent front was eluted and subjected to paper electrophoresis at 1000 V and pH 5.6 for 3 hr, which led to its separation into two fractions: a neutral fraction D-311 and a fraction migrating towards the cathode, D-312. The results of determinations of the composition of peptide D-312 show that it contained one residue each of alanine and lysine and two residues of aspartic acid or asparagine. It was found by the dinitrophenyl method that the N-terminal amino acid was alanine. The C-terminal position of the lysine is not a matter of doubt, since the peptide contains no other amino acids corresponding to the main or "secondary" specificity of trypsin.

Thus, the structure Ala-Asp-Asp-Lys may be proposed for the peptide D-312; however, it is still not clear whether residues of aspartic acid or of asparagine are present in the peptide. Since at pH 5.6 the peptide has a positive charge, it must be concluded that it contains two residues of asparagine and not of aspartic acid. After the incubation of the peptide with leucine aminopeptidase (pH 8.5, 37° C, 20 hr), the following amino acids were found in the hydrolyzate (μmole): alanine 0.31, asparagine 0.43, aspartic acid 0.12 (the lysine was not determined). These results fully agree with the sequence Ala-Asn-Asn. The appearance of a small amount of aspartic acid in the hydrolyzate can be explained by the fact that one of the amide groups of the peptide D-312 is readily split off (cf. [13]). All these results lead to the structure Ala-Asn-Asn-Lys for D-312.

The presence in pepsin of the sequence Asp-Arg-Ala-Asn-Asn-Lys-Val-Gly-Leu has been shown previously [13].

It is obvious that the peptide D-312 is formed in the trypsin hydrolysis of the Arg-Ala and Lys-Val bonds corresponding to the specificity of this enzyme. The isolation of peptide D-312 is one more proof of the existence in pepsin of the

nonapeptide sequence given above. It is interesting to observe that in this fragment of the molecule, of the four basic amino acid residues present in pepsin two are adjacent, which gives some grounds for speaking of a "cluster" of amino acids.

Summary

Peptides of the structure Pyroglu-Tyr-Tyr, Pyroglu-Tyr-Tyr-Tyr, and Ala-Asn-Asn-Lys have been isolated from a trypsin hydrolyzate of reduced carboxymethylated porcine pepsin. The first two of them correspond to the sequence Gln-Tyr-Tyr-Tyr which is present in pepsin and which represents a well-defined cluster of tyrosine residues. The hypothesis has been put forward that this fragment of the structure of pepsin may participate in the enzyme-substrate interaction.

REFERENCES

1. J. C. Bennet and W. J. Dreyer, Ann. Rev. Biochem., **33**, 228, 1964.
2. C. C. Blake et al., Nature., **206**, 757, 1965.
3. J. S. Fruton and M. Bergmann, J. Biol. Chem., **127**, 627, 1939.
4. C. A. Dekker, S. P. Taylor, and J. S. Fruton, J. Biol. Chem., **180**, 155, 1949.
5. B. H. Neumann, G. Levin, A. Berger, and E. Katchalski, Biochem. J., **73**, 33, 1959.
6. J. Tang, Nature., **199**, 1094, 1963.
7. V. M. Stepanov and R. A. Matveeva, KhPS [Chemistry of Natural Compounds], 128, 1965.
8. T. I. Baganova, E. D. Levin, and V. M. Stepanov, Biokhim, **29**, 1070, 1964.
9. Yu. S. Kuznetsov and V. M. Stepanov, Biokhim., **31**, 623, 1966.
10. H. N. Rydon and P. W. G. Smith, Nature, **169**, 922, 1952.
11. D. G. Smyth, W. H. Stein, and S. Moore, J. Biol. Chem., **237**, 1845, 1962.
12. B. Blombäck and R. F. Doolittle, Acta Chem. Scand., **17**, 1819, 1963.
13. Yu. S. Kuznetzov, G. G. Kovaleva, and V. M. Stepanov, Biochim. Biophys. Acta, **118**, 219, 1966.

27 August 1966

Institute of the Chemistry of Natural Compounds,
AS USSR