DETERMINATION OF THE CYSTINE AND CYSTEINE CONTENTS OF PROTEINS WITH THE AID OF AN AMINO ACID ANALYZER

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A method is proposed which permits the determination of the amounts of cystine an cysteine in the form pf systeic acid in various proteins of animal and plant origin – even in those in which, on the analysis of standard hydrolysates, the peaks of cysteine are not detected because of their low concentration.

Cystine and cysteine are sulfur-containing amino acids participating in the formation of disulfide bonds in proteins and, consequently, largely determining their conformation and function. These protons are included among the essential amino acids the amount of which, together with the other essential amino acids, is taken into account in determining the food and fodder value of proteins from various crops.

As a result of the acid hydrolysis of the proteins that is used in the analysis of amino acid composiitons, a considerable breakdown of the cystine and cysteine takes place and it is therefore impossible to determine them quantitatively in hydrolysates. The majority of the methods for determining these amino acids with the aid of amino acid analyzer are based on their oxidation to cysteic acid, which is stable in an acid medium, by various powerful oxidizing agents. Performic acid is most frequently used for this reaction [1, 2] and with its aid the conversion of cystine and cysteine into cysteic acid amounts to 85-90%. This laborious and lengthy method includes the preparation of the performic acid immediately before the reaction, the oxidation of the protein, the destruction of the excess of performic acid, and lyophilization, followed by the standard acid hydrolysis and amino acid analysis.

The development of simpler and faster methods for the quantitative determination of cystine and cysteine not requiring the use of expensive and difficultly accessible reagents and also the modification of known methods is one of the important tasks of modern chemistry. Spenser et al. [3] used dimethyl sulfoxide (DMSO) in low concentration as oxidizing agent. The oxidation of cystine and cysteine under these conditions takes place quantitatively and the method is economical in time.

In an analysis of the amino acid composition of cotton-plant and wheat histones the determination of cysteine is complicated by the fact that it is present in only one of the histone fractions – H3 [4] – in an amount of one or two residues per protein molecule; ordinary amino acid analysis of protein hydrolysates [5] does not detect the cysteine. To determine cysteine in histones and other proteins of animal and plant origin, therefore, we used Spencer's method [3], somewhat modified (see the Experimental part).

We first determined the depth of oxidation of cysteine to cysteic acid, the yield of the latter, and the degree of breakdown of the other amino acids under the conditions of acid hydrolysis performed in 6 N HCl in the presence of 2% of DMSO and without it in tubes sealed in vacuum. For this purpose, a standard set of amino acids was treated with 6 N HCl in two parallel samples to one of which DMSO was added in an amounted of 0.04 ml per 2 ml of a solution of the amino acid in the acid. Then the amino acid analysis of an untreated standard set of amino acids and of both treated samples was performed (see below).

Analysis of the results showed that the treatment of a set of amino acids under the conditions of standard acid hydrolysis led to the partial breakdown of threonine, serine, and tyrosine (by 5-8%), while the cysteine was partially oxidized to cysteic acid. The total yield of cysteine with allowance for the cysteic acid did not exceed 50-60%. Treatment of a

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TABLE 1. Yields of Cysteine and Cysteinic Acid in the Analysis of the Hydrolates of a Number of Proteins Obtained in the Absence and in the Presence of Dimethyl Sulfoxide

Protein	Hydrolysis without DMSO			Hydrolysis in the presence of DMSO					
				calculations on the basis of aspartic acid			calculations on the basis of alanine		
	cysteine	cysteic acid	Sum of cysteine and cysteic acid	cysteic acid		error	cysteic acid		error
	μmole	μmole	mole. %	mole	mole. %	%	μmole	mole. %	%
Phosphoribonuclease from bean leaves	0.0064	0.0061	1.63	0.0143	1.87	12.88	0.0148	1.94	15.98
Ribose 5-phosphate isomerase from spinach leaves	0.0019	0.0016	0.84	0.0061	1.46	42.62	0.0062	1.49	43.62
Protease inhibitors from pea seeds (mutant I)	0.0022	0.0021	0.55	0.0063	0.81	31.74	0.0062	0.79	30.38
Protease inhibitors from pea seeds (mutant II)	0.0026	0.0024	0.59	0.0075	0.89	33.33	0.0074	0.87	32.18
Protein from leather wastes after hydrolysis with HCl	0.0094	0.0070	1.28	0.0224	1.75	26.78	0.0221	1.73	26.01
H_2SO_4	0.0020	0.0015	0.81	0.0049	1.13	28.57	0.0048	1.11	27.03
CH_3COOH	0.0043	0.0027	1.05	0.0086	1.29	18.60	0.0087	1.30	19.23
Protein of triticale flour, AD-205 form	—	0.0062	1.07	0.0100	1.73	38.00	0.0099	1.74	37.43
Protein of barley flour, variety 3613	0.0034	0.0041	0.87	0.0122	1.42	38.51	0.0124	1.44	39.53

mixture of amino acids in the presence of DMSO led to a high loss of tyrosine (more than 80%). At the same time, a new peak appeared on the chromatograms, issuing in 30-50 minutes. A number of other deviations in the yields of amino acids (histidine, serine, methionine, isoleucine, leucine) were also observed. The yield of cysteic acid amounted to 97-100%. The yields of arginine, aspartic acid, threonine, glutamic acid, glycine, alanine, valine, and phenylalanine did not change as a result of the action of the DMSO. The yields of amino acids from samples of the standard mixture (μM) are given below:

Amino acid	Standard set of amino acids	Treated with 6 N HCl	Treated with 6 N HCl containing 2% of DMSO
Lysine	0,1	0,1	0.097
Histidine	0,1	0,098	0.030
Arginine	0,1	0,097	0,101
Cysteic acid	0,0	0,020	0,099
Aspartic acid	0,1	0.100	0.101
Threonine	0,1	0,095	0,101
Serine	0,1	0,093	0,105
Glutamic acid	0,1	0.100	0,099
Proline	0.1	0,099	0,097
Glycine	0,1	0,100	0.101
Alanine	0,1	0,100	0,102
Cysteine	0,1	0,034	—
Valine	0.1	0,101	0 100
Methionine	0,1	0,097	0,031
Isoleucine	0,1	0,100	0,107
Leucine	0,1	0,099	0,125
Tyrosine	0,1	0,094	0,017
Phenylalanine	0,1	0,098	0,102

It is impossible to use the hydrolysate obtained in the presence of DMSO for the complete amino acid analysis of the proteins. However, a combination of the standard acid hydrolysis with hydrolysis in the presence of DMSO (the latter hydrolysate only for the determination of cysteic acid) gave good results.

In the analysis of the standard mixture of amino acids, the integration constants (peak areas) of aspartic acid and alanine are very close to one another and, according to Spencer et al. [3], differ from the constant for cysteric acid by less than 2%. At the same time, after hydrolysis with DMSO the yields of these amino acids remain stable and do not differ from their yields from the standard mixture. Consequently, Spencer et al. [3] proposed to use integration constants of aspartic acid and alanine for determining not only aspartic acid and alaine but also cysteic acid in unknown proteins.

We have tested this method for several proteins the primary structures of which were known (ribonuclease, histone H3 from calf thymus). On the analysis of a hydrolysate of ribonuclease (molecular weight 13,600, eight cysteine residues) obtained by the standard method, we found 3.0 mole% of cysteine and about 1.0 mole% of cysteic acid. After oxidation of the protein with DMSO, the cysteic acid peak was considerably greater (7.9 mole% or 7.84 residues per molecule). Analysis of histone H3 from calf thymus by the standard method did not reveal the presence of cysteine. The yield of cysteic acid after oxidation with DMSO, however, amounted to 1.76 mole%. It is known from the primary structure of this histone [4] that it has two cysteine residues, or 1.80 mole%.

There is literature information [4, 6] on the conservatism of histones H3 from various sources of animal and plant origin. It could therefore be expected that the histone H3 from cotton-plant shoots would also contains two cysteine residues, like histone H3 of calf thymus. In a determination of the amino acid composition of histone H3 from 2-day etiolated shoots of seeds from a cotton plant of variety 108-F (molecular weight 14,300 [5]). 1.49 mole-% of cysteic acid (or 1.55 residues per molecule of histone) was found. Since the histone H3 of the cotton plant is difficult to obtain in the form of an absolutely pure fraction and it contains small amounts of histones H2A and H2B, which do not contain cysteine, as impurities, the somewhat low yield of cysteic acid from the sample of protein taken is completely explicable.

We then used the method of determining cystine and cysteine in proteins after thier oxidation to cysteic acid by low concentrations of DMSO (2%) for estimating other proteins that, on the analysis of ordinary hydrolysates, gave small cysteine peaks. The results are given in Table 1. The yield of cysteine on hydrolysis under standard conditions with allowance for the yield of cysteic acid depends on the protein and is 30-40% lower than the yield of cysteic acid on hydrolysis with DMSO.

Calculations of cysteic acid can be performed with the integration constants for aspartic acid and alanine, which permits cysteic acid to be omitted from the standard mixture. These calculations, performed with the two constants, given fairly close results, and therefore in order to shorten the times of analysis we used only the constant for aspartic acid.

Thus, to determine the complete amino acid compositions of proteins requires the results on the yields of amino acids obtained in the analysis of samples hydrolyzed under standard conditions and those of the analysis of the same substances hydrolyzed in the presence of DMSO, in which only the peaks of the cysteic and aspartic acids are used (the latter is necessary for comparing the samples of hydrolysates with DMSO and without it). Since these two peaks are the first to issue from the column of the analyzer, the working program of the amino acid analyzer is drawn up in such a way that the regeneration of the column is begum immediately after they have issued (70-75 min after the beginning of the working of the column). This permits the time of analysis and the consumption of reagents to be decreased.

EXPERIMENTAL

The complete amino acid compositions of protein hydrolysates, including those containing cystine and cysteine after their oxidation to cysteic acid, were determined on a JEOL JIC-6AH amino acid analyzer with automatic estimation of the peaks. The cysteic acid peak issued at 60-65 min, immediately before the aspartic acid peak.

Amino Acid Analysis. To each of two tubes containing the dry residues after the evaporation of 3 ml of a solution of the standard mixture (with a concentration of 0.1 μM of each amino acid) was added 2 ml of 6 N HCl. One of the tubes also contained 0.04 ml of DMSO. The samples for amino acid analysis were treated in the tubes, after they had been sealed in vacuum, at 100-105°C for 24 h. Then the HCl was distilled off in a vacuum evaporator at a bath temperature of 30-35% in 10-15 min. The residue in each case was dissolved in 1-1.5 ml of water and drying was repeated in order to eliminate the HCl completely. The dry residue was immediately dissolved in 3 ml of 0.2 N citrate buffer, pH 2.2, in order to restore the initial concentration of amino acids (0.1 μM) and was analyzed.

Proteins (0.5-1) were treated in the same way. To prevent the oxidation of tyrosine, serine, and methionine uder the conditions of standard hydrolysis in a tube with pure 6 N HCl, two drops of 5% phenol solution was added. The dry hydrolysis after the evaporation of the HCl was dissolved in 6 ml of 0.2 N citrate buffer, which is sufficient for two parallel analyses.

SUMMARY

Use has been made of a modification of the method for the quantitative determination of cystine and cysteine in proteins with the aid of an amino acid analyzer after their oxidation to cysteic acid in the presence of 2% DMSO, which is faster and less laborious. The conversion of cystine and cysteine into cysteic acid under these conditions amounts to 97-100%. Because of the changes in some amino acids under the action of DMSO, the other amino acids must be determined from parallel samples obtained on hydrolysis in the absence of DMSO.

LITERATURE CITED

1. C. H. W. Hirs, Methods Enzymol., 11, 197 (1967).
2. Methods for the Protein and Amino Acid Analysis of Plants. Methodological Instructions [in Russian], Leningrad (1973), p. 20.
3. R. J. Spenser and F. Wold, Anal. Biochem., 32, No. 1, 185 (1969).
4. I. Isenberg, Annu. Rev. Biochem., 48, 159 (1979).
5. Z. Deyl, K. Macek, and J. Janak, Liquid Column Chromatography, Elsevier, Amsterdam (1975).
6. G. F. Kasymova, T. P. Kopitsya, N. I. Koryakina, and V. K. Burichenko, Khim. Prir. Soedin., 470 (1981).

SYNTHESIS OF THE HEXAPEPTIDE 11-16 OF THE NATURAL SEQUENCE OF HUMAN CALCITONIN

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A new variant of the preparative synthesis of hexapeptide 11-16 of the natural sequence of human calcitonin is described. In several of the stages 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline was used successfully as the condensing agent. The final and intermediate compounds were obtained with good yields in chromatographically homogeneous form. Their purity was checked by TLC and measurements of angles of optical rotation. The final product was identified additionally by ^{13}C NMR. Several physicochemical characteristics of the compounds synthesized (angles of optical rotation, chromatographic mobilities) are given.

Calcitonin is a 32-membered peptide which regulates the calcium and phosphorus metabolism in the human organism. Its synthesis is difficult in view of the fairly large set of amino acids, a number of which are polyfunctional. The synthesis is complicated in the choice of the tactically minimum protection of the amino acids. Nevertheless, such tactics permit a simplification of the scheme of synthesis and the use of cheap and more readily available compounds.

The aim of the present investigation was to synthesize the hexapeptide 11-16 of the natural sequence of human calcitonin in which the hydroxy groups of the hydroxyamino acids (threonine, tyrosine) are present in the unsubstituted state. The preparation of the fragment is usually performed from protected hydroxy amino acids [1] using dicyclohexylcarbodiimide (DCHC) as condensing agent or with the use of activated esters based on N-hydroxysuccinimide or p-nitrophenol. Rittel et al [2] have made wide use of the azide method of condensation. The process of obtaining the hexapeptide amounts to the successive growth of the peptide chain from the C-end beginning with phenylalanine or to the preparation of the tripeptide (11-13) and the dipeptide (15-16). Then glutamine tert-butoxycarbonylhydrazide is added

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