AN ULTRAMICROMETHOD OF FRACTIONATION OF ACID PROTEINS ON POLYACRYLAMIDE GELS

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UDC 577.112.083+612.398-088.1]:543.545

KEY WORDS: microelectrophoresis; acid brain proteins.

Of the methods currently used to fractionate proteins the most sensitive is capillary microelectrophoresis (CMEP), by which nanograms of protein can be determined in a single band [4, 6-9]. However, it is often necessary to determine picograms of protein. The sensitivity of microelectrophoresis (MEP) can be increased by reducing the diameter of the gel, for it is evident that reducing the diameter by 10 times leads to a reduction in the area of cross section by 100 times and, consequently, smaller volumes of the sample are necessary for analysis. However, working with gels under 200 μ in diameter in versions of CMEP is an extremely difficult task.

One of the main factors restricting the further development of the CMEP method in the direction of reducing the concentration of substances for analysis in volumes commensurate with the volume of a single vertebrate cell is that in all the modifications mentioned above it is extremely difficult to use gels under 300 μ in diameter and with a volume of under 2 μl, because the main method of removing gel from the capillary tube after completion of MEP for fixation, staining, and subsequent scanning of the fractionated protein is mechanical expression. If polyacrylamide (PAG) gels are used, however, the possibility of removing them from the capillary tube without injury is very low because of the decrease in elasticity associated with an increase in the ratio of surface area to volume. In addition, the time required to express the gels is long enough for widening of the protein zones through diffusion.

Having used the method of removal of the capillary wall of the separating gel suggested previously [1], the writers have developed an original buffer system for fractionation of acid proteins, by means of which 10^{-11} g of protein can be determined in a single band of the specimen.

EXPERIMENTAL METHOD

The principle of the suggested method is as follows: two blocks of PAG [ratio acrylamide/N,N'-methylene-bis-acrylamide 30:1; catalysts of the polymerization reaction used in concentrations of: 0.0125% for N,N,N',N'-tetramethylethylenediamide (TEMED) and 0.025% for ammonium persulfate] are placed in a chamber (Fig. 1A) made of transparatent plastic, measuring 10 × 10 × 3 cm. The blocks of gel are saturated with 0.07 M Tris solution, pH 10.3.

Proteins are fractionated in 50, 40, 30, and 20% separating gels (SG). The original 53.4% solution of monomers (ratio acrylamide/N,N'-methylene-bis-acrylamide 100:1) contained 0.01% potassium ferricyanide. TEMED (0.079%) and ammonium persulfate (0.1%) are added to the polymerizing mixture. PAG can be formed in capillary tubes down to 40 μ in diameter and 2 cm in length. The capillary tubes are rinsed consecutively with distilled water, chromic mixture, deionized water, 96% ethyl alcohol, and acetone and dried at 120°C for 1 h. After the capillary tubes have been filled with the polymerizing mixture, the gels polymerize on account of capillary forces for 3 h in the saturation chamber at room temperature. The next step is to dissolve the capillary walls in concentrated fluoric acid for 20 min, after which the acid is washed off the gels for 1 h with several changes of deionized water. The gels are

Laboratory of Neuronal Structure, Brain Institute, Academy of Medical Sciences of the USSR, Moscow. Laboratory of Central Mechanisms of Regulation and Control, Institute of Clinical and Experimental Medicine, Siberian Branch, Academy of Medical Sciences of the USSR, Novosibirsk. (Presented by Academician of the Academy of Medical Sciences of the USSR V. P. Kaznacheev.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 95, No. 4, pp. 124-126, April, 1983. Original article submitted November 30, 1981.

0007-4888/83/9504-0559$07.50

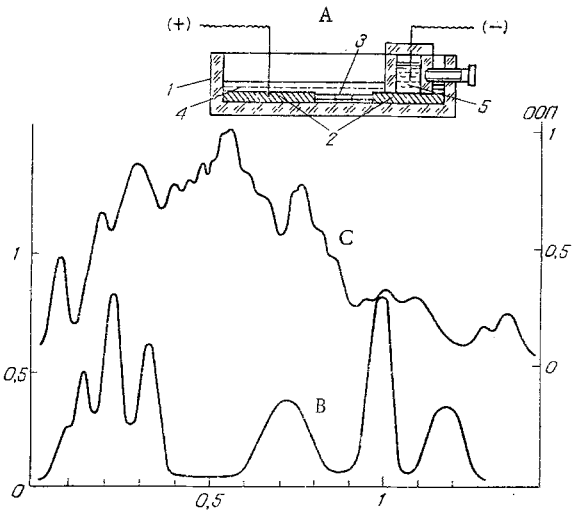

Fig. 1. Scheme of chamber for noncapillary microelectrophoresis (A) and results of fractionation (B, C). 1) Chamber, 2) PAG blocks, cathode and anode; 3) separating gel; 4) paraffin oil; 5) chamber for 0.07 M Tris, saturating electrode block. B) Densitogram of mixture of marker proteins. From left to right: BSA (trimer, dimer, monomer), ovalbumin, carbonic anhydrase, myoglobin, ribonuclease; C) densitogram of extract of proteins from lateral geniculate body. Ordinate, relative optical density (in relative units). Abscissa, distance traveled by bromphenol red during electrophoresis (in mm).

then saturated for 1 h in 0.126 M Tris-sulfate, pH 8.8, containing 1% Triton X-100. To facilitate the manipulations with SG, several crystals of bromphenol red (BPR) are added to the saturating buffer.

The PAG blocks are connected to the electrodes and the chamber filled with paraffin oil. To remove the excess of buffer from the surface of SG, they are dried before electrophoresis on cellophane film. The ends of SG are connected to the PAG blocks (cathode and anode). The contact surfaces of the SG ends and blocks are lined with dialysis films, saturated beforehand with 0.07 M Tris, pH 10.3, to prevent contamination of the block by the test substance. The protein solution is applied at the point of contact between SG and the cathode block, and a current-stabilized voltage is applied to the electrodes [1].

After the end of electrophoresis protein in the gel is stained for 15 min with a solution of 0.33% Coomassie R 250, made up in 45% methanol, 10% acetic acid, and 3.3% sulfosalicylic acid. Excess of dye is washed off with 7.5% acetic acid. The stained gels are again drawn into a capillary tube and scanned on the capillary microspectrophotometer at the Research Institute of Organic Chemistry, Siberian Branch, Academy of Sciences of the USSR, the principle of action of which is based on the two-wave method of detection [3].

Samples of marker proteins are made up in 0.01 M Tris-HCl, pH 7.4, containing 0.001 M EDTA and 1% Triton X-100.

Extracts of protein from rat brain were obtained by the following method: Wistar rats weighing 200 g were decapitated under ether anesthesia. A fragment of tissue from the lateral geniculate body (determined according to Kurepina [2]), 0.5 mm^3 in volume, was homogenized for 2 min with a metal needle 200 μ in diameter, fixed to the shaft of an electric motor revolving at a speed of 1000 rpm, in 25 volumes of homogenizing buffer containing 0.1 M dithiothreitol. The homogenate was centrifuged at 16,000 rpm and 0°C for 1 h. The supernatant was collected and subjected to MEP.

EXPERIMENTAL RESULTS

To determine the sensitivity of the MEP system, bovine serum albumin (BSA; from Calbiochem, USA), made up in a concentration of 1 mg/ml, was fractionated. A solution of albumin in 20% SG, in a volume of 5 nl, was subjected to electrophoresis and the protein was separated into three fractions (monomer, dimer, trimer). The sensitivity of the method, based on this analysis, was thus 10^{-11} g protein per single band.

From left to right three fractions of BSA (trimer, dimer, monomer) can be clearly distinguished on the densitogram of the fractionated mixture of marker proteins (Fig. 1B), followed by peaks for carbonic anhydrase, myoglobin, and ribonuclease. Each protein in the mixture was made up in a concentration of 0.1 mg/ml. To 40% gel with a diameter of 84 μ 10 nl of a solution of marker proteins was applied, and after electrophoresis for 40 sec, they extended over a distance of 1.25 mm along the BPR front. The strength of the current was 10 μA. Differences in amplitudes of peaks for the proteins are explained by their different affinity for the dye. Altogether 20 peaks were distinguishable on the densitogram of the fractionation sample of lateral geniculate body proteins (Fig. 1C). In this case, 10 nl of extract was applied to a gel 100 μ in diameter, the BPR front traveled 1.45 mm, the current was 10 μA, and electrophoresis ended after 40 sec.

The method of fractionation of acid proteins developed by the writers is simple in use and samples are fractionated much more rapidly than by the macromethod, and with very low I values, as a result of the use of gels under 100 μ in diameter. Gels of such diameters can be used to fractionate proteins in a volume of several nanoliters and in low concentrations. Optimal buffers for this electrophoretic system are 0.125 M Tris-sulfate, pH 8.8, containing 1% Triton X-100 for SG and 0.07 M Tris, pH 10.3, for saturating the electrode blocks. The buffer system chosen gives best concentration and fractionation of the samples. Our investigations have shown that the concentration achieved by the leading ions alone is sufficient for fractionating protein taken in a quantity of 10^{-10}-10^{-11} g per gel.

Comparison of our protein fractionation system with that in which PAG blocks were saturated with 5 mM Tris-glycine, pH 8.3 [5] showed that fractionation samples of marker proteins and lateral geniculate body proteins were identical in the two systems, but the zone concentration effect was more clearly observed when our method was used.

Because of the high sensitivity of the method it can be used for analysis of protein composition at the cell level, and also for the diagnosis and prognosis of pathological processes with the aid of ultrabiochemical clinical analyses.

The authors are grateful to their Directors T. A. Leontovich and M. B. Shtark for constant help.

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*Reference 6 missing in Russian original — Publisher.