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Simple approach to eliminating disturbances in isoelectric focusing caused by the presence of salts

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Abstract

It is well known that the resolution in isoelectric focusing (IEF) is impaired by the presence of salts, partly due to a decrease in the width of the pH gradient. In addition, the risk of precipitation increases and the mobilization time for focused protein zones, an important step in IEF in capillary electrophoresis, is prolonged. To eliminate these drawbacks, an on-tube desalting technique for IEF was developed, based on an automatic substitution of the salts with an ampholyte solution in a short focusing step prior to the final analytical isoelectric focusing procedure.

1. Introduction

Capillary electrophoresis (CE) allows rapid separations with high efficiency and resolution. One of the greatest advantages of CE is the use of minute sample amounts, which range from a few nanolitres of sample to the cytosolic fluid of a single cell [1]. Capillary isoelectric focusing (cIEF) has been applied to the separation of haemoglobins [2], transferrins [3] and immunoglobulins [4]. It is known that the presence of salt in a sample changes the pH gradient and confines the protein zone to a small segment of the capillary. This narrow gradient will result in high protein concentrations and consequently an increased risk of precipitation, loss of resolution and long mobilization times [5]. For these reasons, desalting of biological samples prior to IEF is recommended, which usually entails large sample losses when the volume is below 5 μl. So far, no desalting methods are available for sample volumes in the nanolitre range. Therefore, there is a need for a microscale desalting technique. In this paper, we described an on-tube desalting method specific for IEF and its theoretical basis.

2. Experimental and results

2.1. Materials and equipment

An IEF protein standard mixture and Bio-Lyte (pH 3–10) ampholytes were obtained from Bio-Rad Labs. (Richmond, CA, USA). The separations capillary, made from fused silica and obtained from Polymicro Technologies (Phoenix, AZ, USA), was 150 mm × 0.1 mm I.D. with a wall thickness of 0.1 mm. The on-tube detector was a modified Spectroflow 783 from ABI Analytical Kratos Division (Ramsey, NJ, USA) [6].

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The detection point was 15 mm from the cathodic end of the capillary.

2.2. Replacement of salt with ampholytes

The capillary was coated internally with linear polyacrylamide covalently attached to the wall using the method of Hjertén [7]. The IEF protein standard mixture was diluted 1:20 in 1.5% Bio-Lyte (pH 3–10) and to this solution various amounts of solid sodium chloride were added. The coated capillary was filled with this solution. A 3% Bio-Lyte (pH 3–10) solution was titrated to pH 4.0 with 2.0 *M* hydrochloric acid and served as the anolyte. The Bio-Lyte was titrated to pH 11.0 with 2.0 *M* sodium hydroxide and used as the catholyte. Electrophoretic replacement of salt with ampholytes was performed at a 30-μA constant current, and completed when the voltage reached 3000 V. The time for replacement of salt was dependent on the salt concentration (Table 1).

2.3. Focusing and mobilization of proteins

Focusing was performed at a 3000-V constant voltage for 8 min (the time was determined by the use of coloured proteins). Phosphoric acid (0.02 *M*) served as the anolyte and 0.02 *M* sodium hydroxide as the catholyte. The width of the pH gradient was determined by measuring the distance with the aid of a ruler between the two coloured focused protein zones, phycocyanin ($pI = 4.65$) and cytochrome *c* ($pI = 9.6$) (Table 1). Cathodic mobilization was initiated by replacing the 0.02 *M* sodium hydroxide catholyte with 0.02 *M* phosphoric acid. Mobilization was performed at a constant voltage of 3000 V. The migrating zones were monitored at 280 nm as they passed a stationary UV detector. Mobilization occurs in one direction only. Therefore, proteins which focus at a pH around 10 may not be detected by the UV monitor on cathodic mobilization (e.g., cytochrome *c* in Fig. 1A,B), as their steady-state positions may be between the detector window and the cathode. However, on desalting cytochrome *c* can easily be detected (Fig. 1F–H).

For the study of IEF without replacement of salt we were able to avoid the risk of overheating during focusing by using a constant current of 30 μA until the voltage reached 3000 V, and then keeping the voltage constant for 8 min (Fig. 1A–E).

Table 1
Influence of desalting on the width of the pH gradient

Conditions	NaCl concentration in sample (mol/l)	Desalting time (min)	Width of pH gradient (cm)
A	0	0	9.0
B	0.010	0	8.0
C	0.025	0	6.0
D	0.050	0	4.7
E	0.100	0	3.2
F	0.100	5	9.3
G	0.300	15	9.0
H	0.500	30	8.8

3. Theoretical

Ampholytes are small molecules with different pI values. In cIEF, the whole capillary is filled with diluted ampholytes to a concentration of about 1.5%. When a voltage is applied the negatively charged acidic ampholytes migrate towards the anode and decrease the pH at the anodic section, while the positively charged basic ampholytes migrate towards the cathode and increase the pH at the cathodic section. These pH changes will continue until each ampholyte species has come to its isoelectric point, where it will then concentrate. Because each ampholyte has its own buffering capacity, a virtually continuous pH gradient will be formed. To prevent the ampholytes from migrating into the electrode vessels by either diffusion or gradient drift [8], 0.02 *M* phosphoric acid and 0.02 *M* sodium hydroxide were used as anolyte and catholyte, respectively.

Consider the boundary between the anolyte and the medium in the separation tube during the focusing step. The number of protons, N_{H^+},

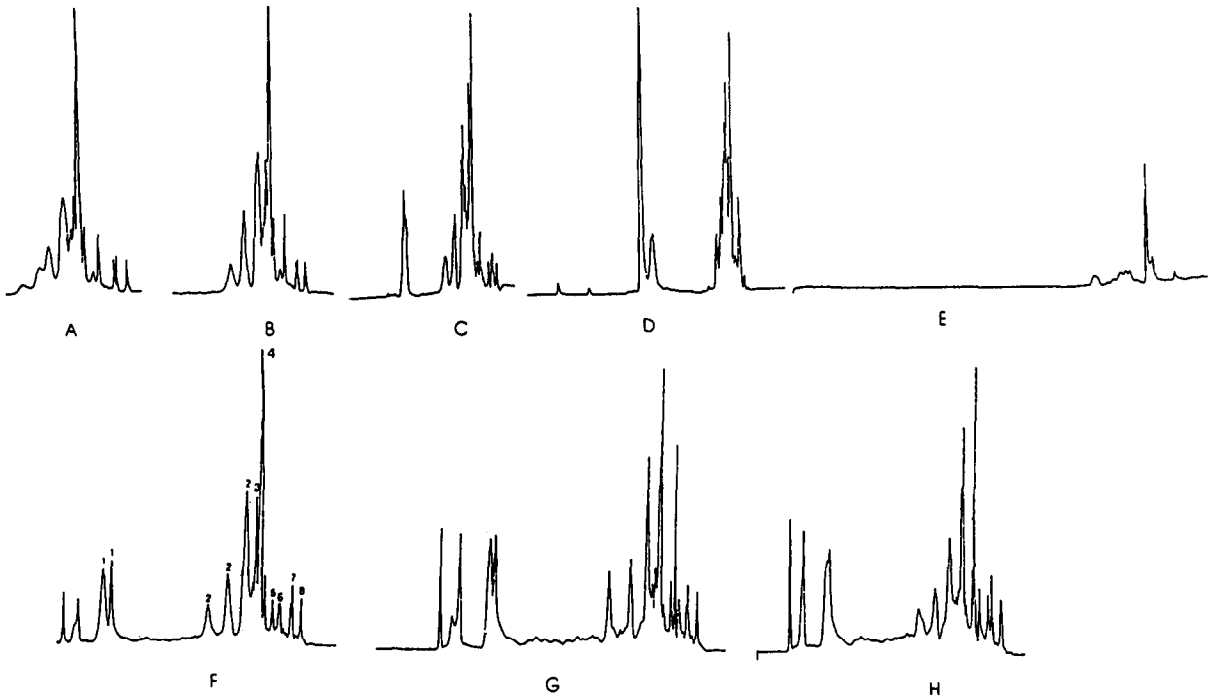

Fig. 1. Isoelectric focusing of IEF protein standard mixture with and without desalting. The protein mixture consisted of cytochrome *c* (peak 1), lentil lectin (2), human haemoglobin A (3), equine myoglobin A (4), human carbonic anhydrase (5), bovine carbonic anhydrase (6), β-lactoglobulin B (7) and phycocyanin (8). Replacement of salt with ampholytes was performed at a 30-μA constant current and completed when the voltage reached 3000 V. A 3% Bio-Lyte (pH 3–10) solution titrated to pH 4.0 and 11.0 served as the anolyte and catholyte, respectively. The letters A–H refer to the experimental conditions listed in Table 1.

passing electrophoretically from the anolyte to the boundary per time unit can be expressed by

$$N_{H^+} = I u_{H^+} n_{H^+} / \kappa \quad (1)$$

where I = current, u_{H^+} = mobility of the protons in the anolyte, n_{H^+} = number of protons per unit volume and κ = conductivity in the anolyte [8]. Ampholytes that are diluted in water have a high ohmic resistance, which increases further during focusing when a voltage is applied. Owing to the low current, the number of protons entering the capillary is limited according to Eq. 1, so the buffering ampholyte pH gradient is not affected.

In the presence of a sample containing salt (e.g. 0.1 *M* NaCl), we obtain a similar expression:

$$N'_{H^+} = I' u'_{H^+} n'_{H^+} / \kappa' \quad (2)$$

where I' = current in the tube in the presence of salt, $u'_{H^+} = u_{H^+}$, $n'_{H^+} = n_{H^+}$ and $\kappa' = \kappa$. In a 0.1 *M* NaCl sample solution the current, I', is about twenty times greater than the current with NaCl is absent.

Combination of Eqs. 1 and 2 gives

$$N_{H^+} / N'_{H^+} = I / I' \quad (3)$$

i.e., $N'_{H^+} = 20\ N_{H^+}$.

The number of protons entering the tube from the anolyte increases about twentyfold, then gradually decreases until most of the Na^+ ions have moved out electrophoretically. This decrease in pH at the anodic section causes the ampholytes to become positively charged and migrate towards the cathode. A similar situation occurs at the cathode, where a large number of OH^- ions enter the tube, giving rise to a pH

increase that forces the ampholytes to migrate towards the anode. As a result, the focused zones will be confined to a distance of 3–4 cm in the central part of the capillary, where a large degree of heat is generated, increasing the risk of protein precipitation [5]. As expected, the length of the pH gradient decreased with increasing amount of salt in the sample (Table 1).

To replace the salts in the capillary with ampholytes and to avoid proteins from either moving out of the capillary or being confined to the centre, 3% Bio-Lyte (pH 3–10) is titrated to pH 4.0 and 10.0 with 2.0 *M* HCl and 2.0 *M* NaOH and employed as anolyte and catholyte, respectively. Eq. 1 now takes the form

$$N''_{H^+} = I''u''_{H^+}n''_{H^+}/\kappa'' \quad (4)$$

where $\kappa'' = 2\kappa'$ (found experimentally) and $n''_{H^+} = (1/200)n'_{H^+}$ (0.0001/0.02) and $I'' = I'$. Accordingly, $N''_{H^+} = (1/400)N'_{H^+}$. Similarly, at the cathodic end $N''_{OH^-} = (1/400)N'_{OH^-}$. Therefore, the amount of H^+ at the anodic end and OH^- at the cathodic end during the removal of salt decreases about 400-fold when the sample contains 0.1 *M* NaCl. The concentrations of H^+ and OH^- decrease further along the capillary as they meet and react with the buffering ampholytes from the electrode vessels. The mobility of the ampholytes is much lower than that of Na^+, so the current will decrease as the Na^+ ions are replaced by ampholytes moving into the capillary tube. The salt replacement is complete when the conductivity in the capillary tubing becomes close to that of a 1.5% ampholyte solution in the absence of salt, e.g., in our experiments when the voltage has increased to 3000 V at a constant current of 30 μA.

4. Discussion

cIEF is a rapid and high-resolution separation technique that can resolve proteins based on small differences in isoelectric points. An obvious disadvantage with all methods based on IEF is that many proteins precipitate at their isoelectric points, particularly at high protein and salt concentrations, and at elevated temperature. The presence of salt in a sample solution will shorten the ampholyte gradient (see Theoretical) and decrease the resolution (see Fig. 1B–D). When the concentration of salt increases above 0.05 *M* the proteins will focus into a very narrow pH gradient (see Table 1) where they tend to precipitate (see Fig. 1E). The mobilization towards the cathode was achieved by replacing the sodium hydroxide with phosphoric acid, which will decrease the pH in the capillary in accordance with the electroneutrality condition [8]. As the proteins and ampholytes become confined towards the centre of the capillary, H^+ and OH^- will accumulate at the margins. The field strength ($\propto 1/\kappa$) at the end of the capillary where phosphate ion enters is much smaller than that in the centre region owing to the higher conductivity at the ends of the capillary. Therefore, the velocity of phosphate ions is lower at the start of the mobilization when the sample contains salt according to the equation

$$v = Eu \quad (5)$$

where v = velocity of the phosphate ions, E = field strength and u = their mobility. Accordingly, the mobilization time is longer. The high pH in the basic region of the capillary hydrolyses the Si–O–Si–C bonds of the inner wall, causing electroendosmosis, which will distort any further IEF experiments.

We shall now show that desalting can be accomplished not only by ampholytes but also by ions of low mobility. From Eq. 1, one recognizes that a decrease in N can be achieved by a decrease in current (I), which is governed by the expression

$$I = V/R \quad (6)$$

and

$$R = l/q\kappa \quad (7)$$

where l = length of capillary and q = its cross-sectional area. Combining Eqs. 6 and 7 gives

$$I = Vq\kappa/l \quad (8)$$

The conductivity, κ, is determined by the expression

$$\kappa = \frac{F}{1000} \cdot \sum C_i u_i \quad (9)$$

where F = the Faraday constant and C = concentration in gram equivalents per litre of solution.

Inexpensive cations and anions (with p$K \geq 11$ and ≤ 3, respectively), that have high molecular masses and low mobilities, may be used for desalting instead of expensive ampholytes in the electrode vessel. Owing to the low ampholyte concentration in the sample solution (below 0.01 M) and neglecting the influence from H^+ and OH^-, the Kohlrausch regulating function, ω, [9] for the phases separated by the moving boundary between Na^+ in the α phase and the displacing ion X^+ in the β phase (migrating into the capillary from the anode) can be written as

$$\omega^\alpha = \frac{C^\alpha_{Na^+}}{U^\alpha_{Na^+}} + \frac{C^\alpha_{Cl^-}}{U^\alpha_{Cl^-}} \quad (10)$$

and

$$\omega^\beta = \frac{C^\beta_{X^+}}{U^\beta_{X^+}} + \frac{C^\beta_{Cl^-}}{U^\beta_{Cl^-}} \quad (11)$$

respectively. As $\omega^\alpha = \omega^\beta$,

$$\frac{C^\alpha_{Na^+}}{U^\alpha_{Na^+}} + \frac{C^\alpha_{Cl^-}}{U^\alpha_{Cl^-}} = \frac{C^\beta_{X^+}}{U^\beta_{X^+}} + \frac{C^\beta_{Cl^-}}{U^\beta_{Cl^-}} \quad (12)$$

Utilizing the conditions for electroneutrality and putting $C^\alpha_{Na^+} \approx C^\alpha_{Cl^-}$, $C^\beta_{X^+} = C^\beta_{Cl^-}$, $U^\alpha_{Cl^-} = U^\beta_{Cl^-} \approx U^\alpha_{Na^+}$ (the mobility of Cl^- is about 50% higher than that of Na^+), one obtains the following equation:

$$C^\beta_{X^+} = \frac{2C^\alpha_{Na^+} U^\beta_{X^+}}{U^\alpha_{Na^+} + U^\beta_{X^+}} \quad (13)$$

Therefore, if the displacing ion has one tenth of the mobility of Na^+, the concentration of this ion will be 2/11 of the Na^+ concentration [$U_{X^+} = (1/10)U_{Na^+}$, $C_{X^+} = (2/11)C_{Na^+}$]. Eq. 9 gives the following approximate value of the conductivity (κ_2) in the capillary following removal of the salt in the sample:

$$\kappa_2 = \frac{F}{1000}\left(\frac{2}{11} \cdot C_{Na^+} \cdot \frac{1}{10} \cdot U_{Na^+} + \frac{2}{11} \cdot C_{Cl^-} \cdot \frac{1}{10} \cdot U_{Cl^-}\right) \quad (14)$$

Similarly, the conductivity (κ_1) prior to removal of salt is

$$\kappa_1 = \frac{F}{1000}(C_{Na^+}U_{Na^+} + C_{Cl^-}U_{Cl^-}) \quad (15)$$

Consequently, $\kappa_2/\kappa_1 = 2/110$. Therefore, the contribution from the displacing ion to the current will also be reduced, as can be seen from Eq. 8. Owing to this lower current there will be a decrease in the number of H^+ and OH^- ions to the extent that the pH gradient will remain unaffected. The advantage of using the displacing ion instead of the ampholyte solution is that the pI of the displacing ion is outside the pH range of the ampholyte gradient. Therefore, this ion will be removed during IEF.

The on-tube desalting technique described here is rapid, highly reproducible and gives a high recovery compared with dialysis.

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