COLUMN CHROMATOGRAPHY OF METHYLTHIOHYDANTOIN DERIVATIVES OF AMINO ACIDS IN THE INVESTIGATION OF THE PRIMARY STRUCTURE OF PORCINE PEPSIN

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We have previously proposed an automatic method for the separation and quantitative determination of the methylthiohydantoin derivatives (MTHs) of acid and neutral amino acids on sulfonated polystyrene resins [1, 2]. This method permits the fairly rapid and simple determination of the amounts of amino acids in the form of their MTH derivatives after stepwise splitting off by Edman's method, and the quantitative treatment of the results is also fairly simple.

The present paper gives the results of experiments on the further improvement of the method that we have proposed and its application in the analysis of short peptides. In the separation of MTH-(amino aicd)s on a column, we used as internal standard MTH-α-aminobutyric acid (MTH-ABA). The MTH-ABA is eluted between MTH-Gly and MTH-Val, without interfering with their determination (Fig. 1). Before deposition on the column a small amount of acetic acid, which absorbs in the 235-nm region and issues with the front, was added to the solution being analyzed, in addition to the MTH-ABA. Its peak was taken as the zero for reckoning the time.

We have isolated a number of short peptides isolated from a thermolysin hydrolyzate of fragment B2 of porcine pepsin by the method described. Figure 2 and Table 1 give the results of an analysis of the pep-

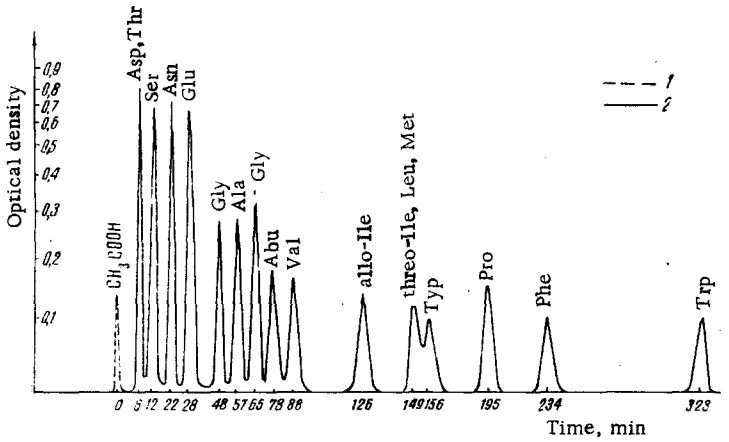

Fig. 1. Separation of a mixture of MTH derivatives of amino acids in a Hitachi 0.34 amino-acid analyzer (conditions: Hitachi resin 2612, column 0.9 × 72 cm, t 18°C ± 0.5°C, eluent: water, pH 3.18 ± 0.03, 0 − 35 min, and then a linear water/ethanol gradient (300 : 300), 35 − 340 min; rate of elution 60 ml/h. Absorption: 1) at 235 nm; 2) at 265 nm.

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TABLE 1. Analysis of the Peptide B2Th4-35

Composition of peptide	Asp 0.17; Ser 0.089; Pro 0.11; Ile 0.079; Leu 0.089					
MTH-(amino acid)s	cycle of separation					
	1	2	3	4	5	6
Isoleucine	0,4*					
Aspartic acid		0,48				
Serine			†			
Leucine				0,38		
Proline					0,16	
Aspartic acid						0,053

*Here and below, the yields of MTH-(amino acid)s are given in micromoles.
†See text.

TABLE 2. Analysis of Peptide B2Th5-312

Composition of peptide	Asp 0.318; Thr 0.188; Ser 0.174; Glu 0.148; Ile 0.173				
MTH-(amino acid)s	cycle of separation				
	1	2	3	4	5
Isoleucine	0,20				
Serine		0,17			
Aspartic acid			0,25		
Serine			0,021		
Threonine				0,11	
Asparagine					0,05

tide B2Th4-35. In the first cycle is split off an amino acid the MTH of which issues at the position of MTH-Ile and MTH-Leu. The small peak of MTH-alloisoleucine is evidence in favor of isoleucine, being obtained, apparently, by the partial racemization of the isoleucine during the Edman splitting-off process. An amino-acid analysis of a hydrolyzate of the shortened peptide showed that it completely retained the leucine, while the isoleucine was present in trace amounts. The third amino acid from the N end is serine, but is proved impossible in this case to estimate the concentration of MTH-Ser. A similar pattern has been observed in other cases of the determination of the serine content when the extraction of the MTH from the reaction mixture was performed with ether. On extraction with ethyl acetate, MTH-Ser issues as a sharp symmetrical peak. Analysis of the reaction mixture after each cycle of separation on a thin layer of silica gel ("Silufol," Czechoslovakia) agreed completely with the results of column chromatography. Consequently, the peptide B2Th4-35 can be assigned the following structure: H_2N-Ile-Asp-Ser-Leu-Pro-Asp.

To determine the reasons for the low yields of MTHs, particularly in the fifth and sixth cycles, we analyzed the benzene fraction collected after all the cycles. In the hydrolyzate we found trace amounts of all the amino acids present in the peptide. Consequently, carbamoylpeptides do not pass into benzene. Considerable amounts of aspartic acid, serine, proline, and leucine were found in a hydrolyzate of the substance that remained in the flask after the sixth cycle. Thus, the low yields of MTHs can be explained by a gradual oxidation of the methylthiocarbamoylpeptides in each cycle of separation.

The peptides B2Th5-312 and B2Th5-4212 contain residues of aspartic acid and threonine (Tables 2 and 3). The MTHs of these amino acids are eluted as a single peak, and therefore they were identified additionally by thin-layer chromatography in the chloroform (with 1.5% of ethanol)–CH_3COOH (80 : 20) system [3]. It was shown with the aid of carboxypeptidase A that the peptide B2Th5-312 has asparagine and glutamine at the C end. Thus, the structure of the peptide can be given as H_2N-Ile-Ser-Asp-Thr-Asn-Gln.

The degree of mobility of the peptide B2Th5-4212 according to Offord is 1. Consequently, one of the aspartic acid residues is present in the peptide in the form of the amide, and the structure of the peptide will be H_2N-Ala-Thr-Pro-Val-Phe-Asp-Asn.

In the peptide B2Th-362, with the composition Asp 0.073 (2), Ser 0.092 (3), Gly 0.035 (1), Val 0.049 (2), Leu 0.053 (2), we determined the sequence of only three amino acids: H_2N-Leu-Ser-Ser. It was impossible to split off a single amino acid in the fourth cycle. Retreatment likewise gave no result.

Column chromatography permits a fairly rapid and reliable identification of the amino acids in the form of their MTHs. In the analysis of peptides, in each cycle of separation, as a rule, the MTH of only

TABLE 3. Analysis of the Peptide B2Th5-4212

Composition of peptide	Asp 0.125; Thr 0.054; Pro 0.075; Ala 0.053; Val 0.052; Phe 0.052					
MTH-(amino acid)s	cycle of separation					
	1	2	3	4	5	6
Alanine	0,42					
Threonine		0,35				
Alanine		Traces				
Proline			0,30			
Threonine			Traces			
Valine				0,14		
Phenylalanine					0,093	
Aspartic acid						0,2

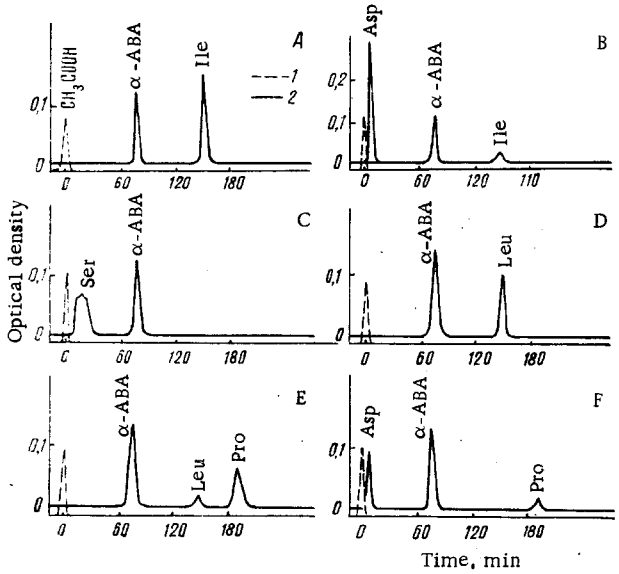

Fig. 2. Determination of the N-terminal sequence of the peptide B2Th4-35: A, B, C, D, E, and F – separation of the reaction mixture after cycles I-VI of separation (conditions the same as in the case of Fig. 1). Absorption: 1) at 235 nm; 2) at 265 nm.

one amino acid appears on the chromatograms. No auxiliary peaks absorbing at 235 and 265 nm and interfering with identification were observed. The deviations from the residence times of the MTHs are small and are easily checked with the aid of the peak of MTH-ABA. The chromatography of aliquots of the solution being analyzed on Silufol, which takes only 20-30 min, permits MTH-Asp to be distinguished from MTH-Thr and is an additional check.

EXPERIMENTAL

The peptides were isolated from a thermolysin hydrolyzate of the B2 fragment obtained in the cleavage of porcine pepsin with BrCN [4]. The hydrochloric acid was prepared by saturating distilled water with hydrogen chloride. The other reagents were purified by procedures described previously [5].

Synthesis of MTH-α-Aminobutyric Acid. To 1 g of α-aminobutyric acid (Reanal, Hungary) in 25 ml of 50% pyridine was added 1 g of methyl isothiocyanate. The reaction was performed at 40°C for 50 min, the pH being kept at 9.0-9.5 with 5 N KOH. Then the reaction mixture was diluted twofold with water, and the excess of reagent was extracted with benzene. The aqueous phase was evaporated in a rotary evaporator, and 10 ml of 1 N HCl was added to the dry residue and it was boiled for 10 min. The white crystals that separated out when the solution cooled were filtered off and recrystallized from ethanol. mp 122-124°C; composition $C_6H_{10}N_2OS$. In chloroform containing 1.5% of ethanol on Silufol the MTH-ABA had R_f 0.4. The MTH-ABA was eluted from a column as one symmetrical peak with a residence time of 78 min and a K value of 12.39. The spectrum of the MTH-ABA coincided completely with that of MTH-Val and had E_{265} 15,660 and E_{235} 9056.

Edman's Method. To a solution of 0.5 μmole of peptide in 0.7 ml of 50% pyridine containing triethylamine (0.01 ml of triethylamine to 10 ml of 50% pyridine) was added 10-15 mg of methyl isothiocyanate. Carbamoylation was performed at 40°C in an atmosphere of nitrogen for 1 h [5]. The solution was evaporated, and the thin film was washed three times with benzene. Traces of benzene were eliminated in the vacuum of an oil pump. Then the dry residue was treated with 0.7 ml of a mixture of hydrochloric and acetic acids (2 ml of 5.7 N HCl + 3 ml of CH_3COOH + 4 ml of H_2O), the tube was filled with nitrogen, and the cyclization process was performed at 40°C for 1.5 h. The reaction mixture was diluted twofold with water, and the MTH was extracted with ether (3 × 2 ml); 1/10 of the combined ethereal extract was analyzed on a Silufol plate and 9/10 on a column.

Analysis of the MTH-(Amino Acid)s on a Column. The MTH-(amino acid)s were separated in a Hitachi 0-34 automatic liquid chromatograph [2]. A column (0.9 × 70 cm) containing resin 2612 (with a particle diameter of 16 μ) equilibrated with water at 18°C was used. After evaporation of the ethereal extract, the dry residue in the flask was treated with 0.15 ml of ethanol, 0.026 ml of 17.5 N CH_3COOH, 0.2 ml of a solution of MTH-ABA (~0.5 μmole), and 0.35 ml of water. This solution was deposited on the column. The column was filled with water, after which elution with water acidified with hydrochloric acid to pH 3.18 ± 0.03 (5 ml) was begun, and this was followed with a linear gradient of ethanol (water–ethanol, 800:300) at the rate of 60 ml/h. The MTH-(amino acid)s were recorded at 235 and 265 nm.

SUMMARY

1. MTH-α-Aminobutyric acid has been synthesized and has been used as an internal standard in the column chromatography of MTH-(amino acid)s.

2. The column chromatography of MTH-(amino acid)s has been used in the analysis of short peptides isolated from a thermolysin hydrolyzate of the B2 fragment of porcine pepsin.

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