MICROELECTROFOCUSING OF PROTEINS IN CAPILLARY GELS

Ulrich Grossbach

Max-Planck-Institut für Biologie, Tübingen (Germany)

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SUMMARY. A microtechnique of isoelectric focusing in acrylamide is described which allows the separation of proteins in the millimicrogram range.

Isoelectric focusing in pH-gradients formed by aliphatic aminocarboxylic acids (1,2) has recently become a widely used method for the separation of proteins (3). For the analysis of microgram quantities of material, isoelectric focusing in gel columns of the type used for disc electrophoresis, has been shown to be a very efficient means (4-8).

Microelectrophoresis in capillary columns (9) has earlier been applied to the separation of proteins in the millimicrogram range (10-12). By use of the equipment devised for microelectrophoresis, it was possible to develop a technique for the electrofocusing of proteins on the same scale. While electrophoresis in capillaries deserves special precautions and manipulations to achieve sharp separation of the sample constituents (9, 12), microelectrofocusing is very easily performed because sharp zones are automatically formed during the run.

METHODS. The separations were performed in quartz capillaries of 50 μ, 100 μ and 300 μ internal diameter. These were obtained from Heraeus Quarzschmelze, Hanau (Germany), and were cut to a length of 65 mm. The acrylamide gel solution prepared was similar to that described by Wrigley (4) and was adjusted to contain 2% ampholyte solution and 20% sucrose. The following stock solutions were prepared weekly: I: 28 g acrylamide; 0.738 g N,N'-methylenebisacrylamide; made up to 100 ml with distilled water.

II: 1.04 ml N,N,N',N'-tetramethylethylenediamine in 100 ml distilled water.

III: 16% ampholyte solution (LKB, Stockholm), pH 3-6.

Immediately before use, 0.4 ml of solution I, 0.1 ml of II and 0.2 ml of III were added to 0.8 ml freshly prepared 40% sucrose solution. The mixture was chilled on ice. Crystalline bovine serum albumin (Armour) and β-lactoglobulin (Serva, Heidelberg) were used as test proteins because they have closely situated isoelectric points of 4.8 (13) and 5.1 (14), respectively. 1 µl of serum albumin solution containing 68 µg protein and 50 µl of 1 mg/ml β-lactoglobulin were added to the monomer solution. Polymerization was initiated by mixing 0.1 ml of 0.14% ammonium persulfate into the solution, which was then filled into the capillaries by capillary attraction.

After polymerization, the capillary columns were attached to the instrument holder of a de Fonbrune micromanipulator. By means of a micropipette operated by a second micromanipulator, the free space at both ends in the capillaries was filled with 2% ampholyte solution (pH 3-6). To prevent evaporation, the capillaries were immediately sealed with a drop of glycerol. They were then detached from the manipulator and, by means of a bored silicone rubber stopper and a short length of glass tubing, connected to a conventional apparatus for disc electrophoresis. The anode buffer compartment was filled with 0.2% H_2SO_4 and the cathode compartment with 0.4% ethanolamine (4). The glycerol seal dissolved when the capillary columns were immersed in the electrode solutions. Care was taken not to dilute the layers of ampholyte solution at both ends of the gels.

A voltage gradient of 100 V was maintained for 10 to 120 min. Higher voltages did not result in superior separations. Because of the high surface-volume ratio of the gels and the short duration of the run, the separations were performed at room temperature. Immediately after

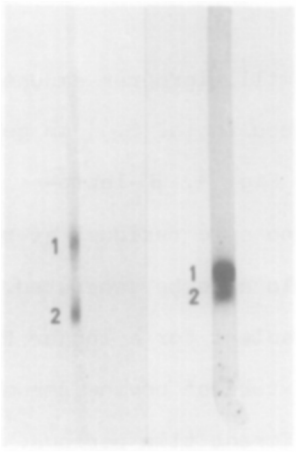

Fig. 1.

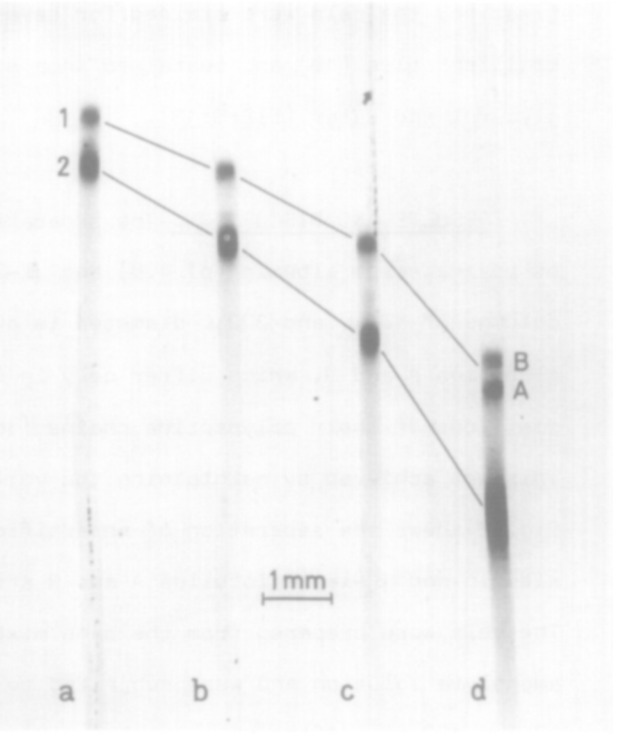

Fig. 2.

Fig. 1: Microelectrofocusing of β-lactoglobulin (1) and bovine serum albumin (2) in acrylamide gels containing 2% ampholyte (pH 3-6). Left: Separation of 16 mμg of (1) and 22 mμg of (2) in a column of 100 μ diameter. Right: Separation of 71 mμg of (1) and 48 mμg of (2) in a column of 300 μ diameter.

Fig. 2: Microelectrofocusing of β-lactoglobulins A and B (1) and bovine serum albumin (2) in gels of 300 μ diameter containing 2% ampholyte (pH 3-6). 50 μg of the β-lactoglobulin sample and 68 μg of (2) were added to 1.6 ml of acrylamide-ampholyte solution, and the capillaries (volume 4.55 μl) were filled by capillary attraction. Time of isoelectric focusing: a: 45 min; b: 55 min; c: 65 min; d: 90 min.

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the run, the gels were pushed out of the capillaries into a drop of 20% trichloroacetic acid on a depression slide. This was easily achieved by means of a piece of tightly fitting steel-wire. For the gels of 50 diameter, rigid tungsten wire was found appropriate. After 1 h of

fixation, the gels were stained for several hours in 0.1% coomassie brilliant blue (15) and destained in a solution of ethanol, acetic acid, glycerol and water (5:2:5:13).

RESULTS AND DISCUSSION. The separation of millimicrogram amounts of bovine serum albumin (pI 4.8) and β-lactoglobulin (pI 5.1) on gel columns of 100μ and 300μ diameter is shown in Fig. 1. β-lactoglobulins A and B, which differ only in the amino acid residues at two positions of their polypeptide chains (16), could also be separated. This was achieved by maintaining the voltage gradient for a longer time. Fig. 2 shows the separation of an artificial mixture of bovine serum albumin and β-lactoglobulins A and B after different time periods. The gels were prepared from the same mixture of acrylamide, protein and ampholyte solution and were subjected to electrofocusing for between 30 and 120 min. After an initial rapid concentration of protein in a short region of the column, the β-lactoglobulin and albumin zones became separated from each other (Fig. 2 a). When the voltage gradient was maintained, the distance between the two zones was increased (Fig. 2 b,c). Separation of components A and B of the β-lactoglobulin zone was achieved after prolonged electrofocusing (Fig. 2 d). At that time, the zone of bovine serum albumin appeared less sharp than before. This seems to indicate that diffusion counteracts the process and maintenance of electrofocusing to a noticeable extent under the conditions applied.

During the course of electrofocusing, a shift of the pattern of proteins along the gel column was regularly observed (Fig. 2). This phenomenon was even more pronounced when lower ampholyte concentrations were used. In runs extended over a period of several hours, the zones migrated to one end of the column and finally ran out of the gel. In gels of 5 mm diameter, this was only observed in runs of more than

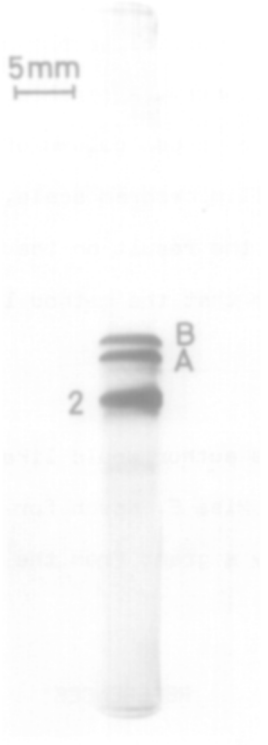

Fig. 3: Isoelectric focusing of the same protein mixture as in Fig. 2 in a gel of 5 mm diameter containing 1% ampholyte (pH 3-6). 22 µg of (1) and 22 µg of (2) were applied to the column. The separation was performed for 2 h at 100 V.

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8 hours. As the length of the columns was 65 mm in both cases, the lower stability of the pH-gradient in the microgels should be due to the reduced volume. The analysis of protein samples by isoelectric focusing on the millimicrogram scale is in good agreement with the results obtained on a larger scale. This is obvious from Fig. 3, which shows a separation of the same artificial protein mixture as in Fig. 2. In this case, however, the diameter of the gel column was 5mm and the protein quantity applied was 220 times higher than in the gels of Fig. 2.

After separation by microelectrofocusing and staining with

coomassie brilliant blue, the relative amounts of β-lactoglobulins A and B in 140 mμg of a commercial sample of β-lactoglobulin were determined by microdensitometry. A parallel determination of 30 μg of the same sample was done on a gel column of 5 mm diameter. According to the evaluation on the millimicrogram scale, the sample contained 60.0% β-lactoglobulin A, while the result on the microgram scale was 57.4%. It would therefore appear that the method lends itself to quantitative evaluations.

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