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Improved IEF method for the separation of proteins

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Abstract: This work is a continuation of our efforts towards developing an improved IEF method for the separation of proteins. Studying the electrochemical reactions occurring on the electrode surface we found that an unusually high electrode current is registered during the early phase of the experiment. We turned our attention to this side effect in an attempt to find a way for effective suppression of the electrolysis of water, which in its turn will bring about a decrease of the current. It was observed that the addition of gelatin to the electrode solutions suppresses the magnitude of the current flowing through the system, which allows the IEF system to approach steady state for a shorter time. In addition we separated the electrode solutions by Nafion® membranes, which selectively restrict the processes of migration and diffusion in the whole electrophoretic system. Under these newly developed conditions the ampholyte pH gradient is strongly affected, behaving as a carrier with very low ionic strength, in which the magnitude of the current flowing through the system rapidly decreases. Thus the isoelectric focusing can be carried out closer to steady state, obtaining sharp protein separation for a considerably shorter run time.

Key words: isoelectric focusing, protein separation, utilization of the electric current

Introduction

A number of studies have revealed that there is a deviation from the idealized model on which Svensson's IEF theory is based [1, 19]. Recently several theoretical treatments of steady state electrolysis and related processes have been published [20, 2, 3, 12, 26, 22, 25]. The theoretical model elaborated in these papers includes the consideration of chemical reactions between the electrolyte subspecies that produce an electric current, with the participation of hydrogen and hydroxide ions. It has long been recognized that there is a certain instability associated with carrier ampholyte pH gradients. It is reflected in the progressive flattening of the gradient in the neutral region (plateau phenomenon) and cathodic, anodic or symmetrical drifts, causing a gradual loss of the basic and/or acidic end of the pH gradient. Through a computer simulation elaborated by Mosher, Thormann and Bier [13, 14] it was revealed that the gradients develop because

a diffusional mass flux is needed to balance the net electrophoretic flux. These authors concluded that the fluxes are due to the ampholytes not being isoelectric, in the sense that the concentrations of their positively and negatively charged species are not equal. Employing phosphoric acid and sodium hydroxide as electrode solutions and varying the concentrations of the solutions, they concluded that "the loss" of the respective extreme regions of the pH gradients mainly depends on the correlation between the initial concentrations of the acid and the base. Applying the concept of the "moving reaction boundary" Pospichal et al. [21], Cheng-Xi Cao [4, 5] and Cheng-Xi Cao et al. [6, 7] use a mathematical approach to describe the transport of particles during IEF. Recently a series of self-coordinated processes occurring under conditions of isoelectric focusing were studied thoroughly [16, 17, 18]. It was established that under electric field a two-way process begins where the migration of the ions produced by water electrolysis is accompanied by diffusion of charge-compensating particles, originating from the electrode solutions and/or some constituents of the carrier matrix. As a result a non-ampholyte (primary) pH gradient is generated in PAG, which can be routinely measured. Because of the continuous flow of ions and particles the primary pH gradient changes with time bringing about a continuous change of the ampholyte pH gradient. It was established that irrespectively of the type of electrode solutions employed when the electrophoresis is carried out in a power mode the current gradually decreases, tending to reach a minimal value and the ampholyte pH gradient is relatively stable [17]. The same correlation was observed for the yield of water ions, which decreases during the process, following a non-linear relationship similar to that of the current. Our experience shows, that while in the electrophoretical system there are conditions allowing the electrolysis of water, i.e. water is present in the system, the current cannot reach a value of zero, the immediate consequence of which is that steady state can be approached, however it cannot be attained. In this context, any substance present in the electrode solutions that can decrease the current will be suitable to bring the system nearer to steady state for a shorter duration. Recently we managed to achieve this by introducing gelatin into the electrode solutions. This result prompted us to extend our studies in this direction by employing the ion exchange membrane Nafion® to restrict the mass transport and electron conduction in the electrophoretic system and to study how it affects the IEF of proteins.

Nafion®

Nafion® is a poly(tetrafluoroethylene) based ionomer, which was developed by Dr. Walther Grot at DuPont in the late 1960's by modifying Teflon® [15]. The ionic properties of Nafion® are created by adding sulfonic acid groups, a chemical with very strong ionic properties, into the bulk polymer matrix. Thus Nafion® combines the physical and chemical properties of its Teflon base material with ionic characteristics. It is found effective as a membrane for proton exchange by permitting hydrogen ion transport while preventing electron conduction [9, 10, 11, 24, 27].

Experimental

Materials, Equipment and Isoelectrophoretic Conditions

Polyacrylamide gel slabs (250×120×2 mm) were prepared using 5% gel concentration and 3% degree of cross-linkage. All of the reagents used for the self-preparation of polyacrylamide gels (PAG) were "puriss" and "for electrophoresis" grade from

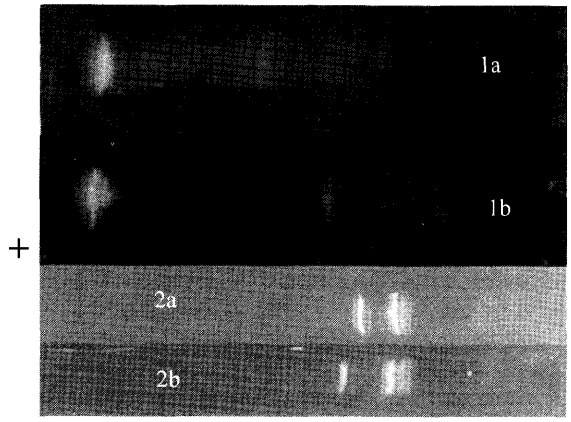

Fig. 1. Effect of gelatin on IEF of a Protein standard mixture (1a, 1b) as well as a hemoglobin standard (2a, 2b). Two types of electrode solutions were employed: 0.1 M phosphoric acid and sodium hydroxide (a) and 0.1 M phosphoric acid and sodium hydroxide in the presence of 1% gelatin (b). PAG was prepared in the presence of carrier ampholytes. The maximum preset voltage is reached 30 min faster when gelatin is introduced into the electrode solutions, and then the ectrophoresis was continued for two hours at 800 V. The focused protein bands have an almost identical separation and sharpness, however, their position along the gel differs, which is caused by the pH gradient drift. Running conditions: 800 V, 20 mA, 15 W. The letters denoting the electrophoregrams correspond to the electrode solutions employed

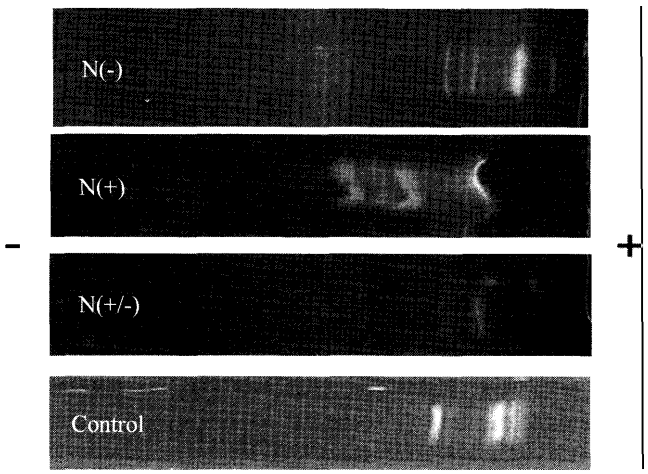

Fig. 2. Effect of Nafion® on IEF of a Protein standard mixture. The electrophoresis was carried out under electrophoretic conditions as in Fig. 1. The focused protein bands have an almost identical pattern, however their position along the gel differs, which is caused by the use of Nafion®. Best result as regards the separation of protein bands and their sharpness was obtained when Nafion® was used to separate only the cathode electrode solutions ( ). As an exception, when Nafion® was used to separate both electrode solutions (N+/-) the proteins are not separated at all

"Fluka" (Germany). 2.2 mL of carrier ampholytes (CA) "Ampholyte high-resolution 3-10" (catalogue No. 39878), Fluka & Riedel, The Sigma-Aldrich Family (USA), per 60 mL gel were introduced, followed by 20 mg ammonium persulfate and 0.06 mL TEMED. Solutions of 0.1 M phosphoric acid (Merck, Darmstadt, Germany) and sodium hydroxide (Reanal, Budapest, Hungary) or distilled water alone (pH=6.75) were used as electrode solutions. The total volume of each electrode solution was 100 mL and was bubbled with argon prior to use for about 15 minutes. In some instances 0.01%, 0.1% or 1% (m/v) gelatin (for electrophoresis, type A, G8150, Sigma) or Triton X-100 (CAS number 9002-93-1, laboratory grade, Sigma Chemical) were added to the electrode solutions. As a separator between the electrode solutions and the gel we used Nafion® 117 perfluorinated membrane, thickness 0.007 in. (catalogue No. 939), which was purchased from Aldrich, USA. As protein standards we used 5 μL 5% (*m/v*) solution of Protein Test Mixture 9 ("wide-range" pI-Marker Proteins), purchased from Serva Electrophoresis GmbH, Heidelberg, Germany (catalogue No. 39206) as well as a hemoglobin standard (Sigma, St. Louis, MO, USA). Staining and destaining procedure of the gel was performed according to the method described by Righetti and Drysdale [23]. Electrophoresis was performed under argon and was carried out using a Pharmacia ECPS 3000/150 Power Supply (Uppsala, Sweden) and an LKB 2117 Multiphor (Uppsala, Sweden) apparatus cooled by running water at a temperature of about 10°C. Platinum electrodes (thin platinum wire – 0.3 mm in diameter, 26 cm length) hanging on a plastic plate (LKB, Sweden) were immersed to the bottom of both electrode solution reservoirs, where the electrode strips were soaked in the corresponding electrode solution. The strips were connected to the gel ends by Whatman 3MM chromatographic paper. To separate the electrode solutions, respectively the electrode strips from the gel, a Nafion® sheet was superimposed on the gel surface selectively: on the anode side (N+), on the cathode side (N-) or on both sides of the carrier gel (N+/-) simultaneously.

The power supply was set to the limiting values of 800 V, 20 mA and 150 W. The duration of the process was read from the moment when the voltage reached the limiting value of 800 V.

Results and discussion

1. Physicochemical influence on the electrode current

Studying the electrochemical reactions occurring on the electrode surface and taking into consideration their relationship with the electrode current we reached the conclusion that under the conditions of IEF the abnormal maximum, which is always registered, is analogous to the same phenomenon observed in polarography. In polarography the abnormal jump of the current is suppressed by addition of small amounts of certain substances like the non-ionic detergent Triton X-100 or gelatin. In this paper we turned our attention to the analogous side effect observed by us, which is registered for both electrode currents in electrophoresis in an attempt to find a way for effective suppression of the electrolysis of water, which in its turn will bring about a decrease of current.

2. Influence of gelatin added to the electrode solutions on the electrode current

We studied the influence of gelatin contained in the electrode solutions on the current flowing through the electrophoretic system. The obtained data show that the decrease of the anode current is proportional to the concentration of gelatin dissolved in the electrode solutions of distilled water. Furthermore, the decrease of the anode current leads to a corresponding increase of the cathode current when the concentra-

tion of gelatin is in the range of 0.01% – 0.1%. However, when the highest feasible 1% concentration of gelatin was employed there was a considerable lowering of both currents. We can now propose that the current flowing through both electrodes can be decreased efficiently by adding 1% gelatin to the electrode solutions, so that steady state is attained for a shorter run time.

3. Influence of Triton X-100 added to the electrode solutions on the electrode current

We studied the influence of 0.01%, 0.1% or 1% concentrations of Triton X-100 introduced in the electrode solutions on the electrode current. Contrary to the results obtained with gelatin containing electrode solutions, in the case of Triton X-100 we found a reciprocal relationship. The magnitude of the current was very high throughout the process.

4. IEF of proteins in the presence of gelatin and Triton X-100 into the electrode solutions

To further verify the influence of gelatin added to the electrode solutions consisting of phosphoric acid and sodium hydroxide, we carried out IEF of a standard protein mixture. For the purposes of comparison we used two types of electrode solutions – the first contained 1% gelatin and in the second gelatin was omitted. It was established that the maximal preset voltage is reached 30 min faster when gelatin is present in the electrode solutions, as compared to the case when gelatin is absent. Electrophoresis was continued for two hours after the maximal preset voltage was reached when the process was interrupted and the electrophoregrams were compared. As can be seen on Fig. 1 the focused protein bands have a very similar separation concerning the number of separated bands, however, their position along the gel is different. Obviously, the presence of gelatin in the electrode solutions brings about suppression of the electrolysis of water, which results in a reduction of the amounts of hydrogen and hydroxide ions liberated in the electrode solutions.

The same electrophoretic system was studied, where gelatin was replaced with 0.01%, 0.1% or 1% concentrations of Triton X-100. Carrying out IEF in the presence of 1% Triton X-100 we observed a most considerable prolongation of the time for which the voltage reaches the preset limiting value, approximately 90 min. In addition we observed that the electrophoresis was accompanied by a considerable transport of water toward the anode, which caused a swelling of the carrier gel. Under these conditions the anodal proteins precipitate, thus compromising the electrophoretic separation.

5. IEF of proteins in the presence of Nafion® as a separator

IEF was carried out with and without Nafion® as described in Experimental. The corresponding results are presented on Fig. 2. A general observation is that the pH gradients are shifted to the more alkaline pH values in respect to Control, where Nafion® is omitted. The maximal shift was observed in two cases: when Nafion® was used to separate both electrode solutions and in the case when Nafion® is applied only on the cathode domain of the carrier. In the case when Nafion® is used to separate only the anode electrode solution the shift is higher in the more acidic region of the gel, but towards the cathode the same pH gradient deviates less from the Control pH values. This result undoubtedly shows that Nafion® selectively restricts the mass transport between the electrode solutions, thus influencing the distribution of the carrier ampholytes along the gel. To verify the expected influence of Nafion®, used to separate the electrode solutions consisting of phosphoric acid and sodium hydroxide, we carried out IEF of a standard protein mixture. For the purposes of comparison four experiments were carried out: without (Control) and with Nafion®. It was established that the maximal preset voltage is reached fastest (for about 150 min) in the cases when Nafion® was used to separate both electrode solutions and when it is on the cathode side of the gel. In the Control,

however, the maximal preset voltage is reached about 30 min slower than "the fastest" runs, under the conditions described above. When Nafion® is used to separate only the anode, the maximal preset voltage is reached slowest (for about 300 min). Electrophoresis was continued for two hours after the maximal preset voltage was reached, then the process was interrupted and the electrophoregrams were compared. As can be seen on Fig. 4 the focused protein bands have an almost similar separation concerning the number of separated bands, however their position along the gel is different. As a result of electroosmosis, on the electrophoregram, which is obtained when Nafion® is used to separate only the anode electrode solution, we observe wavy-shaped protein bands. Undoubtedly the best result was obtained when Nafion® is placed on the cathode domain of the carrier. Concerning the best separation obtained, the presence of Nafion® in the cathode domain of the carrier apparently restricts the migration of hydroxide ions, while the migration of hydrogen ions from the anode is unlimited.

Concluding remarks

The core of the present work is to stress the importance of the electrode solutions for the entire IEF process, which so far appears to be overlooked. In this paper we offer a novel modification of the IEF method, allowing the electrophoresis to be carried out closer to steady state, obtaining sharp protein separation for a considerably shorter run time. This has direct bearing to the improvement of the results obtained in 2-dimensional polyacrylamide gel electrophoresis and thus to proteomics as well. In our opinion the results reported here are a further contribution toward the elucidation of the role of the electrode solutions in the isoelectrophoretic process. In this context a newly discovered method, intended to obviate water electrolysis and related processes occurring on the electrodes under condition of electrophoresis, was recently published [8]. Unfortunately, this study is beyond the scope of our paper, but it is a starting point to make IEF applications not to be a daunting task.

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