CLEAVAGE OF THE PEPTIDE BONDS IN PEPSIN WITH CYANOGEN BROMIDE

V. M. Stepanov, S. P. Katrukha, and V. I. Ostoslavskaya

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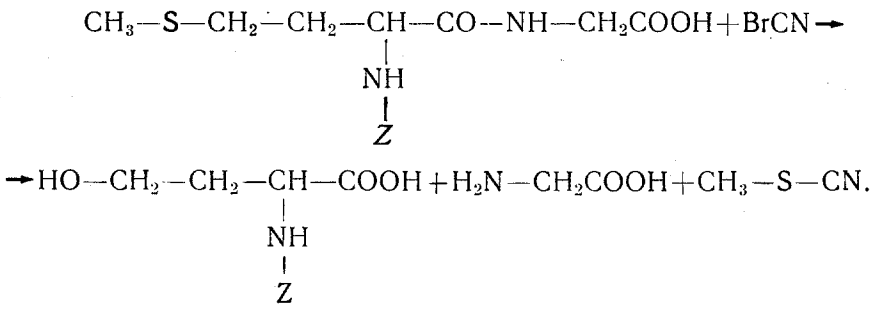
Recently, several chemical methods for selectively cleaving peptide bonds have been proposed [1]. They include the specific cleavage of the peptide bonds formed by the carboxyl group of methionine, which takes place under the action of cyanogen bromide. This method has been used successfully in determining the sequence of the amino acids in ribonuclease, myoglobin, and trypsinogen [2-4]. The molecule of porcine pepsin (whose primary structure is being studied in the protein structure laboratory of OKhPS [Institute of the Chemistry of Natural Compounds]), from the results of an amino acid analysis, contains 4-5 methionine residues [5, 6] with a total chain length of 340 amino acid residues. It may be hoped that the action of cyanogen bromide on pepsin will form comparatively large peptides suitable for further investigation.

The cleavage of proteins with cyanogen bromide is generally carried out in an acid medium at pH 1-2. It was to be expected that the treatment of native pepsin with cyanogen bromide under these conditions would be accompanied by undesirable complications due to the autolysis of the enzyme and also to the possibility of the rapid enzymatic hydrolysis of the fragments formed by the pepsin molecules remaining unattacked. Preliminary experiments with native pepsin confirmed these dangers and therefore the subsequent work was carried out with pepsin inactivated by dissolution in phenol [7]. Since the presence of three disulfide bonds in pepsin may interfere with the separation of the peptides obtained by the cleavage of the methionine bonds, we have studied the action of cyanogen bromide on reduced carboxymethylated pepsin [8].

Denatured and reduced carboxymethylated pepsin is practically insoluble in dilute hydrochloric acid, which is the medium most frequently used for performing the reaction. Consequently, we used 65% formic acid as the solvent. The possibility of carrying out the reaction in this medium was tested on carbobenzoxy-DL-methionylglycine. This dipeptide was treated with a 50-fold excess of cyanogen bromide in 65% formic acid at 20° C for 20 hr. After the elimination of the excess of cyanogen bromide and formic acid in a vacuum desiccator over alkali and dinitrophenylation of the mixture, DNP-glycine was isolated by paper chromatography with a yield of 85%, which showed the complete cleavage of the peptide bond:



To cleave the pepsin, a solution of 20 mg of the reduced carboxymethylated protein in 1.9 ml of 65% formic acid was treated with 30 mg of freshly-prepared cyanogen bromide, which corresponds to an approximately 100-fold excess of the reagent with respect to the methionine residues. After standing at room temperature for 20 hr, the mixture was evaporated to dryness in a vacuum desiccator over solid alkali. In some of the experiments, the solvent and the excess of reagent were eliminated by freeze-drying the mixture.

The composition of the acid hydrolyzate of the mixture of reaction products, determined by Ts. A. Egorov on an automatic amino acid analyzer, showed that the methionine content of the mixture had decreased by a factor of 20 as the result of the cyanogen bromide treatment. Homoserine, which is formed from methionine, was found, its yield being 60% of the theoretical (calculated on the assumption that the pepsin contains four methionine residues). It must be borne in mind that homoserine is partially converted into a lactone, which was not determined.

By means of the hydroxamic reaction [9] it was found that ester linkages appeared in the reduced carbomethoxylated pepsin after the reaction with cyanogen bromide had been carried out under the conditions described. A control experiment showed that the same result was obtained when reduced carboxymethylated pepsin was allowed to stand in 65% formic acid for a day in the absence of cyanogen bromide. It is possible that prolonged incubation with formic acid leads to the partial formylation of the hydroxy groups of the protein. To split off the acyl groups, the pepsin preparation

after treatment with cyanogen bromide was kept for 1 day in 1 N triethanolamine carbonate buffer at pH 8.2.

To determine the number and nature of the peptide linkages cleaved by the action of cyanogen bromide on the pepsin, the N-terminal amino acids formed by the reaction were determined by the dinitrophenylation method. 20 mg of pepsin was treated with cyanogen bromide, after which the reaction mixture was dinitrophenylated and hydrolyzed (40 hr at 105° C), the DNP amino acids were extracted, and were separated by chromatography, the spots were eluted with 5 ml of sodium hydrogen carbonate solution, and the optical density of the solution was determined at 360 mμ in a 1-cm cell (table). The method used is similar to that described previously [7].

Optical Densities of Eluates of DNP Amino Acids

DNP-Amino acids	Denatured pepsin		Carboxymethylated pepsin		
	Method of treatment				
	BrCN, HCOOH	Control HCOOH	BrCN HCOOH	Control HCOOH	Control HCOOH+HBr
Aspartic Acid	1.750	0.170	1.700	0.125	0.117
Threonine	0.290	0.040	0.270	0.050	0.080
Serine	0.030	0.030	0.062	0.018	0.126
Alanine	0.230	0.220	0.245	0.210	0.190
Valine	1.820*	0.075	0.910**	0.045	0.070
Isoleucine		0.960	1.210	1.080	1.170

* The spots of DNP-isoleucine and DNP-valine were not separated.

** The spots of DNP-isoleucine and DNP-valine partially overlapped.

Since, under the usual conditions, the DNP derivatives of asparagine and glutamic acid are not separated, the spot corresponding to them was rechromatographed after elution in 2.5 M phosphate buffer with reference samples. It was found that it contained only DNP-aspartic acid. For a more reliable identification, the DNP derivatives of aspartic acid, valine, and isoleucine were decomposed to give the free amino acids by heating with anhydrous hydrazine, after which the regenerated amino acids were determined in the automatic analyzer.

In order to evaluate the possible nonspecific cleavage of the peptide bonds under the experimental conditions, the N-terminal amino acids in samples of pepsin that had previously been kept in 65% formic acid for 20 hr were determined by the dinitrophenyl method. The possibility of the action of the hydrobromic acid formed in the hydrolysis of the excess of cyanogen bromide was also tested. The results obtained (table) show that nonspecific cleavage is negligible. The sample subjected to the control treatment contained mainly isoleucine as the N-terminal acid, this being the N-terminal amino acid of porcine pepsin [10]. Small amounts of N-terminal alanine are also generally found in samples of pepsin. The somewhat increased content of DNP-aspartic acid can be explained by nonspecific hydrolysis in the acid medium.

The action of cyanogen bromide on inactivated and reduced carboxymethylated pepsin gave the same N-terminal amino acids, valine and aspartic acid, with a small amount of threonine. The yield of DNP amino acids calculated on 1 mole of pepsin used in the reaction was 0.40 mole of isoleucine, 0.30 mole of valine, and 0.57 mole of aspartic acid. The introduction of accurate corrections for the decomposition of the DNP amino acids during the 40-hour acid hydrolysis and chromatography is complicated by the fact that the degree of decomposition may depend on the nature of the accompanying amino acid residues. Since isoleucine is a N-terminal amino acid of pepsin, it may be assumed that the DNP-isoleucine content of the hydrolyzate corresponds to 1 mole of N-terminal amino acid.

In the determination of the N-terminal amino acids in pepsin in our laboratory, similar yields of DNP-isoleucine were obtained. If the yield of 0.40 mole corresponds to the rupture of one bond, about 1 mole of N-terminal valine and somewhat less than 2 moles of aspartic acid are formed in the cleavage of pepsin with cyanogen bromide. The low yield of DNP-aspartic acid is probably due to its lower resistance to hydrolysis.

The results obtained enable us to assume the presence of a methionyl-valine bond and two methionyl-aspartic acid (or methionyl-asparagine) bonds in porcine pepsin. The amino acid composition of the pepsin shows the presence of four peptide bonds containing methionine, which could not be determined by dinitrophenylation. By using the methylthiohydantoin method of determining N-terminal amino acids [11] we have been able to show that after the action of cyanogen bromide on the pepsin N-terminal glycine appears, in addition to the valine and aspartic acid mentioned above. It is known that DNP-glycine is very readily decomposed on acid hydrolysis. Consequently, it cannot be found

in our experiments. The detection of N-terminal glycine gives some grounds for assuming that the methionyl-glycine bond is present in pepsin; however, this fact requires additional confirmation.

Summary

1. Conditions for the cyanogen bromide cleavage of the peptide bonds of pepsin formed by methionine have been found.

2. Pepsin contains a methionyl—valine bond and two methionyl—aspartic acid (or methionyl—asparagine) bonds.

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Institute of the Chemistry of Natural Compounds, AS USSR