AMINO ACID ANALYSIS OF PROTEINS AND PEPTIDES BY THIN-LAYER ELECTROPHORESIS IN ASSOCIATION WITH CHROMATOGRAPHY

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In the investigation of the structure of proteins and peptides, rapid information on their amino acid composition is frequently required. The development of thin-layer chromatography (TLC) has considerably accelerated the acquisition of the data mentioned [1, 2] as compared with paper chromatography. However, to separate complex mixtures of amino acids it is necessary to carry out TLC in several systems which, as is well known, complicates the work. Consequently, the method of thin-layer electrophoresis (TLE) [3, 4], differing from TLC, has been used.

The TLE method, not recognised until 15 years after the publication by Consden, Gordon, and Martin of the results of the TLE of amino acids and peptides in silica gel [5], has proved to be extremely convenient and effective in the analysis of a number of substances. In recent years, this method has been used for the separation of phenols, phenolcarboxylic acid, and naphthols [6, 7], iodates and periodates [8], nucleotides [11], a number of amines and amino acids [9], various dyes [10], and also, in association with TLC [13, 14] to obtain peptide "maps."

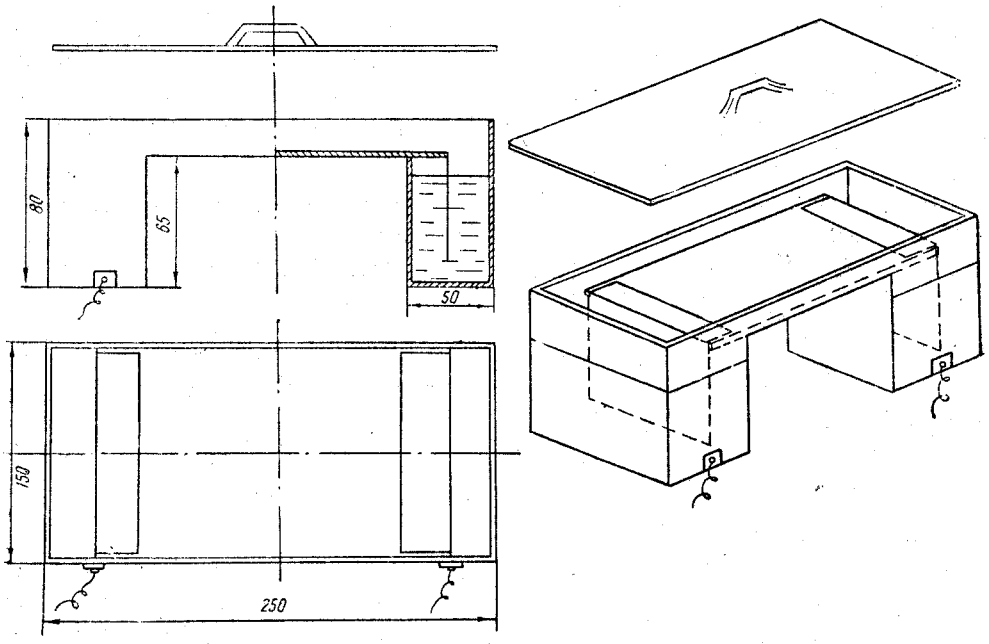

Fig. 1. Sketch of the apparatus for electrophoresis in a thin layer of cellulose.

There are also investigations reported in the literature on the characteristics of the flow of liquid during TLE in various carriers [14] and, in addition, there is information on the considerable advantage of this method, since it requires less time and material and ensures greater clarity of separation [3, 15].

To determine the amino acid composition of proteins and peptides, we have used the TLE method in association with TLC. Only a few studies on the separation of amino acids by the TLE method in association with TLC have been mentioned [9, 12, 16]. However, in all cases synthetic mixtures of amino acids were used and not more than 10–12 amino acids were separated. As carrier we selected cellulose powder, which is readily prepared from chromatographic paper under laboratory conditions.

Electrophoresis was carried out in the first direction in the apparatus (material, transparent plastic, 5 mm thick; cover, 10 mm; electrodes, platinum) shown in Fig. 1. The most effective electrolyte for separation of amino acids was 1 N acetic acid.

Chromatography was carried out in the second, perpendicular, direction, after the complete elimination of the electrolyte from the carrier, using a mixture of butan-1-ol, acetic acid, and water (4 : 1 : 1) as the main solvent system (Fig. 2a). Good separation of leucine plus isoleucine, phenylalanine, methionine, tyrosine, and tryptophan was achieved in the tert-amyl alcohol-methyl ethyl ketone-water (4 : 4 : 2) system [17] (Fig. 2b).

In chromatography, it proved to be extremely convenient to add 0.15 –0.20% of ninhydrin directly to the chromatographic system [18]. This excluded the additional coloration of the plate by the reagent solution. The amino acids appeared directly after the chromatographic system had been dried. The spots obtained were clearer with sharp edges and, which is important, the R_f values of the amino acids did not change, although the sharpness was reduced on spraying with a solution of ninhydrin [19]. It is also possible to use the ninhydrin reagent with 2, 4, 6-collidine for the qualitative detection of the amino acids [20]. In this case the amino acids appear in the form of spots with different colors: aspartic acid, pale blue; glycine, brown; serine, green; tryptophan, purple; and so on.

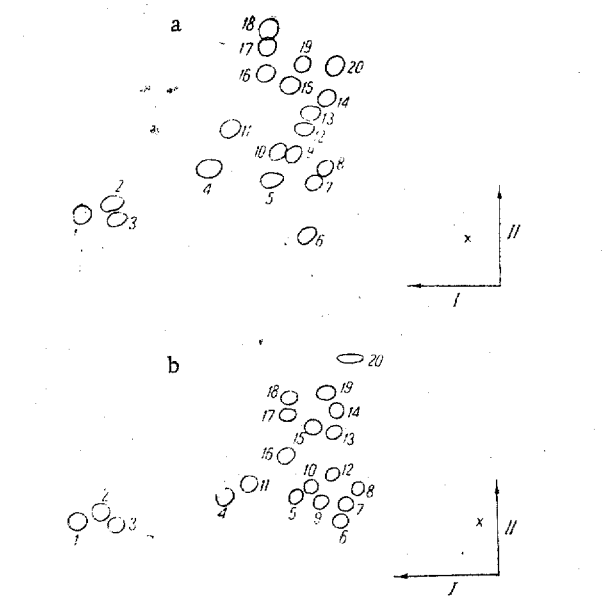

Fig. 2. Amino acid "maps" obtained by electrophoresis in association with chromatography in a thin layer of cellulose. a) Mixture of amino acids. I) Electrophoresis: 1 N CH_3COOH, 470 V, 50 min; II) chromatography: butan-1-ol–acetic acid–water (4:1:1) + 0.2% ninhydrin. 1) Lysine; 2) histidine; 3) arginine; 4) glycine; 5) serine; 6) cystine; 7) aspartic acid; 8) 4-hydroxyproline; 9) glutamic acid; 10) threonine; 11) alanine; 12) proline; 13) tyrosine; 14) tryptophan; 15) methionine; 16) valine; 17) leucine; 18) isoleucine; 19) phenylalanine; 20) ε-DNP-lysine. b) Mixture of amino acids. I) Electrophoresis: 1 N CH_3COOH, 470 V, 50 min; II) chromatography: tert-amyl alcohol–methyl ethyl ketone–water (4:4:2) + 0.15% ninhydrin (symbols for the amino acids as above). c) Insulin hydrolysate. I) Electrophoresis: 1 N CH_3COOH, 460 V, 50 min; II) chromatography: butan-1-ol–acetic acid–water (4:1:1) + 0.2% ninhydrin (symbols for the amino acids as above).

To obtain clear and reproducible results certain experimental rules must be strictly observed:

1) before electrophoresis the plate must be washed with the electrolyte from one end (the anode), since washing from both ends or spraying distorts the electrophoregrams;

2) a solution of ε-DNP-lysine [DNP = 2, 4-dinitrophenyl] must be added to the mixture to be analyzed so that the dimensions of the spots deposited on the moist plate do not exceed 1–2 mm in diameter;

3) after TLE and TLC the plate must not be dried at a high temperature, since under these conditions there is a marked displacement of the amino acids to the edges of the plate (edge effect) which affects the reproducibility of the results;

4) the potential gradient applied to the ends of the plate in the apparatus (Fig. 1) must not exceed 25–26.5 V/cm; and

5) the chamber for TLC must be of rigidly fixed dimensions (see Experimental).

When the above rules are observed, it is possible to achieve satisfactory reproducibility of the results and from the distribution of the spots with respect to the standard ε-DNP-lysine it is easily possible to identify the amino acids in a hydrolysate of a peptide or protein. ε-DNP-lysine proved to be the most convenient compound as a standard in TLE and TLC: under the conditions described it does not coincide with any of the amino acids and it is easy to detect on the "map" by its yellow coloration.

In addition to this, to characterize the amino acids we have used the magnitude

$$R_{\varepsilon DL} = \frac{a}{b.c} \cdot 10^3,$$

where a is the distance from the amino acid to the point of deposition; b is the distance from the ε-DNP-lysine to the point of deposition; and c is the distance from the amino acid to the ε-DNP-lysine.

The magnitude $R_{\varepsilon DL}$ is characteristic for each amino acid in a given chromatographic system of solvents and its value gives an idea of the hydrophilic and lipophilic properties of the amino acid to be determined.

The table gives the mean $R_{\varepsilon DL}$ values of the twenty most important amino acids. The $R_{\varepsilon DL}$ values for amino acids from protein hydrolysates agree well with the values in the table. Although some amino acid pairs have similar $R_{\varepsilon DL}$ values on chromatography in the butanol system (for example, serine and glycine, glycine and hydroxyproline, valine and tyrosine), their $R_{\varepsilon DL}$ values differ on chromatography in the system containing tert-amyl alcohol. Moreover, these amino acids are located on the "map" at different and characteristic distances both from one another and from the reference ε-DNP-lysine, which facilitates and simplifies their identification.

$R_{\varepsilon DL}$ Values of the Most Important Amino Acids

Substances investigated	A*	B*
Cystine	9.6	9.6
Ornithine	13.0	17.1
Aspartic acid	14.9	9.8
Lysine	16.3	17.0
Histidine	16.5	17.2
Arginine	16.6	17.5
Serine	18.7	14.7
Glycine	19.5	16.7
4-Hydroxyproline	19.8	11.3
Glutamic acid	21.6	12.0
Threonine	24.0	15.2
Alanine	26.3	18.0
Proline	37.8	15.6
Valine	48.3	21.4
Tyrosine	49.4	30.0
Leucine	55.6	34.5
Isoleucine	55.7	42.0
Methionine	67.7	31.5
Tryptophan	76.0	49.7
Phenylalanine	106.1	62.5

*Arithmetical mean values from eight amino acid "maps." Chromatography in the butanol system.

**Arithmetical mean values from four amino acid "maps." The chromatography after the electrophoresis was carried out in the system containing tert-amyl alcohol (cf. Experimental).

The procedure described enables four to six amino acid "maps" of protein or peptide hydrolysates to be obtained in a working day.

Experimental

Starting materials. Acetic acid, butanol, methyl ethyl ketone, and tert-amyl alcohol of chemically pure or analytical grade were used for the chromatography and were additionally purified by distillation before use; the ninhydrin was a preparation of the Voikov factory additionally purified by recrystallization from 0.1 N hydrochloric acid or a preparation of the firm of Reanal (Hungarian People's Republic) without recrystallization; the trypsin was a product of the firm of SPOFA, Czechoslovak Socialist Soviet Republic; and the insulin, with an activity of 24 units/mg, was obtained from the Scientific Research Institute of the Meat and Milk Industry. For amino acid analysis, 5 mg of protein was hydrolyzed with 5 ml of twice-distilled hydrochloric acid in sealed tubes at 110° C for 24 hr. The solution was evaporated twice with water, and the residue was dissolved in 0.1 ml of a mixture of acetic acid, 85% formic acid, and water (28:20:52), and 3 drops of a 0.1 M solution of ε-DNP-lysine was added. The colored solution was deposited on the plate with a fine capillary (one contact). Work was also carried out with amino acids (from the Soyuzreaktiv combine and from the firm of Hitachi) in the form of 0.1 M solutions in a mixture of acetic and formic acids. A standard mixture of twenty amino acids was prepared by mixing approximately 0.04 ml (3 drops) each of 0.1 M solutions of the amino acids and ε-DNP-lysine. Thus, 0.80 ml of the mixture contained 0.4–0.8 mg of each amino acid and with a single contact (about 2 μl) about 0.5–1.0 of each amino acid was deposited at the spot.

Preparation of the paper powder. Ten sheets of chromatographic Leningrad paper of type "M" were cut into small pieces and covered with 3 *l* of a 5% solution of nitric acid. The mixture was boiled for 50–60 min. After cooling, the paper pulp was washed with distilled water to pH 6–7, dried at 80°–100° C, and ground in a ball mill. The powder was sieved through a kapron No. 64 sieve and was used (without additives) for the preparation of a thin layer. It was stored in a closed bottle.

Deposition of a thin layer. The glass plates were boiled for 20 min in a 20% solution of sodium carbonate, rinsed with water, kept for 1 hr in dichromate, and carefully washed with distilled water, and, before the deposition of the layer, with alcohol. The deposition of a layer on 7 plates (14 × 18 cm) required 22 g of cellulose powder in 100 ml of water. The mixture was carefully shaken in a closed bottle for 1 min and was spread by hand (14 ml of the mixture per plate) or by means of Stahl's apparatus [1]. The plates with the deposited layer of cellulose were left overnight at room temperature and were then stored in a closed vessel.

Technique of thin-layer electrophoresis. The electrode vessels of the electrophoresis apparatus (see Fig. 1) were filled with about 480 ml of 1 N acetic acid and the prepared plate was placed on the horizontal table. The surface of the electrolyte had to be not more than 1 cm distant from the plate. From the anode side, a moist bridge of chromatographic paper (5 × 14 cm), previously wetted with 1 N acetic acid, was applied to the surface of the layer with a width of 0.7–1.0 cm. The other end of the bridge dipped into the electrolyte. After the electrolyte front had migrated through the whole plate, a similar bridge was applied from the cathode side, the apparatus was closed with the cover, and after 5 min the direct current was switched on (470 V). Then the current was switched off and with a single contact of a fine capillary the sample was deposited at a corner of the anode side of the plate, 2 cm from its edge. It was necessary that the diameter of the spot not exceed 2 mm. Electrophoresis was carried out at 460–470 V and a current of 5–7 mA for 50–55 min. After the electrophoresis was complete, the bridges were removed, the plate was dried for 30 min at room temperature and then under a lamp (500 W) at a distance of 25 cm from the plate (40°–45° C) until the odor of acetic acid had completely disappeared (about 40 min).

Chromatographic technique. Chromatography was carried out in a glass chamber (15 × 20 × 25 cm) with a ground cover. The walls of the chamber were lined with chromatographic paper previously moistened with the chromatographic system. The amino acids were separated with a mixture of butan-1-ol, acetic acid, and water (80:20:20) + 240 mg of ninhydrin or a mixture of tert-amyl alcohol, methyl ethyl ketone, and water (40:20:20) + 200 mg of ninhydrin. 100 ml of the system was poured onto the bottom of the chamber. To obtain a uniform solvent front, a thin layer of the carrier 5 mm wide was removed from the top and side edges of the plate. Chromatography was carried out at room temperature for 50–60 min, and then the plate was dried under a lamp. The spots of the amino acids appeared after the complete drying off of the solvent. To color each amino acid specifically, the plate can be sprayed with a developing agent containing 0.3 g of ninhydrin, 80 ml of collidine, 40 ml of acetic acid, and 120 ml of ethyl alcohol [20] and kept at 60°–70° C until the spots appear.

The amino acid "maps" obtained in this way were photographed or copied onto paper. To calculate a $R_{\varepsilon DL}$ value, the distances from the point of deposition to the amino acid and to the ε-DNP-lysine and also the distance between the amino acid and the ε-DNP-lysine were measured (cf. table and Fig. 2a–2c).

Summary

A rapid and simple method for the qualitative determination of the amino acid composition of proteins and peptides by thin-layer electrophoresis in combination with thin-layer chromatography has been developed which permits a "map" with the separation of 18–20 amino acids to be obtained in 3–4 hr.

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