ON THE TRYPTOPHAN CONTENT OF HOG PEPSINOGEN, PEPSIN AND S-SULFO-PEPSIN

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The tryptophan content of the S-sulfo derivative of hog pepsin, of native pepsin and pepsinogen was determined because the available data were controversial. The results of the determination by independent methods show that both pepsinogen and pepsin contain 5 residues of tryptophan per molecule.

One of the fundamental characteristics of a protein intended for sequential work is its amino acid composition. The methods of amino acid analysis have been maximally standardized[1] by now save for a few amino acids; among their number falls also tryptophan. The degradation of tryptophan under the conditions of conventional acid hydrolysis does not permit its determination to be carried out on the same sample simultaneously with other amino acids. The tryptophan content must therefore be determined on acid hydrolysates obtained under modified conditions[2,3] or on alkaline[4] or enzymatic[5] hydrolysates, or, alternatively by one the numerous methods of other types. The nonuniformity of methods used for the determination of the tryptophan content of proteins often leads to controversial results. Hog pepsin may be adduced by way of a good example.

Brand[6] was the first to study the tryptophan content of hog pepsin and reported 4 tryptophan residues per molecule of the enzyme. Later, Blumenfeld and Perlmann[7] found 6 tryptophan residues in pepsin and the same content postulate Arnon and Perlmann[8] also in pepsinogen. The problem of the tryptophan content was studied later by Van Vunakis and Herriott[9], who found 4 residues, similarly to Beaven and Holiday[10]. Special attention was focused on the tryptophan residues of hog pepsin by Dopheide and Jones[11]. These authors determined the tryptophan content of pepsin from pepsinogen[12] and complemented their analysis by a sequential study on the neighborhood of the tryptophan residues. They concluded from their experiments of both types that hog pepsin contains only 4 tryptophan residues per molecule.

In the early stage of our sequential work on hog pepsin in this Laboratory, we considered necessary to examine the tryptophan content of our starting material. In view of the data on the heterogeneity of commercial crystalline pepsin[12], used by us for the preparation of S-sulfo-pepsin, we decided to determine also the tryptophan content of pepsinogen, which is not contaminated by autolytic products[12]. The results of these experiments are summarized in the present paper.

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EXPERIMENTAL

Material

Hog pepsinogen (2 x crystallized and lyophilized) and pepsin (2 x crystallized and lyophilized) were commercial products of Worthington Biochemical Corporation, Freehold, N.J., U.S.A. S-Sulfo-pepsin was prepared by sulfitolysis[13] of pepsin and freed of low molecular weight products as described elsewhere[14]. Bovine trypsin, used as a standard, was prepared by three-fold crystallization of a commercial product of Léčiva, Prague. 2-Nitrophenylsulfenyl chloride was obtained from the Research Institute for Pharmacy and Biochemistry, Prague. Guanidine hydrochloride was from the Institute of Chemical Process Fundamentals, Czechoslovak Academy of Sciences, Prague. The solution of guanidine hydrochloride was purified before use as recommended[15]. L-Tryptophan was a product of Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Sulfuric acid 35·4N was prepared by distillation with $FeSO_4$ of a commercial product of Lachema, Brno. The remaining chemicals were of a purity prescribed for the individual methods.

Methods

The ultraviolet and visible spectra were measured in 1 cm quartz cells in OPTON PM-QII Spectrophotometer (Feintechnik Oberkochen G.m.b.H., West Germany).

Spectrophotometric determination[15]. The samples of pepsinogen, pepsin, S-sulfo-pepsin, and trypsin of defined dry weight and ash content were dissolved in 6M guanidine hydrochloride in 0·02M phosphate buffer, pH 5·5 to give 10^{-5}M solutions. The spectra of these solutions were measured in the wavelength range 250—300 nm. The values of ε_{280} and ε_{288} were calculated from the measured values of A_{280} and A_{288}, taking a molecular weight of 38944 for pepsinogen[12], 34163 for pepsin[12], 34649 for S-sulfo-pepsin, and 23950 for trypsin[16]. The content of tryptophan in mol/mol protein was calculated from the formulas derived by the author of the method. Five parallel determinations were always made.

Colorimetric determination[17]. Samples (17—20 mg) of pepsinogen, pepsin and trypsin were dissolved in water (final volume 10 ml) and the concentration of the solutions obtained was determined spectrophotometrically using empiric formulas[18]. One ml was taken from each solution and pipetted in the mixture of 8 ml of 23·8N-H_2SO_4 and 1 ml of *p*-dimethylaminobenzaldehyde solution (30 mg/ml 2N-H_2SO_4). The reaction mixtures were incubated at 30°C, 48 h in the darkness. After this period, 0·1 ml of 0·04% $NaNO_2$ solution was added to each sample and the color allowed to develop 30 min. The absorbance of the solutions was then measured at 600 nm against a blank solution in which the sample had been replaced by water. The tryptophan values were read off a calibration curve obtained with an authentic sample of L-tryptophan. Six parallel determinations were always made.

Determination by the substitution method[19]. Samples corresponding roughly to 1 μmol of pepsinogen, pepsin, and trypsin were dissolved in 2 ml of 98—100% HCOOH (neither pepsinogen nor pepsin are soluble in acetic acid used by the authors of the original method). A 20-fold molar excess of 2-nitrophenylsulfenyl chloride in 1 ml of HCOOH was added to these solutions. The reaction mixture was shaken 1 h. A 20-fold excess (v/v) of a mixture of acetone and 1M-HCl (ratio 39 : 1) was then added at −5°C. The precipitate of the 2-nitrophenylsulfenyl derivative was set aside for 16 h at 4°C and then centrifuged off. The excess of the reagent was removed by washing the precipitate 4-times with acetone. The precipitate was subsequently washed with ether and dried *in vacuo* over KOH overnight. The yield was 85%. The labeled proteins were dissolved

to 10^{-6}M solutions in 98–100% HCOOH. The spectra of these solutions were measured in the wavelength range 250–400 nm. The value of the standard molar absorptivity $\varepsilon_{stand} = 5272$ was calculated from the ε_{365} value for labeled trypsin, assuming the presence of four labeled tryptophan residues in the molecule. Six parallel determinations were always made.

RESULTS AND DISCUSSION

This investigation was undertaken as a part of the basic characterization of the starting material for our sequential studies, of hog pepsin. We determined the tryptophan content of both the native protein and its S-sulfo derivative, and of pepsinogen which is known[12] to be essentially homogeneous. To check the reliability of the methods used, we also determined the tryptophan content of bovine trypsin (4 residues) which had been confirmed by the determination of the complete amino acid sequence of the protein[16]. The results of our experiments are summarized below.

Protein	Method used		
	spectrophotometric	colorimetric	substitution
	mol Trp/mol protein		
Pepsinogen	4·9	4·8	5·4
Pepsin	5·2	5·1	4·6
S-Sulfo-pepsin	4·5	—	—
Trypsin	4·3	3·8	—

The data obtained, which represent the mean values obtained by 6 (or 5 respectively) independent measurements, lead us to conclude that the tryptophan content of both pepsinogen and pepsin is 5 mol/mol of protein. The same tryptophan content is assumed also for S-sulfo-pepsin, even though the determination could be carried out by one method only. (S-sulfo-pepsin was insoluble under the conditions required for the colorimetric test or for the labeling with 2-nitrophenylsulfenyl chloride). The methods of tryptophan determination by amino-acid analysis of acid hydrolysates prepared under modified conditions of hydrolysis[2,3] seem therefore to be more suitable for tryptophan analysis of substituted and denatured protein derivatives.

The problem of the tryptophan content of hog pepsin was devoted special attention by Dopheide and Jones[11]. These authors determined the tryptophan content of reduced and carboxymethylated pepsin from pepsinogen[12] by a substitution method (using 2-hydroxy-5-nitrobenzyl bromide[20]) and found 4 mol/mol protein. When they used the same method[20] for native pepsin from pepsinogen[12] and for urea-denatured pepsin, they found 2 and 3 residues, respectively, of tryptophan per mol

of pepsin. By spectrophotometric analysis[15] (the method used by us in this study) of native pepsin, Dopheide and Jones found 5·6 mol of tryptophan/mol of protein. These strikingly controversial results led the authors to make conclusions on the reliability of the methods used as well as on the accessibility of the individual tryptophan residues in the three-dimensional structure of pepsin. They complemented therefore the analytical part of their study by sequential analysis of peptides selectively isolated from the neighborhood of tryptophan residues (4 peptides). We have presented in one of our early papers[21] the sequence of 37 residues in the C-terminal region of the pepsin molecule. This sequence also involves one tryptophan residue. The amino acid sequence around this residue, however, is not reconcilable with any of the four tryptophan peptides isolated by Dopheide and Jones. This finding together with our present knowledge of the neighborhood of the remaining residues[22] permit us to conclude that the actual tryptophan content of hog pepsinogen and pepsin is 5 mol/mol of protein, as evidenced by our analyses presented in this paper.

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