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Journal of Chromatography A, 768 (1997) 307–313

JOURNAL OF CHROMATOGRAPHY A

Evaluation of phytic acid as a buffer additive for the separation of proteins in capillary electrophoresis

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Received 26 October 1995; revised 10 December 1996; accepted 11 December 1996

Abstract

The use of phytic acid to improve protein analysis by capillary electrophoresis (CE) is becoming more and more popular. Due to its size and number of negative charges (up to 12) it provides a high ionic strength combined with a low conductance resulting in an efficient decrease of wall adsorption for proteins. Because of its twelve acidic groups, phytic acid can be used as a buffer over a wide pH range (pH 2–11). The limited wall adsorption of proteins using phytic acid-containing buffers is observed for buffers with a pH of 5.5 and higher. With a monoprotic buffer, most of the investigated proteins show wall adsorption at the pH values studied. In case of a phytic acid buffer, wall adsorption is reduced by a factor of 2–4. The use of phytic acid both as a modifier and as a pH buffer results in more pronounced differences between the various protein mobilities compared with the use of monoprotic buffers. As a result this feature can be used to improve resolution in protein separations.

Keywords: Buffer composition; Phytic acid; Proteins

1. Introduction

Capillary electrophoresis (CE) is a still developing technique in protein separation. Unfortunately, adsorption onto the walls of the capillary results in severe peak distortion which frequently deteriorates system selectivity and sensitivity. For this reason several approaches, such as static coating of the capillary wall with apolar molecules, dynamic coating both with polar and apolar compounds and increasing the ionic strength have been considered. In the present study the effect of the ionic strength is extensively investigated. When relatively small ions are used to enhance the ionic strength, the associated increment of the electric current is rather dramatic, resulting in Joule heating effects. Therefore, large polyionic molecules can be used, which provides high ionic strengths at relatively low currents. This because the current is more or less linearly related to the charge-to-size ratio of the ion and the ionic strength which increases quadratically with the charge of the molecule [1]. Phytic acid is an example of a large polyionic molecule (see Fig. 1) and since it can have up to twelve negative charges, its contribution to the ionic strength is relatively large compared with its concentration. The following data illustrate this: a 20 m*M* solution of phytic acid at pH 9.5 provides an ionic strength of 1.3 *M*, while a 20 m*M* sodium chloride solution has an ionic strength of only 20 m*M*, i.e., 65 times lower [2]. In addition it has been shown that CE separations of proteins can indeed be improved significantly by adding phytic

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PII S0021-9673(96)01099-0

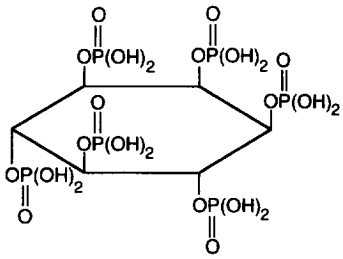

Fig. 1. Structure of phytic acid.

acid to the separation buffer [3,4]. The same holds for peptides, amino acids and organic acids [2,5,6].

The buffer capacity, another characteristic feature of phytic acid, has not been fully explored yet. The availability of twelve acidic groups with pK_a values ranging from 1.9–9.5 [6] provides the possibility to use phytic acid not only as an additive to suppress wall adsorption effects, but also to control the pH.

In the present study the potential use of phytic acid as a pH buffer and/or buffer additive is studied. In order to do so the buffer capacity and pH limitations of phytic acid are compared with the use of a monoprotic buffer and a mixture of a monoprotic buffer and phytic acid.

2. Experimental

2.1. Chemicals

The 40% (w/w) phytic acid solution was purchased from Janssen Chimica (Tilburg, Netherlands). α-Chymotrypsinogen A (bovine pancreas), ribonuclease A (bovine pancreas), cytochrome *c* (horse heart) and myoglobin (horse heart) were delivered by Sigma (St. Louis, MO, USA). Lysozyme (hen egg white) and sodium azide were obtained from Merck (Darmstadt, Germany) while formamide, hydrochloric acid, sodium hydroxide, boric acid, acetic acid and phosphoric acid were purchased from J.T. Baker (Deventer, Netherlands). Water was demineralized and distilled before use.

2.2. Spectrophotometric experiments

The 40% phytic acid solution was diluted with water to 5 m*M*. Subsequently, solutions of different pH were prepared by adding a 5 *M* sodium hydroxide solution. The pH was measured with a Metrohm pH-meter, type 691 (Herisau, Switzerland).

Extinction coefficients were determined by recording the spectra from 200 to 400 nm at a speed of 500 nm/min with a DU-40 spectrophotometer (Beckman, Fullerton, CA, USA) and recorded on a Apple Classic computer (2 points/nm). A home-made data acquisition programme (DV64, Free University, Amsterdam, Netherlands) was applied. The quartz cuvette had an optical length of 10.00 mm and water was used as a blank.

2.3. Buffer capacity

1.21 g of the 40% phytic acid solution was diluted with 75 ml of water. The pH was set at the required value with a 8 *M* sodium hydroxide solution.

Using 0.1 ml aliquots of a 0.83 *M* hydrochloric acid solution, delivered by a Metrohm Dosimat (Model 6650), the pH of the previous solutions was decreased stepwise, and measured after each addition. Similar experiments were performed by adding 0.1 ml aliquots of a 1.17 *M* sodium hydroxide solution. The volume of the added acid or base solution, which effected a pH-change of 0.1 unit was determined.

2.4. Electrophoresis

2.4.1. Buffer preparation

All buffers were prepared daily and filtered over a 0.45 μm filter (Schleicher and Schuell, Dassel, Germany). The three buffer combinations involved were: (i) 15 m*M* phytic acid+150 m*M* pH buffer, (ii) 15 m*M* phytic acid and (iii) 150 m*M* pH buffer.

The pH buffers were prepared with acetic acid (pH 5.5), phosphoric acid (pH 6.5, 7.5), and boric acid (pH 8.5, 9.5, 10.5). The buffers were set at the desired pH by adding solid sodium hydroxide.

2.4.2. Sample preparation

Protein stock solutions of 4 g/l containing 6 m*M* of sodium azide were prepared weekly in water and stored in the dark at −20°C. The actual working solutions (200 μg/ml) were prepared daily by diluting the stock solutions with water.

The electric-osmotic flow (EOF) marker was 0.67% formamide in water.

2.4.3. Electrophoresis system

The CE experiments were performed on a modified Prince system (Lauerlabs, Emmen, Netherlands). The capillary was placed for 70% in a PVC tube of 6 mm I.D. through which temperature controlled air was blown (35 m s^{-1}). The air was thermostated in a copper tube, placed in a water bath, and transported via an isolated PVC tube with 8 mm I.D. The water bath consisted of a cryostat and a temperature controlled heater with a water jet. The system was equipped with a fused-silica capillary of 375 μm O.D. and 75 μm I.D. The total length of the capillary, purchased from LC-Service (Emmen, Netherlands) was 850 mm and the injection to detection length was 660 mm. The capillary was conditioned by flushing subsequently with 0.1 *M* sodium hydroxide (5 min), water (15 min) and finally the buffer under investigation (30 min).

The temperature was set at 20°C. Detection was performed at 210 nm. Between the experiments with different buffers, the capillary was subsequently rinsed with 0.1 *M* sodium hydroxide (5 min), water (10 min) and buffer (15 min). Between runs with the same buffer the capillary was only rinsed with the buffer solution for 2 min. Rinsing of the capillary was always performed with a pressure of 2000 mbar.

2.4.4. Electrophoretic analysis

A plug of the EOF marker was injected by using a pressure of 10 mbar pressure for 0.1 min whereafter electrophoresis was performed at 10 kV for 15 min. Thereafter, the protein sample was injected at 10 mbar for 0.2 min and the electrophoretic process was continued using a voltage of 10 kV. This was done to avoid effects of the EOF marker on the separation of the proteins.

3. Results and discussion

Phytic acid can only be used as a buffer and/or additive in CE in case the concentration does not interfere with the UV absorbance detection of the analyte. For this reason first of all the extinction coefficient will be studied as a function of the pH.

Secondly, the buffer capacity of phytic acid as a function of the pH will be investigated. The data obtained will be compared with the calculated buffer capacity of monoprotic buffers.

Finally, the influence of phytic acid on the separation of proteins will be examined.

3.1. Molar absorptivity of phytic acid

The molar extinction coefficients of phytic acid solutions in the range of pH 2–11 were measured as described in Section 2.4. All molar absorptivities are smaller than 72 dm^3 mol^{-1} cm^{-1} for wavelengths between 200 and 400 nm at a pH of 11. This number is even lower at a pH of 2 (32 dm^3 mol^{-1} cm^{-1}). These values are in agreement with the data given in the literature [2,3] where a value of 10 dm^3 mol^{-1} cm^{-1} is given for non-dissociated phytic acid at a pH of about 1 [6]. The result is that at concentrations of 10 m*M*, which are normally used, phytic acid can be used in CE.

3.2. Buffer capacity

Because phytic acid has as many as twelve acidic groups with pK_a values ranging from 1.9 to 9.5 [6], it is expected that in the pH titration curve the individual pK_a values can not be distinguished. This means that over a wide pH range the buffer capacity (β in mol dm^{-3}), which will be equal to the inverted first derivative of the titration curve, will be almost constant.

To measure β as a function of the pH, a number of 10 m*M* phytic acid solutions were prepared. The pH of these solutions was set by adding solid sodium hydroxide. In the next step an exactly known volume of concentrated hydrochloric acid (or sodium hydroxide) was added until the pH decreased (or increased) with 0.1 of a pH-unit. The measured β values were corrected for differences in the phytic acid concentrations of the individual solutions. For small differences in the buffer concentration, the buffer capacity is linearly related to the phytic acid concentration. The buffer capacity can be written as the number of moles of a strong acid (hydrochloric acid) that have to be added to 1 dm^3 of the buffer to realize a pH change ΔpH [7]:

$$\beta_{H^+} = \frac{C_a}{-\Delta pH} = \frac{\left(\frac{V_{HCl} M_{HCl} 10^{-3}}{V_{tot}}\right)}{-\Delta pH} \quad (1)$$

In Eq. (1) a direct relation between β_{H+} and V_{HCl}, the volume of hydrochloric acid added is given. C_a is the amount of strong acid added per 1 dm^3 buffer solution, M_{HCl} is the molarity of the hydrochloric acid solution and V_{tot} the volume before the addition of hydrochloric acid. The volume increase after the addition of hydrochloric acid can be neglected. An equation similar to Eq. (1) can be derived if sodium hydroxide instead of hydrochloric acid is used to determine the buffer capacity.

It should be noted that Eq. (1) provides the experimental value of β_{H+}. A correction has to be made if the phytic acid concentration (C_{pa}) deviates from 0.010 *M*, which results in the following equation:

$$\beta_{H^+} = \frac{\left(\frac{V_{HCl} M_{HCl} 10^{-3}}{V_{tot}}\right)}{-\Delta pH} * \left(\frac{0.010}{C_{pa}}\right) \quad (2)$$

where

$$C_{pa} = \frac{\frac{m_{pa}}{MW_{pa}} \times f_{pa}}{V_{tot}} \quad (3)$$

In the above equations m_{pa} is the amount of phytic acid (g), MW_{pa} its molecular weight (g/mol) and f_{pa} the weight fraction in the original phytic acid solution. A further simplification can be realized by substituting MW_{pa} by 650.04 g/mol and f_{pa} by 0.40 (see experimental Section 2.3) and by taking ΔpH as −0.1, leading to Eq. (4):

$$\beta_{H^+} = 0.1625 \frac{V_{HCl} M_{HCl}}{m_{pa}} \quad (4)$$

In the same way, the buffer capacity determined by measuring the pH increment after the addition of a strong base (sodium hydroxide) is given by:

$$\beta_{OH^-} = 0.1625 \frac{V_{NaOH} M_{NaOH}}{m_{pa}} \quad (5)$$

To decide whether the determined buffer capacities of phytic acid are sufficient, these values are compared with the buffer capacity of a monoprotic buffer. In order to do this similar calculations should be made. The necessary equations can be found in a number of textbooks [8]. The buffer capacity of a solution containing a weak monoprotic acid (HA) and its conjugated base (A^-), can be described by the concentrations in the solution before and after the addition of an amount of acid to this solution, resulting in a distinct pH decrease (Eq. (6)).

$$\beta_{H^+} = \frac{C_{after} - C_{before}}{-\Delta pH} \quad (6)$$

where

$$C_{after} = \left(10^{-pH} - 10^{-(14-pH)} + \frac{A_{tc}}{\frac{10^{-pK_a}}{10^{-pH}} + 1}\right)_{after}$$

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where the subscripts refer to the situation after and before the addition of acid and A_{tc} is the molar buffer concentration of the weak acid and its conjugated base. Eq. (6) provides the number of moles of hydrochloric acid required for the shift in pH (ΔpH). β_{OH}^- can be described in the same way [8].

In Fig. 2 the β values calculated, taking $-\Delta pH = -0.1$, are given for various monoprotic weak acids with dissociation constants varying from 2 to 10 and using a total buffer concentration of 10 m*M*. As expected, at pK_a values below 3 and over 11 there is no effect of the amount of buffer added anymore.

An important question to be answered is, if the experimentally determined buffer capacities of 10

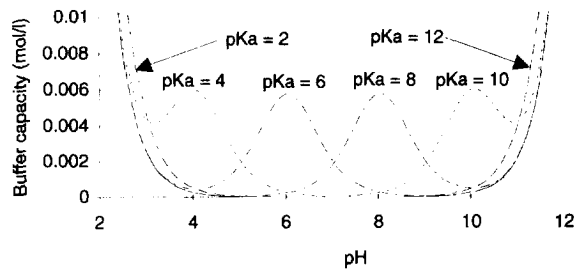

Fig. 2. Calculated buffer capacity of monoprotic buffers with pK_a 2, 4, 6, 8 and 10 as a function of pH.

m*M* phytic acid solutions are higher than the buffer capacities calculated for monoprotic buffers (see Eq. (6)). To answer this question it should be kept in mind that monoprotic buffers can be applied in the pH range from $pK_a - 1$ to $pK_a + 1$. As can be seen in Fig. 2, the maximum buffer capacity can be found at a pH equal to the pK_a of the acid, while the minimum acceptable buffer capacity is found close to $pK_a - 1$ and to $pK_a + 1$. This can be seen in Fig. 2 for a buffer with a pK_a value of 6 where the lowest applicable buffer capacities can be found at pH values of 5 and 7. Substitution of the maximum pH shift with -0.1 and setting $pK_a = pH_{before} + 1$ into Eq. (6) results in:

$$\beta_{H^+} = \frac{C'_{after} - C'_{before}}{0.1} \quad (7)$$

where

$$C'_{after} = \left(10^{-pH} - 10^{-(14-pH)} + \frac{A_{tc}}{\frac{1}{10^{1.1}} + 1} \right)_{after}$$

$$C'_{before} = \left(10^{-pH} - 10^{-(14-pH)} + \frac{A_{tc}}{\frac{1}{10^{1}} + 1} \right)_{before}$$

This equation can be used for a buffer solution with a pH value equal to or higher than 7. For pH values lower than 7 a similar equation can be derived using the addition of a base to the buffer solution.

The minimum acceptable values of β, calculated for monoprotic buffers for pH values between 1 and 13 are depicted in Fig. 3. It can be seen that for pH values below 4 and higher than 10 the buffer capacity increases. This can be explained by the influence of the concentration of the H^+ and OH^- ions, respectively.

Fig. 3 also shows the measured buffer capacity of a 10 m*M* solution of phytic acid. The most important conclusion is that all measured buffer capacities of phytic acid are higher than those of 10 m*M* solutions of monoprotic buffers. Therefore, it can be concluded that phytic acid can be used as a pH buffer.

3.3. Protein separation

It is obvious from Fig. 4, which shows the electropherograms of lysozyme and ribonuclease at a pH of 10.5, that phytic acid indeed influences the peak shape in the CE separation of proteins. Significantly less peak distortion is observed when phytic acid is added to, or used as the buffer which probably is due to a limited adsorption of the proteins onto the wall of the CE capillary.

In addition to these adsorption effects, stacking effects may be important. Both effects do not influence the peak shape of the EOF marker, and although, it is difficult to distinguish between both

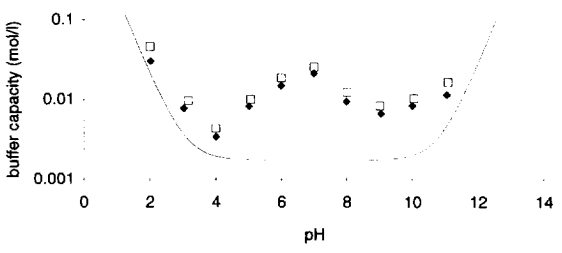

Fig. 3. Measured buffer capacities of a 10 m*M* phytic acid solution versus pH for the addition of strong acid (♦) or strong base (□), compared with the calculated minimum buffer capacity of 10 m*M* monoprotic buffers (solid line).

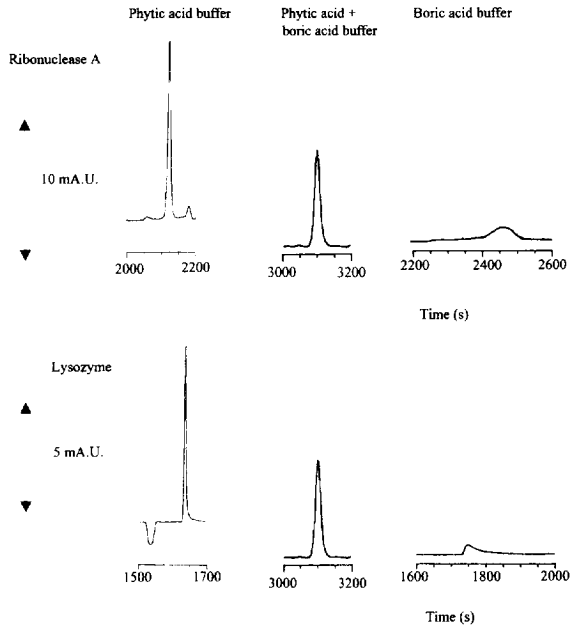

Fig. 4. Electropherograms of lysozyme and ribonuclease at pH 10.5 utilizing different buffer systems, i.e., phytic acid, phytic acid combined with boric acid buffer and boric acid buffer. For CE conditions see Section 2.4.

effects to explain the improvement of the peak shapes, some remarks can be made.

The overall effect on the peak distortion (broadening) for five proteins is shown in Fig. 5 where the number of theoretical plates (N) is plotted as a function of the pH for three buffer systems. These N values are calculated according to [9] from the peak area (A_p), peak height (h_p) and migration time (L) (Eq. (8)). In this way the whole peak profile is taken into account.

$$N = 2\pi\left(\frac{Lh_p}{A_p}\right)^2 \tag{8}$$

In a system without wall adsorption the peak distortion depends on the diffusion coefficient. Therefore, it can be expected that the peak of the proteins are narrower than those of the EOF marker. Because even a weak adsorption of a protein onto the capillary wall already will have a dramatic effect on the peak distortion [1] it can be concluded that for proteins (the open circles in Fig. 5) with N values lower than the EOF marker (the dashed lines in Fig. 5) wall adsorption is significant. When only a pH buffer is used and no phytic acid (Fig. 5A) the situation holds for only about 25% of the proteins studied. Using phytic acid this fraction is increased to 66% (Fig. 5B) and becomes even higher (80%) when a combination of a pH buffer and phytic acid is used (Fig. 5C).

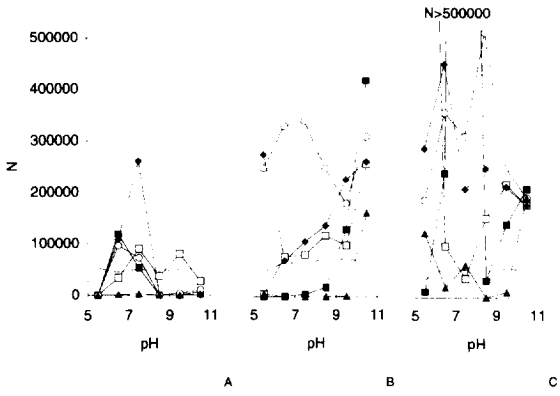

Fig. 5. Number of theoretical plates as a function of the pH for five proteins lysozyme (■), myoglobin (□), α-chymotrypsinogen (♦), ribonuclease (○) and cytochrome c (▲), applying three different buffer systems, i.e., 150 mM monoprotic buffer (A), 15 mM phytic acid (B) and the combination of a 150 mM monoprotic buffer and 15 mM phytic acid (C). The dotted line indicates the number of theoretical plates for the EOF marker. The monoprotic buffers used were acetic acid (pH 5.5), phosphoric acid (pH 6.5, 7.5) and boric acid (pH 8.5, 9.5 and 10.5). For more details see Section 2.3.

Two effects mainly determine the broadening: (i) stacking, which results in peak sharpening, and (ii) wall adsorption resulting in peak broadening. The conductivity of the sample is about the same in all experiments. The conductivities of the buffers, however, vary and are related to the current. Fig. 6 shows that for pH values of 5.5, 6.5, 7.5 and 10.5 the currents observed when only a monoprotic pH buffer is applied, are larger than or equal to those buffers where only phytic acid is present, resulting in more efficient stacking. Thus, if the peak sharpening effect of stacking is larger than the peak dissortion caused by the effect of wall adsorption, a lower efficiency is expected when phytic acid is present. However, this effect is not observed. In fact the N values for the proteins analysed with phytic acid as buffer (Fig. 5B), are higher than those analysed with a monoprotic pH buffer (Fig. 5A). Therefore, probably reduction of wall adsorption by using phytic acid in the buffer is the predominant factor limiting the peak distortion.

To summarize, the combination of a monoprotic buffer and phytic acid leads to the highest N values, probably due to the fact that adsorption of proteins onto the capillary wall is strongly reduced.

In Fig. 7 the influence of the composition of the buffer on the mobility of the proteins is shown. For all proteins (except lysozyme) a decrease in the

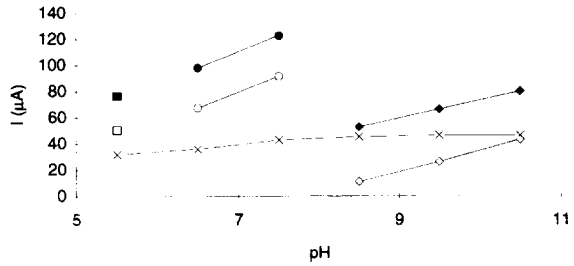

Fig. 6. Measured electrical current as a function of pH application on different buffer systems, i.e., 15 mM phytic acid (X), 150 mM acetate (□), 150 mM acetate + phytic acid (■), 150 mM phosphate (○), 150 mM phosphate + 15 mM phytic acid (●), 150 mM borate (◇) and 150 mM borate + 15 mM phytic acid (♦).

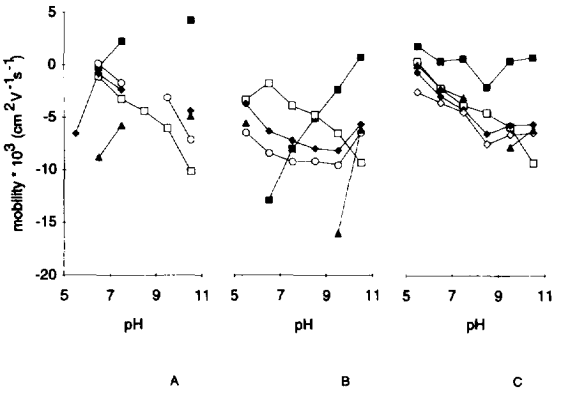

Fig. 7. Mobility of five proteins as a function of the pH, i.e., lysozyme (■), myoglobin (□), α-chymotrypsinogen (♦), ribonuclease (○) and cytochrome *c* (▲), applying three different buffer systems, i.e., 150 m*M* monoprotic buffer (A), 15 m*M* phytic acid (B) and the combination of a 150 m*M* monoprotic buffer and 15 m*M* phytic acid (C). The same monoprotic buffers were used as in Fig. 5. For more details see Section 2.3.

mobility is observed at higher pH values, because of an increased number of negative charges on the proteins (Fig. 7). Some data points are missing in the graph because the proteins could not be determined because of extreme wall adsorption.

Moreover, it can be seen that best resolution is obtained by using only phytic acid. This because the most pronounced differences are found between the various protein mobilities (Fig. 7B).

The deviating behaviour of lysozyme (Fig. 7B) can be probably explained by an ion-pairing effect. As described by Okafo et al. [3] this effect predominantly occurs with basic peptides and proteins where the negatively charged groups of phytic acid can form an ion-pair with the positive charges of the proteins. This feature can be used to improve resolution in protein separation in those cases where the mobilities of the analytes are the same and only monoprotic pH buffers are used, since not all proteins show this ion-pairing effect. This effect might even be increased by adding ion-pairing cations (i.e., zinc) to the buffer, since these cations are able to bind with the negative sites of the protein and of phytic acid will would result in protein-positive ion–phytic acid complexes [10,11].

4. Conclusions

It is shown that phytic acid is a suitable buffer additive for protein analysis using CE. Because of its low UV absorbance, it can be used in concentrations up to 10 m*M*. The most striking feature of this solute is that it provides a high ionic strength combined with a low electric conductivity resulting in a relatively low current and so avoiding extensive peak distortion and other Joule heating effects.

The buffer capacity of a 10 m*M* phytic acid solution is larger than the buffer capacity of monoprotic buffer solutions over a wide pH range (pH between 2 and 11) and therefore, this solute can be used both as a modifier and as a pH buffer additive. For the five proteins examined, applying phytic acid as a buffer additive in CE buffers with pH values of 5.5 and higher, a significant reduction of wall adsorption is achieved. Finally, if a phytic acid solution is also utilized as a pH buffer, it influences the mobility of the proteins in such a way, that separations are possible now, that can not be achieved by using only monoprotic buffers. These results underline the potential of phytic acid to improve CE protein separations.

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