THE SEPARATION OF 1-DIMETHYLAMINONAPHTHALENE-5-SULFONYL DERIVATIVES OF PEPTIDES BY TWO-DIMENSIONAL ELECTROPHORESIS IN A THIN LAYER OF CELLULOSE

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In the analytical chemistry of the peptides, highly sensitive, rapid, and simple methods of determining the number of peptides in a mixture are frequently required. A great step forward in the analysis of such mixtures was the introduction into analytical practice of the DNS* label for the amine groups of peptides [1, 2] with subsequent separation of the DNS-peptides by the TLC method in a thin layer of silica gel [3, 4]. By this method it is possible to separate substances on the basis of differences in their hydrophobic and hydrophilic properties. For DNS-peptides such differences are levelled out to a considerable extent by the large DNS residue, which affects the separating capacity of the method.

In a study of the electrophoretic properties of the DNS derivatives of amino acids and peptides in a number of electrolytes, we found a similarity in the behavior in an electric field to that of the free amino acids under the same conditions. The results obtained enabled us to propose a new method for separating DNS-peptides which is based on two-dimensional electrophoresis in a thin layer of cellulose. In the first direction, electrophoresis is carried out in a pyridine-acetate buffer with pH 6.5, and in the second direction in an electrolyte with pH 2.0. Since at pH 6.5 the carboxyl groups possess a negative charge and the dimethylamino groups are practically electroneutral, DNS peptides differing from one another in molecular weight will migrate toward the anode with different velocities. Furthermore, in the acid medium (pH 2.0) the $N(CH_3)_2$ group of the DNS residue acquires a positive charge close to +1, while the negative charge of the carboxyl group, determined by the pK_a value, is considerably less than −1. The resultant charge becomes positive and the DNS peptides migrate toward the cathode with different velocities.

Thus, the separation is based on the two main differences in the properties of the DNS-peptides, namely: the difference in the magnitude of their negative charges and molecular weights on electrophoresis in a pH 6.5 buffer and the difference in the magnitude of their positive charges on electrophoresis in the acid pH region. Such a marked change in the polarity of the DNS-peptides leads to a change in the direction and rate of their migration and permits the separation and identification of DNS derivatives of peptides of similar size, composition, and properties. This conclusion is confirmed by the "peptide maps" that we obtained in the separation of a model mixture of seven DNS-peptides (Fig. 1) and of a pepsin hydrolysate of the S-sulfo-(chain A) of insulin treated with DNS chloride (Fig. 2). As was to be expected, in Fig. 1 all seven spots of the mixture of DNS-peptides can be seen and in Fig. 2 sixteen peptides from the enzymatic hydrolysate of the S-sulfo-(chain A) of insulin.

It must be mentioned that after dansylation the excess of DNS acid formed as a result of the hydrolysis of the DNS chloride must be removed, since it adversely affects the separation of the DNS-peptides on electrophoresis.

The number of substances deposited on the plate and the time of electrophoresis also depend on the composition and structure of the peptides and the experimental procedure selected. The proposed method permits the "peptide map" to be obtained in 2.5–3 hr with an amount of the order of only 2×10^{-9}–4×10^{-9} mole of the initial protein or peptide.

The method of separating DNS peptides that we have developed has been used to investigate an incomplete acid hydrolysate of the DNS derivative of the Russian antibiotic A-128-OP (acid) [5]. Partial hydrolysis was carried out under conditions permitting the maximum number of fluorescing derivatives to be obtained [concentrated HCl–CH_3COOH (1 : 1), 50° C, 4 hr]. It can be seen from Fig. 3 that this hydrolysate contains eight DNS-peptides. Each of the DNS peptides was subsequently separated by the TLE method in systems with pH 6.5 and 2.0. The homogeneity of the peptides was checked by the TLC method in various systems. The results of the investigation will be given later.

*Here and below, the following abbreviations are adopted: DNS) 1-dimethylaminonaphthalene-5-sulfonyl-; TLC) thin-layer chromatography; TLE) thin-layer electrophoresis.

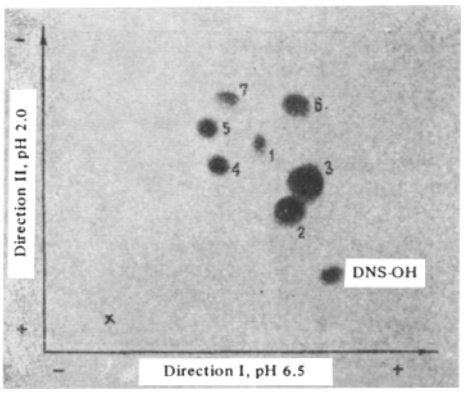

Fig. 1. Model mixture of DNS-peptides (direction I: buffer with pH 6.5, 600 V, 70 min; direction II: buffer with pH 2.0, 600 V, 40 min.): 1) glycyl-L-proline; 2) L-histidyl-L-leucine; 3) glycylphenylalanine; 4) γ-aminobutrylglycine; 5) 3-glycylaminopyrrolid-2-one; 6) L-α, γ-diaminobutyryl-L-leucine; 7) L-α, γ-diaminobutyrylglycine.

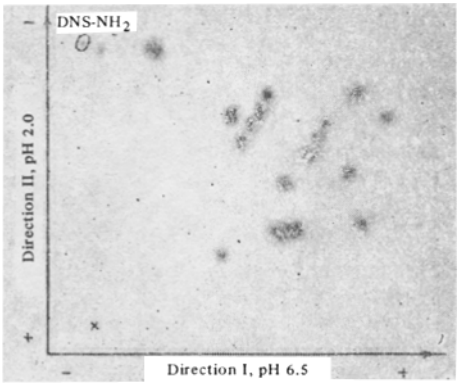

Fig. 2. Pepsin hydrolysate of the S-sulfo-(chain A) of insulin (direction I: buffer with pH 6.5, 600 V, 60 min; direction II: buffer with pH 2.0, 600 V, 50 min).

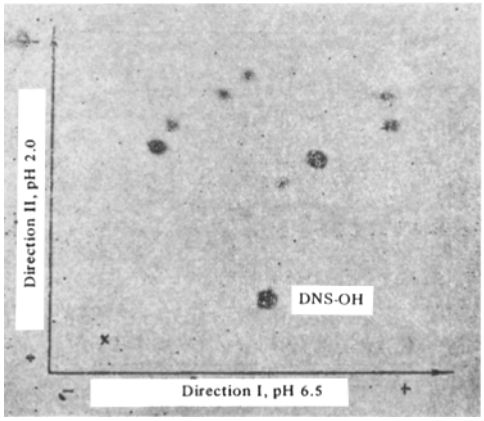

Fig. 3. Incomplete acid hydrolysate of the DNS derivative of the antibiotic A-128-OP (acid) (direction I: buffer with pH 6.5, 600 V, 55 min; direction II: buffer with pH 2.0, 400 V, 45 min).

EXPERIMENTAL

DNS chloride with mp 69° C was obtained by a published method [6]. The peptides glycyl-L-proline, L-histidyl-L-leucine, and glycyl-L-phenylalanine were supplied to us by the firm "Reanal" (Hungarian People's Republic), and the peptides L-α,γ-diaminobutyrylglycine, L-α, γ-diaminobutyryl-L-leucine, 3-glycylaminopyrrolid-2-one, and γ-aminobutyrylglycine were synthesized by one of the authors [7]. The S-sulfo-(chain A) of insulin was obtained as described by Zahn et al. [8]. A highly-purified pepsin preparation was kindly given to us by V. M. Stepanov (IKhPS AN SSSR [Institute of the Chemistry of Natural Compounds, AS USSR]). The antibiotic A-128-OP was that obtained previously [5]. The preparation of the cellulose powder and its deposition in a thin layer on plates was carried out as reported previously [9]. Electrophoresis was performed on an instrument with water cooling [10]. The source of direct current was a high-voltage stabilized rectifier of the VVC-1 or ÉFA-1 type. In electrophoresis in the first direction a buffer with pH 6.5 was used: pyridine–CH_3COOH–H_2O (50 : 2 : 1448), and in the second direction the mixture 85% HCOOH–CH_3COOH–H_2O (34 : 10 : 1200) with pH 2.0. Chromatographic paper impregnated with 1 N acetic acid and water was used to form the bridges between the buffer and the plate. The positions of the DNS-peptides on the plate were determined by their luminescence in reflected UV light on examination in the Brumberg UB-1 ultrachemiscope.

Preparation of the DNS-peptides. To 5 mg (2 μM) of the S-sulfo-(chain A) of insulin treated with pepsin were added 4 ml of 3% aqueous sodium bicarbonate solution and a solution of 15 mg (50 μM) of DNS chloride in 3 ml of acetone. The mixture was shaken at room temperature for 3 hr and was kept at 37° C for 1 hr. The completeness of dansylation was monitored by paper electrophoresis in 1 N CH_3COOH, 750 V, 1 hr 15 min, with subsequent treatment of the electrophoregrams with a 0.4% solution of ninhydrin in ethanol. If ninhydrin-positive peptides were still present, another 0.5 ml of sodium bicarbonate solution and 5 mg of DNS chloride were added and the mixture was kept at 37°C for another hour. Then, to precipitate the salt, 8–9 ml of acetone was added, the mixture was cooled, the precipitate was separated off, and the solution was evaporated. The residue was dissolved in 3 ml of 0.01 N acetic acid and was deposited on a column (1 × 5 cm) of the cation-exchange resin Dowex 50 × 4 (200–400 mesh) in the H^+ form washed with 0.01 N acetic acid [4]. The DNS acid was eluted with ~200 ml of 0.01 N acetic acid until a sample taken from the column with a capillary and deposited on paper did not luminesce in UV light. The column was washed with water and the DNS-peptides were eluted with ~200 ml of a 1 N solution of ammonia in 25% acetone until a sample no longer fluoresced in UV light. The eluate was evaporated in vacuum at 37° C. The residue was dissolved in 0.4 ml of the same solution of ammonia and the resulting solution of DNS-peptides was used for the electrophoretic investigations.

The DNS derivative of the antibiotic A-128-OP and the model mixture of DNS-peptides were obtained similarly.

Technique of two-dimensional thin-layer electrophoresis. Cellulose powder passed through a No. 64 sieve was used as the carrier in TLE. The solution under investigation was deposited on the dry plate with a thin capillary in the form of a spot with a diameter of not more than 2 mm, 3.2 cm from the cathode and 2.2 cm from the edge of the plate closest to the worker. The plate was carefully sprayed with the pH 6.5 buffer solution from a finely-dispersing atomizer, the region with the spot of deposited sample being sprayed last. Then the plate was placed on the table of a cooled instrument for TLE and, beginning from the anode end, paper bridges (5 × 13 cm) moistened with the same electrolyte were applied, and the current was switched on (the voltage and the time of electrophoresis are given in the figures). After the end of TLE, the plates were dried under a 500-W lamp until the odor of pyridine had disappeared completely. The cooled plate was carefully sprayed with the pH 2.0 electrolyte and electrophoresis was carried out in the direction perpendicular to the original one. In the first case, the region with the DNS-peptides was sprayed last, and the bridges (5 × 18 cm) were applied first from the cathodic side of the plate remote from the peptides (the current strength in TLE in instruments with cooling must not exceed 0.7–1.0 mA/cm). Then the plate was dried under a lamp and was kept at 110° C for 20 min and in an atmosphere of ammonia for 5 min. The DNS-peptides were detected by examining the plate in UV light. For documentation, the plates were photographed in reflected UV light.

CONCLUSIONS

1. A new method for the separation of DNS-peptides has been proposed which is based on the use of two-dimensional electrophoresis in a thin layer of cellulose and which, in combination with photodocumentation, enables clear satisfactorily reducible "peptide maps" to be obtained with only 2–4 × 10^{-9} mole of protein (peptide) in a time not exceeding 3.5 hr.

2. The method described has been used to determine the number of DNS peptides in an incomplete acid

hydrolysate of the DNS derivative of the antibiotic A-128-OP (acid).

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