# Electroendosmotic Preparative Electrophoresis: a One-Step Method for the Purification of Nearly All the Proteins from Complex Mixtures.

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**Abstract**

Electroendosmotic Preparative Electrophoresis (EPE) is a protein purification procedure that couples the high resolving power of polyacrylamide gel electrophoresis (PAGE) to a very effective elution system, which is made possible by the exploitation of the electroendosmotic flow generated during the electrophoresis. By the use of this technique it is possible to obtain the contemporary purification of nearly all the proteins present in complex mixtures in only one step. The EPE apparatus and its use are described in detail and some applications in different purification problems briefly rewieved.

**Key words:** Preparative electrophoresis, Electroendosmosis, SDS PAGE, Protein purification.

*BAM 3 (3): 239-244, 1993*

Protein purification is a major problem in biological research, particularly when high purity of molecules is required for biochemical characterization and antibodies production.

A variety of procedures are in use for this purpose, including different types of column chromatography. Such methods are often time consuming and/or expensive and, moreover, the purification of a single protein in only one step is rarely achieved.

The use of electrophoretic systems for protein purification has been proposed many times, since electrophoresis allows the best resolution of complex proteic mixtures in a relatively simple and inexpensive way. The main limitation in the use of electrophoresis as a preparative method derives from the difficulties in the recovery of the fractionated protein bands. In fact, in most of the cases, the elution mechanism does not allow to maintain the sharpness of separated bands, impairing the overall effectiveness of the system.

Here we report on a preparative purification technique which is able to fully exploit the high resolutive power of polyacrylamide gel electrophoresis (PAGE), due to the simplicity and efficiency of the elution mechanism. The latter is based on the exploitation of the electroendosmotic flow generated during the electrophoresis. By the use of the described procedure the purification of nearly all the proteins present in a mixture can be obtained in only one step.

## Materials and Methods

Electroendosmotic preparative electrophoresis (EPE) is carried out with an ELFE apparatus (Genofit, Geneva; Switzerland), connected to a fraction collector.

The apparatus consists essentially of two electrodic chambers, a cylindrical gel tube (2 X 10 cm) and a capillary, which is placed in the middle of the tube. The upper end of the capillary is connected to a teflon catheter, while its lower end is positioned on a glass fiber filter (Wathman GFC), which, in turn, is in contact with a dialysis membrane (Millipore type VS 0.025 µm), tightly held on the lower surface of the gel. A schematic overview of the apparatus is shown in fig. 1.

When an electrical field is applied to the system, the electroendosmosis phenomenon is amplified by the large liquid-solid interface present within the dialysis membrane. This causes the generation of an upward buffer flow which, after rinsing the glass fiber filter, is canalized through the capillary to the exterior. In this way each electrophoresed protein band emerging from the bottom of the gel, as soon as enters the glass fiber filter, is eluted, and can be collected separately.

The operations for the use of the described apparatus can be summarized as follows (refer to fig. 1).

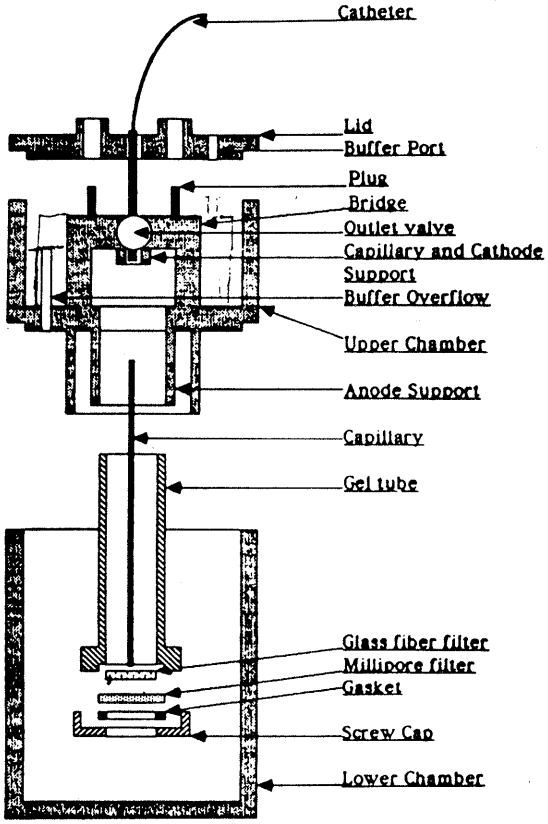


*Figure 1: Overview of the electroendosmotic preparative electrophoresis unit (by courtesy and permission of Genofit, Geneva, Switzerland).*

After the connection of the capillary to the teflon catheter, the gel tube is inserted into the upper buffer chamber. The position of the tube must be regulated in such a way that its lower end be perfectly aligned with the lower end of the capillary. The gel tube is plugged by inserting the plastic disk with the gasket and screwing the screw cap. The catheter is closed with the outlet valve or with a clamp, in order to prevent the ascent of the gel solution into the capillary. At this point the desired amount of gel solution can be poured into the gel tube. Continuous or discontinuous alkaline PAGE systems with different acrylamide concentrations can be used.

In discontinuous Laemmli system [7], which can be carried out also in non denaturing conditions (i.e. in the absence of the detergent SDS) we routinely use 10 ml of resolving gel solution overlaid by 12 ml of stacking gel. The latter quantity should be varied according to the volume of the sample to be loaded (a ratio between sample and stacking gel volumes lower than 1:3 gives the best results).

After gel polymerization, the plastic disk is removed from the bottom of the gel and the catheter is released. With the apparatus upside-down, and after wetting with a few drops of running buffer the bottom of the gel, the latter is covered subsequently with the glass fiber filter and the Millipore membrane. The glossy side of the membrane must be towards the gel surface. It is essential to avoid the entrapment of any air between the gel and the filter and between the filter and the membrane. Both filter and membrane must be wetted and disaerated before use.

After fixing the filter and the membrane by placing the gasket and screwing the screw cap, the apparatus is inserted into the lower chamber, filled with disaerated running buffer. It is very important to avoid the entrapment of air bubbles under the gel tube.

After filling the upper chamber, the electrophoretic run is started. The amount of current to be used should be regulated according to the concentration of acrylamide in the gel: "soft" gels (i.e. 5-7% T) tend to be pushed upward due to the electroendosmotic overpressure generated by the membrane. For this reason we use 30 mA constant current only with gels containing an acrylamide concentration higher than 10%, whereas no more than 10-15 mA should be used for gels with lower acrylamide content.

Few seconds after the current has been turned on, an outflow of buffer from the catheter is observed. If not, the air present in the capillary has to be sucked with the aid of a syringe and/or the elution system controlled for proper assembly.

After checking the regularity of the buffer flow, up to about 2 ml of the solution containing the proteins to be fractionated and a drop of tracking dye is loaded onto the stacking gel surface.

When the tracking dye has reached the bottom of the resolving gel the fraction collection can be started. In order to obtain the best separation among the electrophoresed protein bands it is a good practice to collect a large number of low volume fractions (i.e. 0.2-0.6 ml).

The preparative electrophoresis is completed in a time which varies according to the mobility of the slowest moving protein that has to be purified (5-8 hours).

Collected fractions are then examined for purity by analytical PAGE and pooled as desired.

## Results and Discussion

The EPE technique, performed using the above described apparatus, has been used for the fractionation of different protein mixtures [2, 3, 4, 8, 9, 10, 11]. On the contrary, the most of the preparative apparatus types described in the literature have never been used on any serious separation problem or have been applied only once to the single problem for which they were developed, as already noted by Chrambach and Nguyen [1]. This difference can be considered a first evidence of the effectiveness and versatility of EPE. Moreover, the fractionation of multicomponent systems by successive elution of electrophoresed bands, resulting in the purification of several proteins in only one step can be achieved by the use of the technique here described. This desirable goal has been considered quite impossible to reach, due to the difficulties in electrophoretic parameters (pH, pore size etc.) optimization [1]. With respect to this point, the electrophoretic conditions to be used in performing EPE, have to be determined preliminarly by carrying out analytical PAGE experiments. The analysis of the results so obtained, in most of the cases, is sufficient to give valuable informations on the best set of conditions to be adopted for the preparative electrophoresis, in order to have the best separation among the bands of interest. The only point to be taken into account is that the resolution between two adjacent bands has to be evaluated on a distance corresponding to the entire length of the preparative resolving gel, since the resolution of the proteins in the collected fractions is that of the moment in which the bands are eluted from the bottom of the gel tube, if a sufficiently low fraction volume is provided.

The adjustment of the elution speed, a major problem in preparative electrophoresis, in the case of EPE is automatically achieved. In fact, the electroendosmotic flow is strictly dependent on the electrical field intensity, which, in turn, determines the migration speed of the electrophoresed protein bands. In this way the electroendosmotic flow increases according to the speed of bands outcoming from the bottom of the gel. This self-regulating elution mechanism results in an optimal arrangement between dilution and resolution of separated proteins.

The amount of protein that can be loaded on the preparative gel is up to 30 mg. The protein recovery is total, although the amount of one purified band is determined, besides its quantitative presence in the original extract, by the distance with the nearest migrating contaminant. This distance can be maximized, as noted above, by adjusting electrophoresis parameters.

As previously mentioned, a variety of gel types can be used in performing EPE. The sole limitation concerns the use of electrophoretic systems at acidic pH, in which the direction of the electroendosmotic flow is the same of that of electrophoresed protein bands (i.e. towards the cathode).

Figure 2 gives an idea of the results obtainable with EPE. The figure shows the analytical SDS-PAGE pattern of the proteins in every third fraction subsequently eluted from the preparative electrophoresis of the water-soluble barley grain proteins. The separation was performed according to Laemmli [7] with an acrylamide concentration of 12%. An excellent purification of a large number of proteins has been achieved, although, with an acrylamide concentration of 12%, the faster moving bands (left side of fig. 2A) are less well separated in comparison to those of lower mobility (right side of fig. 1A and fig. 1B). A better purification of the former is obtained by increasing the acrylamide concentration of the preparative resolving gel up to 16 or 18 % (not shown). It is noteworthy that in the separated fractions it is possible to find some proteins that are only barely detectable in the pattern of the original extract (lane O, compare, for example, with the band arrowed in fig. 2B). Thus, proteins present only in trace amounts in a given mixture can be recovered pure with minimal dilution.

The technique has been used for the first time in 1988 for the separation of the various cellulase isoforms produced by *Streptomyces* [2]. That work is an example of the possibility to achieve a good separation of a complex mixture in non-denaturing conditions (i.e. in the absence of the detergent SDS) (fig. 3), allowing the purification of at least 6 active cellulase isoforms in one step, which required only 7/8 hours to be performed.

Examples of the application of EPE carried out in the presence of SDS are reported in a higher number of papers. The first concerns the purification of the wheat kernel storage proteins, namely High Molecular Weight (HMW) subunits of glutenin [3]. The latter are a group of 3 to 5 polypeptides showing an apparent M.W. varying from 95 to 136 kDa [12]. The purification of all the subunits present in a cultivar was considered for a long time a very difficult goal due to the insolubility of the polypeptides in water and to their similarity in both molecular mass and physicochemical characteristics. However, the application of EPE to this problem allowed the contemporary purification of all the 5 subunits present in the cultivar Clara in only one step (fig. 4). It is noteworthy that two of these subunits (subunit 1 and 5) show only minimal difference in M.W., resulting in a migration rate difference that often is hardly

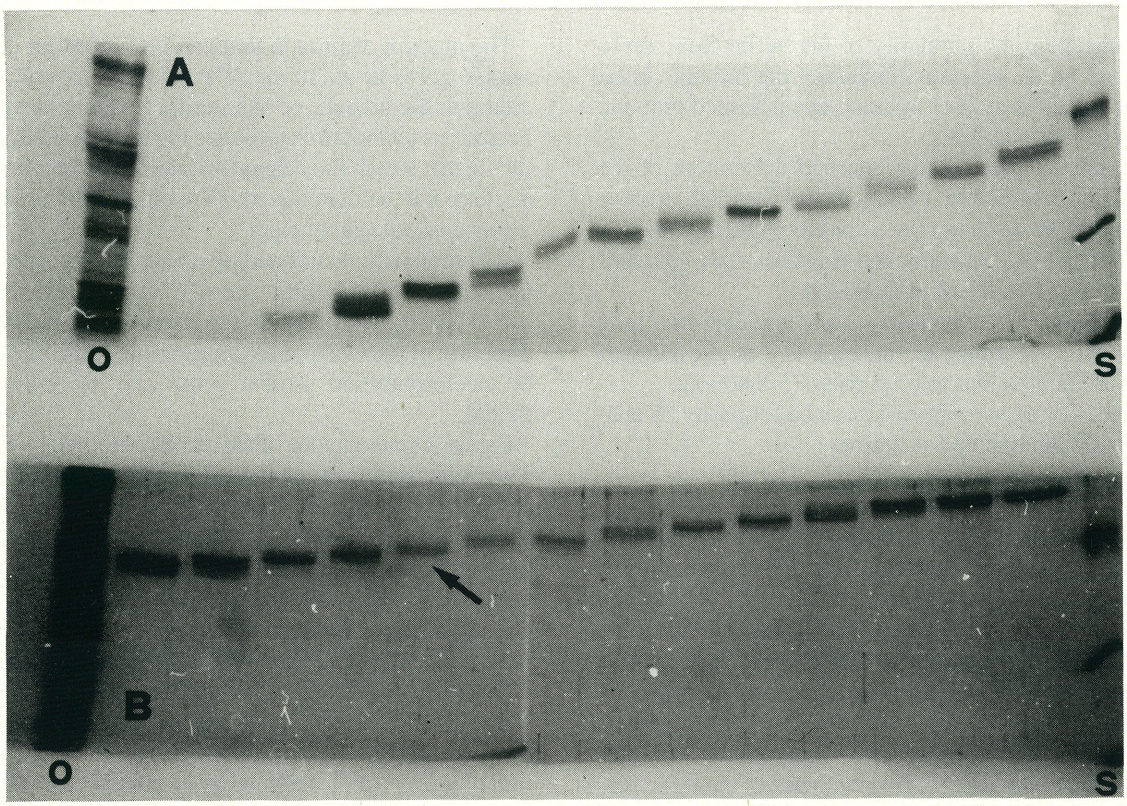


*Figure 2: Analytical SDS gel electrophoresis of every third fraction obtained from the fractionation of the water soluble barley grain proteins. O: whole water extract. S: molecular weight standards of 45, 31 and 21 kDa (from the top to the bottom). The arrow indicates a minor purified band.*

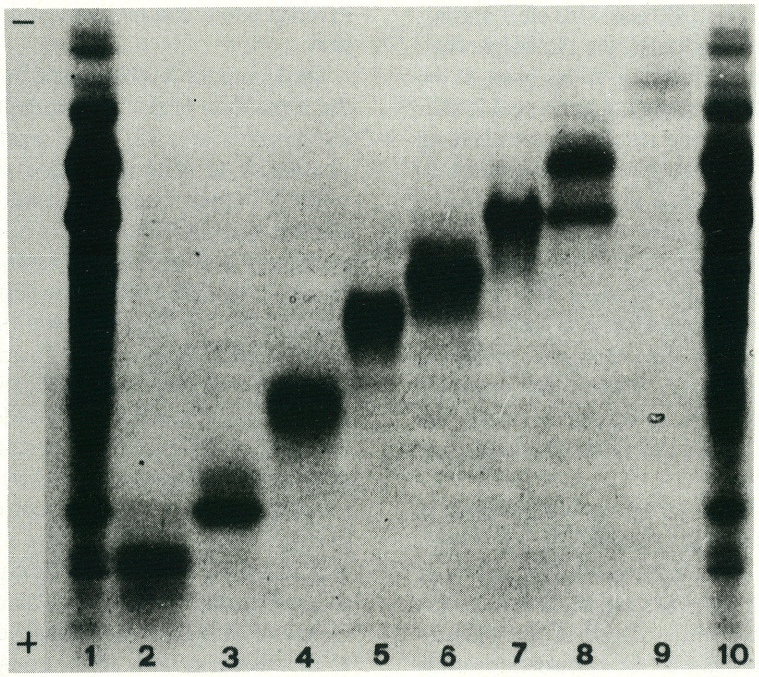


*Figure 3: Analytical gel electrophoresis in the absence of SDS of the cellulase isoforms obtained by EPE performed in non-denaturing conditions. Lane 1 and 10: crude cellulase from Streptomyces strain A 20. Lanes 2 to 9: pooled purified fractions. (Reprinted, with permission, from ref. 2).*

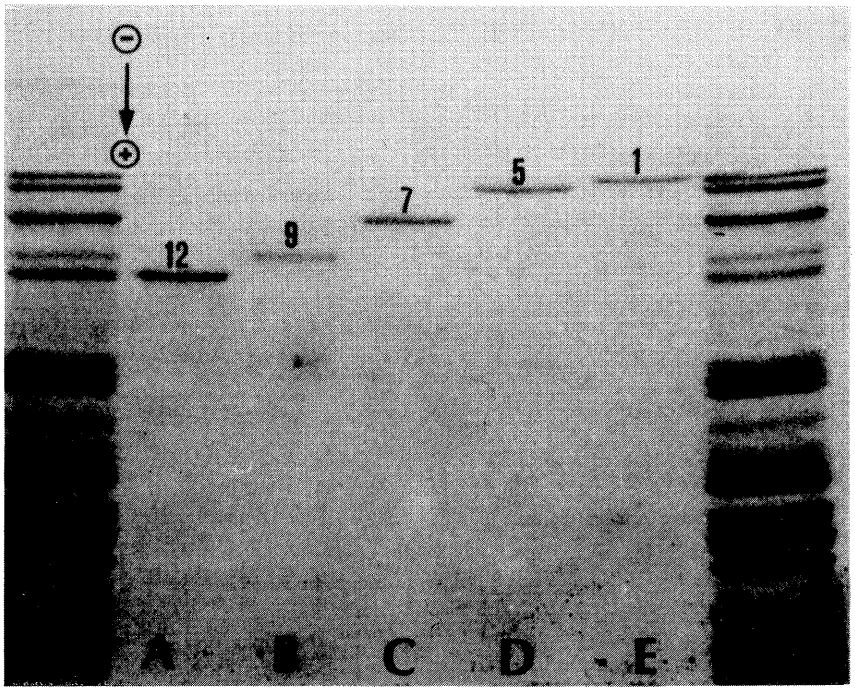


*Figure 4: Analytical SDS gel electrophoresis of the five High Molecular Weight Glutenin Subunits of the wheat cultivar Clara, fractionated by EPE. Lanes A to E: pooled purified bands, numbered according to the international nomenclature. On the left and right sides of the gel are the patterns of total reduced proteins from cultivar Clara. (Reprinted, with permission, from ref. 3).*

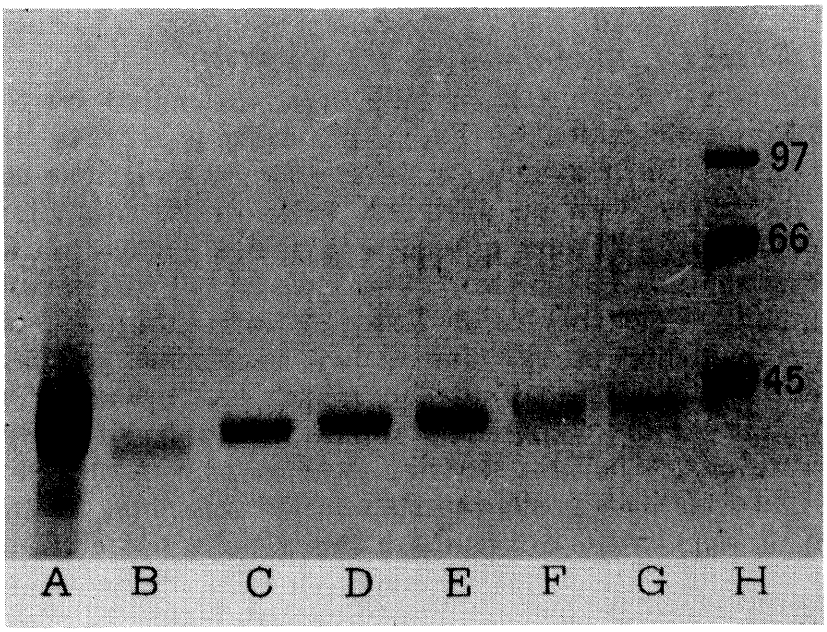


*Figure 5: Analytical SDS gel electrophoresis of some fractions obtained from the fractionation of beer proteins by EPE. Lane A: total proteins from beer. Lanes B to G: purified proteins in the 40-43 kDa region. Lane H: molecular weight standards. Molecular masses in kDa are indicated on the rightside. (Reprinted, with permission, from ref. 9).*

detectable even in analytical SDS gels. A comparison of the results obtainable with the described technique and those achieved by RP-HPLC separation of HMW glutenin subunits has been reported by Kawa et al [6].

In addition, EPE has been successfully applied to the purification of Myosin Heavy Chains [11]. In this case two subsequent preparative runs with different acrylamide concentration and gel size were carried out, in order to obtain the complete separation of the isoforms.

Other applications include the purification of three polypeptides, differing each other only for about 1 and 2 kDa in M.W., present in the light harvesting complex of thylakoid membranes [4], the fractionation of wheat kernel water soluble proteins in non-denaturing conditions [8], the purification of a 32 kDa protein secreted by *Lactobacillus plantarum* and involved in the aggregation of bacterial cells (10), the separation of egg yolk proteins (Pressi G, Curioni A, Dal Belin Peruffo A, Furegon L, Zamorani A: Determination of egg content in egg pasta by an indirect ELISA procedure, submitted) and the purification of all the proteins and polypeptides down to about 5 kDa from beer [9]. In the latter paper it has been demonstrated that the resolving power of EPE is even higher than that of analytical electrophoresis, since several polypeptides, forming a sole diffuse band in analytical SDS-PAGE, were collected in distinct fractions (fig. 5). Moreover, the use of EPE for the separation of 2 DNA fragments differing in size by only 5% with a recovery over 95% [5] must be cited.

The described electroendosmotic preparative electrophoresis unit is not available on the market at the moment. However, a skilful technician should be able to build a similar apparatus without excessive difficulties.

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