QUANTITATIVE DETERMINATION OF AMINO ACIDS IN PROTEIN HYDROLYZATES BY THIN-LAYER CHROMATOGRAPHY

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A number of methods of determining amino acids without the use of amino-acid analyzers exists [1-4]. The main disadvantages of these methods are the length of time for performing an analysis [1-3] or a qualitative approach [4]. In the present paper we describe a method for the quantitative analysis of amino acids in 4-5 h, i.e. in a time similar to that of amino-acid analyzers.

The method that we proposed consists of the following stages: hydrolysis of the protein; determination of the concentration of amino nitrogen in the hydrolyzate by Folin's method [5] and deposition of a definite amount of amino nitrogen at the start; two-dimensional chromatography on microplates (7 × 7 cm) in a thin layer of cellulose by the modified method of Jones and Heathcote [6]; quantitative evaluation of the chromatogram by eluting the spots [3] and measuring the absorption of light on a MKMF-1 microcolorimeter; and quantitative determination of the tryptophan and proline with the aid of specific test-tube reactions.

The optimum amount of hydrolyzate used for analysis is 50-70 nmoles of amino nitrogen, which corresponds to about 10-15 μg of amino-acid mixture. The amino acids were separated by thin-layer chromatography on cellulose. Of all the systems tested, that of Jones and Heathcote proved to be the best, ensuring the separation of all the amino acids with the exception of leucine and isoleucine. At the same time, we changed the size of the plates (7 × 7 instead of 20 × 20 cm), the composition of the solvents (acetone instead of methyl ethyl ketone, 12% ammonia instead of 34%, ninhydrin added directly to the solvent), and also the order of passage of the solvents, since the complete elimination of ammonia is necessary before separation in the second solvent. Even a very small amount of ammonia gives a highly colored background with ninhydrin which prevents the measurement of the colorations of the amino acids.

The extinctions of each amino acid were determined in the analysis of a synthetic equimolar mixture of amino acids and also of hydrolyzates of insulin and lysozyme. Below we give the absorption factors of five nmoles of amino acids after their chromatography and treatment with ninhydrin: cysteine 0.09; aspartic acid 0.12; glutamic acid 0.17; arginine 0.18; histidine 0.13; lysine 0.185; glycine 0.11; serine 0.16; threonine 0.18; alanine 0.165; tryosine 0.11; valine 0.16; methionine 0.16; phenylalanine 0.105; leucine + isoleucine 0.161.

Special conditions are required for two of the amino acids – tryptophan and proline. Tryptophan is determined before the hydrolysis of the protein sample and its amount is calculated by making use of a calibration curve after the colorimetry of the sample [7]. The determination of proline is performed in a separate sample of the hydrolyzate by means of the reaction with isatin [8]. The values of the corrections in the determination of proline, which are due to the contribution of the neutral amino acids are given in Fig. 1d. On the basis of the presumed values of the proline content for hydrolyzates rich in this amino acid, about 0.5 μmole of amino nitrogen must be analyzed, and if the amount of proline is small about 1 μmole of amino nitrogen.

Table 1 gives the results of quantitative determinations of the amino acids of insulin, lysozyme, and antithyroid phytoprecipitin. The accuracy of the analysis averages ± 10%; for amino acids present in small amounts, the accuracy of the determination is lower.

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TABLE 1. Amino-Acid Compositions of Hydrolyzates Determined by the Proposed Method

Amino acids	Lysozyme		Insulin		Thyreoprecipitin	
	M±σ, %	calc. figures	M±σ,%	calc. figures	M±σ, %	Literature figures [9]
Cysteine	4,8±1,0	5,8	9,9±0,3	10,8	0,0	0,0
Aspartic acid	16,7±1,4	17,0	5,6±0,8	6,0	12,2	13,9
Glutamic acid	5,1±0,6	4,55	15,2±0,4	13,2	8,45±	8,39
Arginine	10,9±1,1	12,0	2,7±0,7	3,2	2,9	2,77
Histidine	1,1±0,2	0,98	4,5±0,1	5,7	1,7	1,78
Lysine	3,9±0,6	5,4	2,5±0,5	2,8	4,75	5,63
Glycine	5,2±0,4	5,0	5,3±0,8	4,5	3,8	3,45
Serine	6,2±0,5	6,1	4,9±0,3	4,7	7,3	7,3
Threonine	5,1±0,6	5,0	1,8±0,1	1,9	9,05	11,58
Alanine	7,1±0,4	6,0	4,2±0,5	4,0	5,45	5,44
Tyrosine	4,1±0,7	3,44	11,2±0,2	10,9	5,45	4,58
Valine	5,4±0,9	4,2	8,0±0,4	8,7	9,15	7,81
Methionine	2,3±0,2	1,8	0	0	0	0
Phenylalanine	3,7±0,5	3,1	8,1±0,8	7,4	5,55	7,92
Leucine + isoleucine	10,8±0,2	10,3	13,9±1,0	13,6	9,5	8,81
Proline	1,6±0,3	1,4	1,9±0,3	1,8	2,6	2,89
Tryptophan	5,6±0.4	7,9	0	0	2,7	4,21

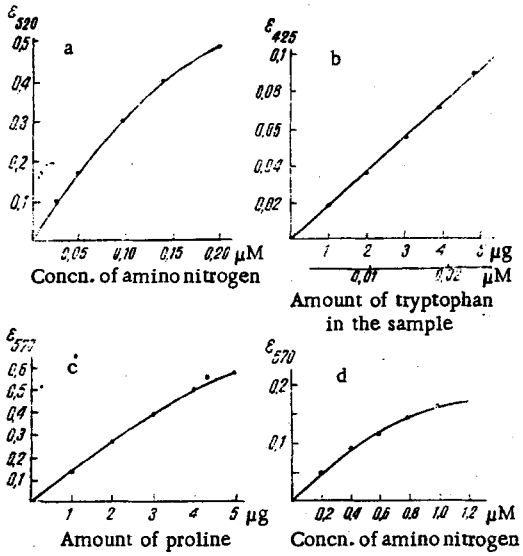

Fig. 1. Calibration curves for the determination of amino nitrogen (a), tryptophan (b), and proline (c), and corrections in the determination of proline (d).

EXPERIMENTAL

Hydrolysis of the Protein. The mixture of 0.5-1 mg of the protein and 0.15 ml of 6 N hydrochloric acid was sealed into a tube with a diameter of 3 mm and heated at 105°C for 18-20 h. Then the tube was opened and the hydrolyzate was dried in a vacuum desiccator over KOH.

Determination of the Concentration of Amino Nitrogen in the Hydrolyzate. The hydrolyzate was dissolved in water to a concentration of about 10-15%, a sample of 0.05-0.1 μl* was taken and this was added to 0.1 ml of a 1% solution of bicarbonate in a test tube (7× 100 mm). Then 0.02 ml of 0.5% sodium naphthoquinonesulfonate was added and the mixture was heated on the water bath for 10 min. After cooling, 0.02 ml of acetate buffer [50% acetic acid and 5% sodium acetate (1:1)], 0.02 ml of 4% sodium sulfite, and 0.4 ml of water were added. The degree of coloration was determined on a MKMF-1 microcolorimeter at 520 nm in a 10-mm cell. The number of nanomoles of amino nitrogen was found from a calibration graph, which was plotted for a mixture of amino acids of known molarity imitating the protein hydrolyzate (Fig. 1a). An amount of material corresponding to 50-70 nmoles of amino nitrogen was deposited at the start.

Thin-layer Chromatography. Plates (7× 7 cm) were prepared on the day before the analysis. Cellulose was deposited by the pouring method at the rate of 0.5 g per plate, and drying was performed on a horizontal surface at room temperature for 18-24 h. The cellulose powder was prepared as described by Katrukha et al. [4]. Commercial preparations of cellulose can be used after they have been washed with hydrochloric acid and particles not bigger than 5-7 μ have been removed.

The two-dimensional separation of the amino acids was performed in the following systems: 1) isobutanol-acetone-12% ammonia (15:9:6, twice) and 2) isopropanol-water-formic acid (20:5:1) with 0.2% of ninhydrin. After the first system had been run, the plate was heated at 105°C for 5 min and was blown with air by a fan for 30 min. After the separation had been completed, the solvent was eliminated by evaporation and the plates were heated at 105°C for 6 min. The ninhydrin remained in the sorbent layer.

* The pipette for 0.05-1.1 μl consists of a capillary fixed with sealing wax in a glass tube. The capillary is calibrated with water, making use of a 0.25-μl pipette prepared from a thermometer capillary calibrated with mercury.

Determination of the Amino Acids. The spots of the amino acids were accurately scraped into test tubes (5 × 50 mm) not more than 2 hours after the chromatogram had been run. An adjacent section of equal area was scraped off as control. The color was eluted with 0.1 ml of an ethanolic solution of copper sulfate [3]. The suspension was stirred and centrifuged, and the optical density was measured in a microcolorimeter with a 520-nm filter in a 3.5-mm cell. The differences in parallel chromatograms did not exceed 0.01 unit. The number of nanomoles of each amino acids was calculated by drawing up a proportion in relation to the extinction for 5 nmole of the appropriate amino acid (see above).

Determination of Tryptophan. The solution under test, containing 50-100 μg of protein, was transferred to a test tube (7 × 100 mm), and 0.05 ml of a 2.5% solution of ninhydrin in a mixture of formic and concentrated hydrochloric acids (3 : 2) was added. The mixture was heated on the boiling water bath for 10 min, cooled, and treated with 0.3 ml of ethanol. The light absorption was measured on a MKMF-1 instrument in an 8-mm cell with a 425-mm filter. After deducting the blank, the amount of tryptophan was found from a calibration curve.

Determination of Proline. In a test tube (15 × 150 mm), 10-30 μl of sample containing 0.5-1 μmole of amino nitrogen was treated with 0.05 ml of a 0.5 M solution of citrate buffer, pH 4.1, and then with 0.1 ml of a 0.2% solution of isatin in ethanol and 1 ml of ethanol. The mixture was heated in the boiling water bath for 10 min. After this time, the solvent was boiled off, and a dirty gray coating remained on the walls. The test tube was cooled in a stand protected from the light, the contents were dissolved in 3 ml of acetone–water (2 : 1), 1 ml of toluene was added, and the tube was shaken vigorously for 15-20 sec. After the emulsion had separated, the lower layer was taken off, and toluene extract was washed with 3 ml of water to remove the excess of unchanged isatin. The mixture was shaken for 15-20 sec and was then centrifuged for 1-2 min to break the emulsion. The light absorption of the toluene layer was measured on a microcolorimeter in a 10-mm cell with a 570-nm filter. The value of the extinction due to the reactions of the other amino acids of the hydrolyzate, which was found from a graph according to the amount of amino nitrogen taken for analysis, was deducted from the figure found. Then the amount of proline in the sample was determined from a calibration curve.

CONCLUSIONS

A micro method for the quantitative determination of amino acids in protein hydrolyzates has been proposed which permits the analysis of two hydrolyzates to be performed in 4-5 h. After the separation of the amino acids by TLC in cellulose, they are determined quantitatively by the colorimetric method using a MKMF-1 microphotocolorimeter. Proline and tryptophan are determined separately with the aid of specific test-tube reactions. The sensitivity of the method permits the analysis of 10-15 μg of amino-acid mixture.

LITERATURE CITED

1. N. I. Berezovskaya and E. D. Goryachenkova, Vopr. Med. Khimii, No. 2, 155 (1957); A. I. Kharat'yan and G. A. Feigin, Klinich. Meditsina, No. 5, 102 (1964).
2. G. N. Zaitseva and N. P. Tyuleneva, Lab. Delo, No. 3, 24 (1958); M. K. Medvedev, P. V. Kugenev, and V. P. Shchuka, Lab. Delo, No. 9, 3 (1961); V. R. Makarova and M. A. Torban, Tr. Moskovskogo Instituta Vaktsin i Syvorotok, 18, 188 (1963).
3. T. S. Paskhina, The Quantitative Determination of Amino Acids by Means of Paper Chromatography through the Formation of Copper Derivatives of Amino Acids with Ninhydrin [in Russian], Moscow (1959).
4. G. S. Katrukha, Zh. P. Trifonova, and A. B. Silaev, Khim. Prirodn. Soedin., 348 (1966).
5. I. Todorov, Clinical Investigations in Pediatry [in Bulgarian], Sofia (1968), p. 739.
6. K. Jones and J. Heathcote, J. Chromat., 24, 106 (1966).
7. M. Gaitond and T. Dowey, Biochem. J., 117, 907 (1970).
8. Boctor N. Foad, Analyt. Biochem., 43, 66 (1971).
9. G. Entlicher, J. Kostir, and J. Kocourek, Biochim. Biophys. Acta, 221, 272 (1970).