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Capillary electrophoretic separations of proteins using carrier ampholytes

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Abstract

Capillary separations of proteins using carrier ampholytes are performed between an anolyte and a catholyte of same pH (pH 3). Depending upon the concentration of carrier ampholytes used, two different separation processes take place. At a 10% concentration, the high-resolution separation of six model proteins is achieved, which can be described as a transient capillary isoelectric focusing (cIEF) system moving isotachophoretically. The isotachophoretic (ITP) behaviour of the system is evidenced by the influence of the catholyte concentration on the separation. The separation is neither pure cIEF nor pure cITP and the migration order of the proteins results from the influence of both their isolelectric points and their mobilities. © 2002 Published by Elsevier Science B.V.

Keywords: Ampholytes; Isoelectric focusing; Isotachophoresis; Proteins

1. Introduction

Carrier ampholytes are mixtures of a large number of amphoteric species characterised by slightly different isoelectric points [1]. These mixtures were first designed and synthesised for use in isoelectric focusing in gels [2] and were later applied to capillary isoelectric focusing (cIEF) [3,4]. cIEF separations are usually carried out between a strong base and a strong acid forming a ''pH cage''. In a simplified explanation of the cIEF process, a voltage is applied across the capillary and, thereupon, the carrier ampholytes establish a stable linear pH gradient in which the sample analytes are separated according to their isoelectric points (p*I*).

Carrier ampholytes have also been used as spacer ions for isotachophoretic separations. In isotachophoresis (ITP) [5,6], the sample is introduced between a leading and a terminating buffer and, under the influence of an electric field, ionic components arrange in zones in the order of their mobilities. Under steady-state, the components are focused into adjacent square-wave zones and their identification is therefore difficult. In order to overcome this limitation, carrier ampholytes can be used which are focused between the analyte zones and thus act as

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spacers of intermediate mobilities. This last approach has found extensive application using commercially available carrier ampholytes for the separation of proteins [7–13]. The technique has been referred to as ITP with carrier ampholytes, displacement electrophoresis with spacers or isotachophoretic-electric focusing. Most of these applications involved anionic systems (i.e., the proteins are separated as anions) and the migration behaviour of the proteins was explained based on a separation mechanism similar to that of IEF. More recently, Charlionet et al. [14] described a method for the isotachophoretic separation of proteins on a thin gel slab in a polyampholyte background with a field strength gradient. The method was called ''isotachophoretic focusing'' (ITF) and was described based on the existence of a stable field strength gradient with constant pH under the steady-state [14]. However, both pH and conductivity gradients are generated in such a system [15]. Slais derived equations to describe ITF separations in these combined pH and conductivity gradients [15] and the model was experimentally verified for strong and weak electrolytes using commercially available carrier ampholytes mixtures [16,17]. However, the model was not extended to such complex polyelectrolytes as proteins.

In this report, we describe a separation system in which model proteins dissolved with carrier ampholytes are separated between an anolyte and a catholyte buffer both having the same pH (pH 3). The influence of the initial concentration of carrier ampholytes on the separation was examined and the results showed that two different separation processes take place at low and high concentrations. When using 10% carrier ampholytes, the high-resolution separation of six model proteins is achieved. This separation was further characterised as a ''transient cIEF moving isotachophoretically'' and, in particular, the influence of the catholyte/leading buffer concentration on the separation was observed to follow isotachophoretic rules. The migration order of the proteins can be explained based on both their isoelectric points and their mobilities at their respective positions. The system was compared with cIEF with electrophoretic mobilization using mass spectrometry detection and the results show the similarity of the two separation modes.

2. Experimental

2.1. Chemicals

De-ionised water from a Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare all solutions. Acetic acid and formic acid were from Baker (Deventer, The Netherlands) and methanol was from Rathburn (Walkerburn, UK). The model proteins: lysozyme (chicken egg white), α-chymotrypsinogen A (bovine pancreas), myoglobin (horse heart), carbonic anhydrase II (human erythrocytes), β-lactoglobulin A (bovine milk) and β-lactoglobulin B (bovine milk), as well as 2-(*N*-morpholino)ethanesulfonic acid (MES), aspartic acid (Asp) and potassium acetate (KAc) were all purchased from Sigma (St. Louis, MO, USA). For each protein, a stock solution of 1 mg/ml in water was prepared. Carrier ampholytes Pharmalyte 3–10 and Pharmalyte 5–8 were obtained from Pharmacia (Uppsala, Sweden).

2.2. Capillaries and capillary coating

Fused-silica capillaries (75 μm I.D.×375 μm O.D.) were obtained from Polymicro Technologies (Phoenix, AZ, USA). In order to prevent protein adsorption to the capillary walls and to reduce the electroosmotic flow, the capillaries were internally coated. The two-layer siloxanediol–polyacrylamide coating procedure used was as described in the literature [18,19].

2.3. Experimental conditions

Experiments were performed using a programmable Prince apparatus (Prince Technologies, Emmen, The Netherlands) equipped with a high-voltage power supply and allowing hydrodynamic injection. A solution of potassium acetate (KAc) adjusted to pH 3 with formic acid was chosen as the catholyte buffer, and a solution of aspartic acid (Asp) of same concentration and pH served as the anolyte buffer. In a transient cIEF process, the potassium ion K^+ has a higher mobility than any of the sample proteins and plays the role of leading ion, while aspartic acid is a neutral zwitterion at the pH used

and serves as terminating species. The sample solution consisted of six model proteins at a concentration of 50 μg/ml each, dissolved with carrier ampholytes in the anolyte buffer. The separation capillary was 90 cm long. UV detection was carried out at 280 nm at a distance of 26 cm from the capillary outlet, using a commercial UV detector (model Spectra 100, Spectra-Physics, Mount View, CA, USA). The procedure of an experiment was the following: the capillary was first flushed with the catholyte buffer; a plug of the protein–ampholytes mixture, representing 70% of the total length of the capillary, was injected between the catholyte buffer and the anolyte buffer; and a 25-kV constant voltage was then applied across the capillary to start the separation. Preliminary experiments were performed to optimise the sample plug length so as to provide maximal sample loadability without loss of separation efficiency.

2.4. Mass spectrometry measurements

All mass spectrometric measurements were performed using a LCQ-DECA XP Ion trap mass spectrometer (Thermo-Finnigan, San Jose, USA) equipped with a custom-made electrospray ionization source in a coaxial sheath-flow configuration. The sheath liquid consisted of 20 m*M* formic acid in methanol–water (50:50) and was delivered at a flow-rate of 2 μl/min. The electrospray voltage was 5 kV and the voltage applied at the capillary inlet was increased to 30 kV so that the effective separation voltage across the capillary was 25 kV. As previously, on-line UV detection at 280 nm took place at 26 cm from the capillary outlet.

3. Results and discussion

3.1. Amount of carrier ampholytes

The influence of the carrier ampholytes concentration on the separation of six model proteins was studied. Fig. 1 shows the different electrophorograms obtained for concentrations of carrier ampholytes from Pharmalyte 3–10 varying from 0 to 10%. In the absence of carrier ampholytes (Fig. 1a), the model proteins are detected as contiguous square-wave zones with plateau concentration profiles, which are typical to conventional cITP separations. A small amount of carrier ampholytes added to the sample produces zones with a Gaussian distribution with the carrier ampholytes acting as spacers (Fig. 1b). However, the six proteins are well-resolved only when a concentration of 10% carrier ampholytes is used (Fig. 1e). The current was monitored during the experiments and two distinct profiles were observed, as seen in Fig. 2. For 0, 1 and 2% carrier ampholytes, the current increases steadily over the analysis time, whilst for a higher concentration of 10%, the current first decreases then increases to a higher value than the starting current and further decreases again exponentially. The profile observed for 5% carrier ampholytes is intermediate. These two different profiles indicate that two different separation mechanisms take place for low and high concentrations of carrier ampholytes. It is important to realise that the capillary is almost entirely filled with the protein–ampholytes solution at the beginning of an experiment. This solution thus constitutes the initial ''background electrolyte'' and most probably has an influence on the separation process. In particular, the pH of the protein–ampholytes solution greatly influences the electrical charge state of ampholyte species, such as proteins, whose net charge at any given pH depends on their isoelectric point. The pH of Pharmalyte 3–10 solutions of different concentrations in 10 m*M* Asp was measured and reported in Table 1. It can be assumed that the sample proteins have little influence on the pH and that the pH measured thus corresponds to the pH of the sample solution used for the experiments in Figs. 1 and 2. At the opposite, the Pharmalyte 3–10 initial solution is very highly concentrated and does influence the pH very strongly, causing an increase from pH 3 at 0% to pH 6.9 at 10%. The six model proteins separated have p*I* values ranging from 5.15 to 11 (see Table 2) while the carrier ampholytes cover the p*I* range 3–10. Hence, depending on the pH of the protein–ampholytes mixture, the ampholyte species (both carrier ampholytes and proteins) initially possess a positive, zero, or negative net charge. At pH 3 (0% Pharmalyte 3–10), all ampholytes have a net positive charge but above pH

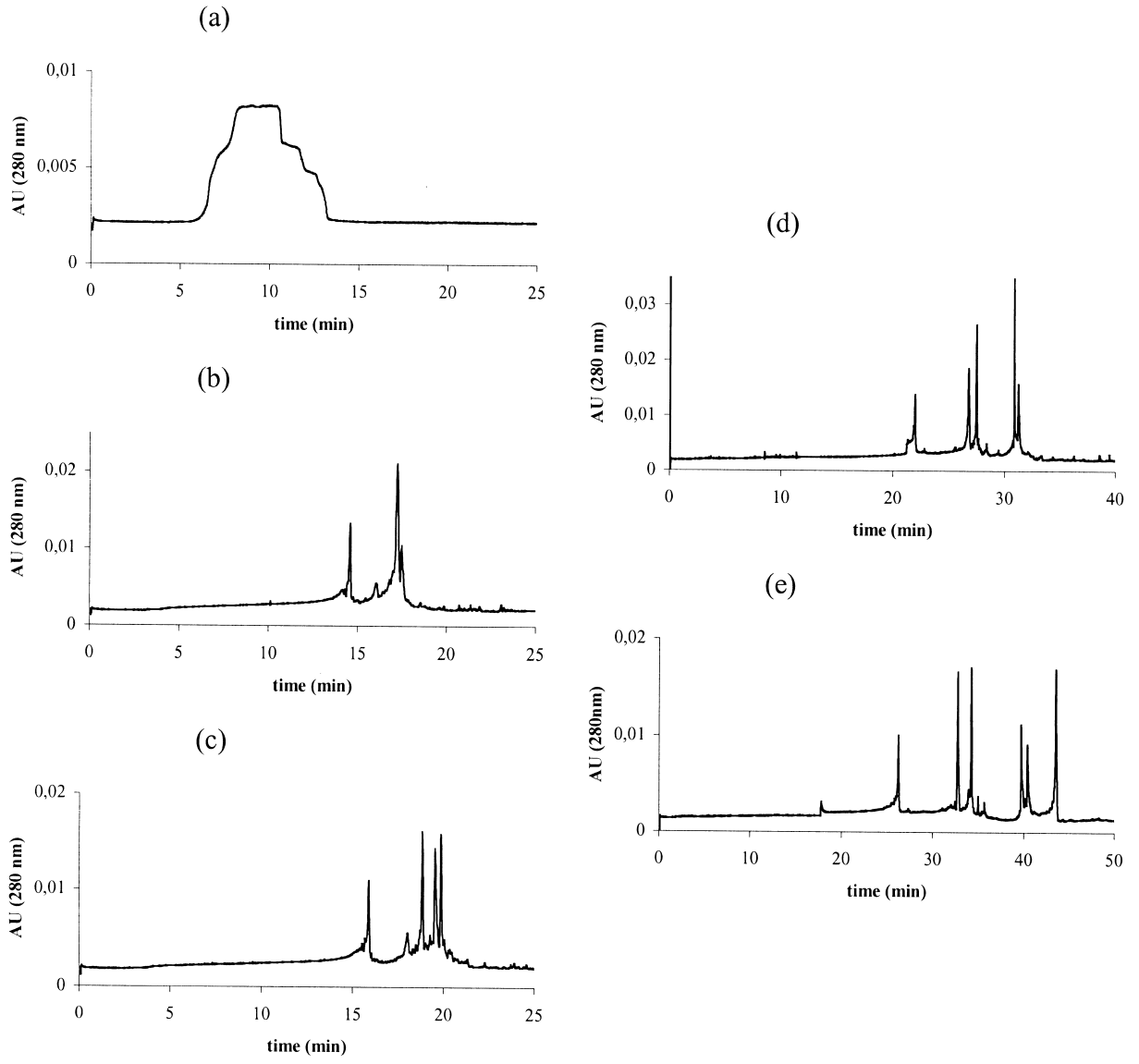

Fig. 1. Separations of six model proteins using varying concentrations of carrier ampholytes from Pharmalyte 3–10 in the sample: (a) 0%, (b) 1%, (c) 2%, (d) 5%, (e) 10%. Catholyte buffer, 10 m*M* KAc adjusted to pH 3 with formic acid; anolyte buffer, 10 m*M* aspartic acid pH 3.

4.1 (1% Pharmalyte 3–10), an increasing proportion of the ampholytes will have a negative net charge. Let us now consider the consequence of this on the separation process. From 0 to 2% Pharmalyte 3–10 (pH 3–5), the sample proteins are all positively charged since their p*I* values lie above 5. When the voltage is applied across the capillary, the proteins and other positively charged carrier ampholytes all migrate electrophoretically in the same direction (towards the cathode) according to their respective mobilities. The potassium ion, K^+, contained in the catholyte has a higher mobility than any of the ampholyte species in the sample and thus plays the role of leading ion. The separation can be seen as a

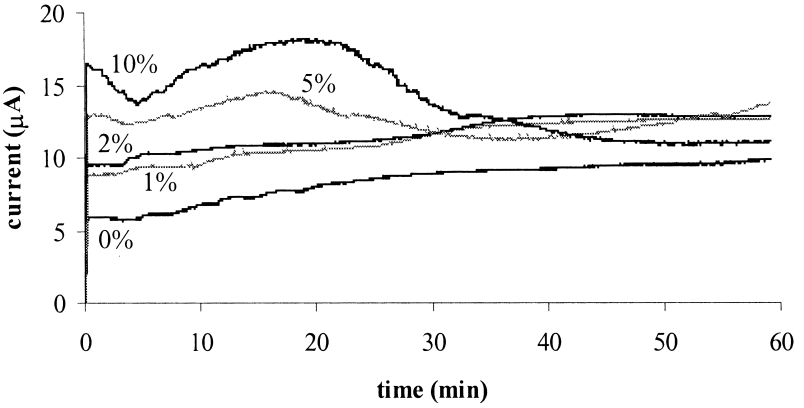

Fig. 2. Current profiles observed for varying concentrations of Pharmalyte 3–10. Experimental conditions are the same as in Fig. 1.

Table 1
pH of Pharmalyte 3–10 solutions in 10 m*M* aspartic acid

% Pharmalyte 3–10	pH
0	3.0
1	4.1
2	5.0
5	6.2
10	6.9

pseudo-ITP system in which proteins are separated according to their mobilities and carrier ampholytes serve as spacer ions of intermediate mobilities. The current increase observed over the experiment can be attributed to the migration of the acetate and formate counter-ions present in the catholyte towards the anode and the resulting migration of H^+ ions towards the cathode, owing to electroneutrality. When the concentration of carrier ampholytes reaches 10%, the pH in the initial protein–ampholytes mixture is 6.9 and there is a big proportion of ampholyte species possessing a negative net charge, in particular three of the model proteins are negatively charged. The current profile shows a distinctive drop during the first 5 min of the experiment, which suggests that a transient cIEF takes place (this current decrease suggests that the total amount of charges decreases). Presumably, the high concentration of carrier ampholytes leads to the establishment of a pH gradient within the sample plug. However, the current does not drop to the low value usually observed in conventional cIEF, which indicates that the ampholytes do not reach their isoelectric points. The progressive migration of acetate and formate counter-ions towards the anode causes increasingly more H^+ ions to enter the capillary and migrate towards the cathode, resulting in a decrease in pH within the capillary and an increase in current.

Table 2
Properties of the model proteins and carrier ampholytes

Ampholyte species	μ_{ep} ($\times 10^{-8}$ m^2 V^{-1} s^{-1})[a]	p*I*	M_r[b]	$\lvert[dz/d(pH)]_{pI}\rvert$
Lysozyme	3.22	11	14 313	4.73
Chymotryspsinogen A	2.56	8.7	25 666	6.71
Myoglobin	2.70	7.2	16 951	0.38
Carbonic anhydrase II	2.68	5.9	29 115	2.28
β-Lactoglobulin B	2.93	5.3	18 281	11.86
β-Lactoglobulin A	2.92	5.15	18 367	13.52
Pharmalyte 3–10	1.42–6.62	3–10	<1000	

[a] Electrophoretic mobility measured in 10 m*M* KAc–formic acid, pH 3.
[b] Molecular mass obtained from Ref. [21].

In contrast with cIEF, the proteins are not focused at their isoelectric points but at a pH below their p*I*, at which they possess a positive net charge that causes them to migrate electrophoretically under the influence of the applied electric field. The carrier ampholytes and proteins form a stack that is dragged isotachophoretically towards the cathode by the K^+ leading ion. As the ampholyte zones progressively exit the capillary, the current decreases. Thus the separation taking place at a 10% concentration of carrier ampholytes can be characterised as a transient cIEF moving isotachophoretically.

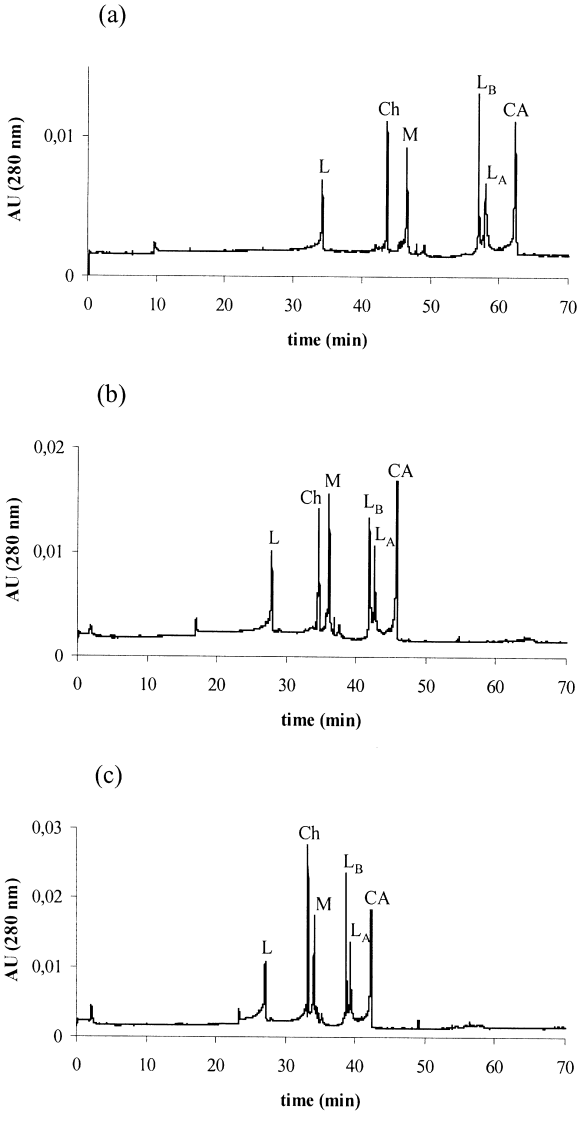

Fig. 3. Electrophorograms obtained at different concentrations of the catholyte buffer: (a) 5 m*M*, (b) 10 m*M*, (c) 15 m*M*. Catholyte buffer, KAc adjusted to pH 3 with formic acid; anolyte buffer, 10 m*M* aspartic acid, pH 3. The sample consists of six model proteins dissolved in the anolyte buffer with 10% Pharmalyte 3–10. L, lysozyme; Ch, chymotrypsinogen A; M, myoglobin; L_B, β-lactoglobulin B; L_A, β-lactoglobulin A; CA, carbonic anhydrase II.

3.2. Influence of the catholyte buffer concentration

The isotachophoretic behaviour of the separation was examined by studying the influence of the concentration of the catholyte/leading buffer. Experiments were performed using 10% carrier ampholytes in the protein–ampholytes mixture and varying concentrations of the catholyte buffer. As shown in Fig. 3, an increasing concentration of the leading buffer results in a decrease of resolution when the same amount of carrier ampholytes is used. This observation is in agreement with the isotachophoretic rules [20]. According to the Kohlrausch regulating function, the concentration of the ampholyte zones is determined by that of the leading electrolyte. Thus, if the concentration of the leading electrolyte is increased while the amount of carrier ampholytes is kept unchanged, the length of each ampholyte zone is adjusted to maintain the concentration fixed by the leading electrolyte. A higher concentration of the leading buffer results in shorter ampholyte zones and thereby in a compression of the whole stack. Hence, the space between two separated proteins decreases, i.e., the resolution decreases. This also applies to the protein zones and sharper peaks are indeed observed in Fig. 3 for higher leading buffer concentration. It is also interesting to note that the decrease in resolution is not even along the whole stack. For instance, the resolution decrease is more accentuated between myoglobin (M) and β-lactoglobulin B (L_B), which indicates that a higher number of carrier ampholytes is present in this area.

3.3. Migration order of the proteins

The proteins migrate in the following sequence: lysozyme>α-chymotrypsinogen A>myoglobin>β-lactoglobulin B>β-lactoglobulin A>carbonic anhydrase II (L>Ch>M>L_B>L_A>CA). In cIEF, the position of a protein is determined by its p*I* but this

is not the case here since the proteins migrate electrophoretically and are focused out of their isoelectric points. It can be assumed that during the first ''transient cIEF'' phase of the separation (corresponding to the decrease in current observed in Fig. 2), the proteins get ordered according to their p*I* in a pH gradient. This gradient is then disrupted due to the decrease in pH resulting from the progressive migration of the counter-ions from the catholyte buffer within the capillary. Each ampholyte zone in the system thus has a pH different from its p*I*, which is determined by the counter-ions. Assuming that a pH gradient is maintained, each protein experiences a different pH and migrates according to its electrophoretic mobility at the pH of its position in the gradient. The net result is that the proteins get rearranged according to their net mobilities and the separation becomes isotachophoretic.

The protein mobilities were measured in the catholyte buffer with constant pH 3 and are reported in Table 2. The values obtained show that the proteins are not ordered by their decreasing mobilities at pH 3, which indicates that a pH gradient is still present. If the pH gradient had vanished completely, the pH would be uniform across the whole capillary and presumably equal to the pH of the end-buffers (pH 3), and therefore the proteins would be expected to migrate in the order of their decreasing mobilities at pH 3. Most probably, the ITP ''steady-state'' is not reached before the proteins leave the separation capillary. The order of migration of the first three proteins (L>Ch>M) corresponds indeed to their decreasing p*I* values. However, for the most acidic proteins which spend more time in the separation capillary, an inversion in migration order occurs since carbonic anhydrase II with a p*I* of 5.9 comes out after the two β-lactoglobulins B and A with p*I* values 5.3 and 5.15, respectively.

Due to the ITP migration of the gradient, the ampholytes are focused out of their isoelectric points and the pH at the position of a protein is different from its p*I*. The magnitude of the deviation from the p*I* was not measured but it can be assumed that it is not large and in that case, knowledge of the mobility–pH curve around the p*I* $\{[d\mu/d(pH)]_{p_I}\}$ should be useful to interpret the results. Unfortunately, this information is not available for proteins. Nevertheless, we have obtained the theoretical titration curves of the proteins from the internet [21] and, based on these curves, we have estimated the variation of charge with pH around the p*I*, $|[dz/d(pH)]_{p_I}|$, for the six proteins (see Table 2). This value gives an indication of how many charges a protein can acquire at a pH close to its p*I* value. A high value corresponds to the acquisition of a high amount of charges. The electrophoretic mobility of an ion is related to its charge to mass ratio so that for two proteins with similar molecular mass, the one with the highest value of $|[dz/d(pH)]_{p_I}|$ has the highest mobility close to its p*I*. From Table 2, it can be seen that the two β-lactoglobulins (A and B) possess higher $|[dz/d(pH)]_{p_I}|$ values than carbonic anhydrase II and since their molecular masses are also lower than that of carbonic anhydrase, they should have higher mobilities. This explains the fact that they migrate before carbonic anhydrase II.

3.4. Mass spectrometry measurements

The separation system was coupled to an ion trap mass spectrometer using a sheath liquid flow to establish the electrical contact needed at the outlet of the capillary. Using the same conditions as in Fig. 3b, the influence of the interface on the separation was first examined. Fig. 4a shows the UV trace obtained and Fig. 4b the corresponding mass electrophorogram. Obviously, the sheath liquid flow results in band broadening and loss of resolution. However, the separation pattern is maintained and, in particular, the migration order of the protein analytes is unmodified. Mass spectrometry has the advantage to allow for unambiguous identification of the separated components and was used to examine the similarity of the ''transient cIEF'' system with cIEF using electrophoretic mobilization. For this purpose, a sample consisting of three proteins only (myoglobin, carbonic anhydrase II and β-lactoglobulin B) was used and Pharmalyte 3–10 was replaced by Pharmalyte 5–8.

Transient cIEF was applied to the three proteins dissolved in 10 m*M* Asp with 10% carrier ampholytes from Pharmalyte 5–8, and resulted in the mass electrophorogram shown in Fig. 5a. The migration order of the proteins observed is the same as previously. cIEF separations were carried out on a

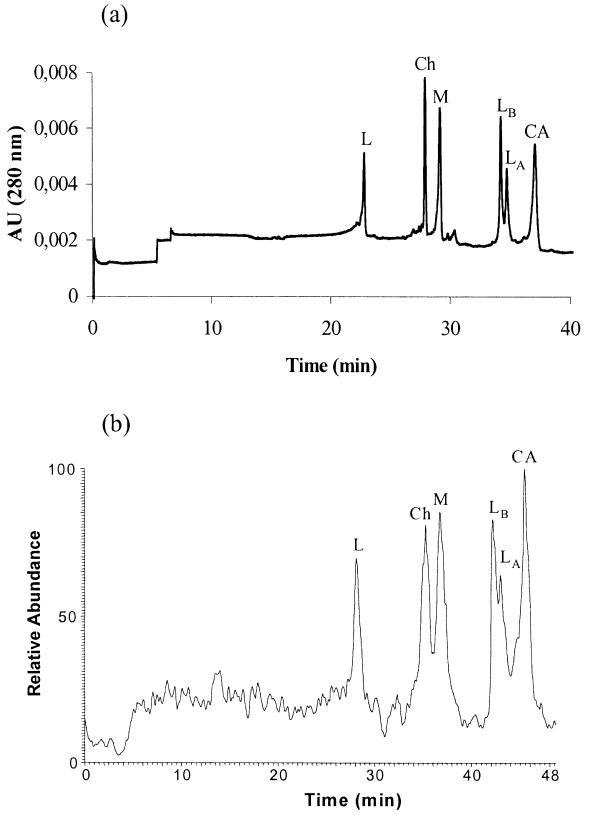

Fig. 4. Separation conditions are the same as in Fig. 3b. (a) UV trace at 280 nm observed at 26 cm from the capillary outlet and (b) mass electrophorogram obtained by electrospray ion-trap mass spectrometry. L, lysozyme; Ch, chymotrypsinogen A; M, myoglobin; L_B, β-lactoglobulin B; L_A, β-lactoglobulin A; CA, carbonic anhydrase II.

sample containing the same three proteins dissolved in water with 10% Pharmalyte 5–8, using a 50 m*M* MES solution with pH 8.5 as catholyte, and a 100 m*M* acetic acid solution with pH 2.9 as anolyte. In addition to providing electrical connection, the sheath liquid contains formate ions that can migrate within the capillary and induce electrophoretic mobilization of the pH gradient. The simultaneous application of a pressure at the inlet of the capillary permits to control the extent of migration of the formate ions within the capillary and thus to examine the influence of these counter-ions on the migration order of the proteins. cIEF separations were performed using 0-, 2- and 5-mbar pressure mobilization in combination with electrophoretic mobilization and the resulting mass electrophorograms are shown in Fig. 5b–d. In order to avoid fouling of the source by the MES catholyte solution, the electrospray was started only 10 min after the first peak (M) appeared in the UV trace and, thus, the time scale in Fig. 5b–d is not significant. Except from the different sample solutions and anolyte and catholyte solutions, the experimental conditions (sample plug length, separation voltage, ESI voltage, sheath-liquid flow and composition) used in Fig. 5a–d were identical. Fig. 5b–d shows that the migration order of carbonic anhydrase II and β-lactoglobulin B is reversed when no pressure is used as compared with a 5-mbar pressure mobilization. With a 5-mbar mobilization pressure (Fig. 5d), carbonic anhydrase II (CA) comes out before β-lactoglobulin B (L_B) and the two proteins are base-line resolved. In that case the migration of formate ions within the capillary is minimal and mobilization is mainly due to the pressure applied. The pattern observed thus corresponds to decreasing isoelectric points of the proteins. With a 2-mbar mobilization pressure (Fig. 5c), the two proteins CA and L_B are no longer separated and co-migrate in one peak. This indicates that electrophoretic mobilization by the formate ions is more effective, causing the proteins to acquire a charge and migrate according to their electrophoretic mobilities. Because L_B has a higher mobility than CA, it catches up with it. When no pressure is applied (Fig. 5b), mobilization is purely electrophoretic and the analysis time is consequently longer. Hence, the proteins spend more time migrating at their respective mobilities. The net result is that β-lactoglobulin B overtakes carbonic anhydrase II and comes out first. The separation pattern obtained in Fig. 5b, by cIEF with no mobilization pressure (i.e., electrophoretic mobilization by the formate ions only) is similar to the separation pattern obtained by ''transient cIEF'' in Fig. 5a, although in this last system both acetate and formate counter-ions are present. Because the formate ion has both a higher mobility and a lower pK_a (pK_a=3.75) than the acetate ion (pK_a=4.75) it has undoubtedly the greatest influence on the pH change in the capillary and thus on the separation. It should be noted that the current in the experiment of Fig. 5b follows the same profile as the one of Fig. 5a except that it first decreases to a very low value, as usual in cIEF.

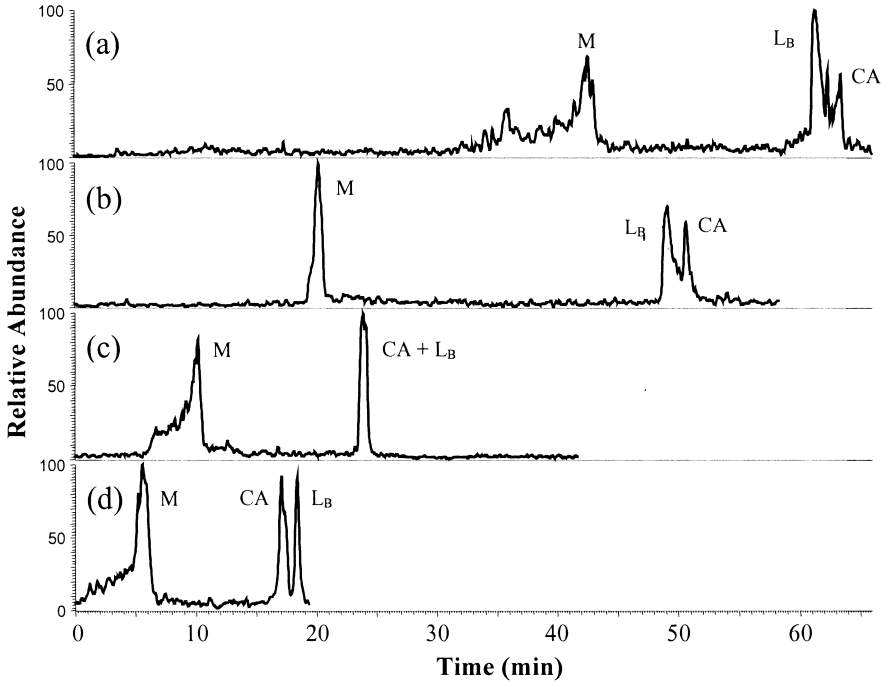

Fig. 5. Mass electrophorograms obtained by electrospray ion-trap mass spectrometry. (a) Three proteins at 50 μg/ml each dissolved in anolyte with 10% Pharmalyte 5–8; catholyte, 10 m*M* KAc/formic acid, pH 3; anolyte, 10 m*M* aspartic acid, pH 3. (b–d) CIEF of three proteins dissolved in water with 10% Pharmalyte 5–8; catholyte, 50 m*M* MES, pH 8.5; anolyte, 100 m*M* acetic acid, pH 2.9, using varying mobilization pressures: (b) 0 mbar, (c) 2 mbar, (d) 5 mbar. M, myoglobin; L_B, β-lactoglobulin B; CA, carbonic anhydrase II.

4. Conclusions

In this work, it was shown that when the separation of proteins using carrier ampholytes is performed between an anolyte and a catholyte both with pH 3 (which thus do not form a ''pH cage''), two separation processes can take place depending upon the initial concentration of carrier ampholytes in the sample. At a high concentration of 10%, the high-resolution separation of six model proteins is achieved. In that case, the separation process can be described as ''transient cIEF moving isotachophoretically'', in which the catholyte buffer is the leading electrolyte. Such a system follows ITP rules as evidenced by the influence of the catholyte/leading buffer concentration on the separation, and resolution can be increased by decreasing the leading buffer concentration. The separation is neither pure cIEF nor pure cITP but rather intermediate. The migration order of the proteins is the result of the influence of both their p*I* values and their mobilities at their respective positions. Accordingly, the technique showed similarities with cIEF using electrophoretic mobilization. A clear disadvantage of the technique is that the migration order of the protein analytes cannot be predicted and information such as p*I* value is lost. However, the system offers a certain flexibility and might permit to separate proteins with very similar p*I* values that are not separated by cIEF. In particular, the selectivity of the separation can be adjusted by the choice of the catholyte counter-ion. In addition, the technique obviates the need for mobilization of the proteins as in cIEF. Another advantage is that the risk of precipitation of the proteins should be minimised in such a system, as compared with cIEF, since the proteins are charged.

In future research, further experiments will be performed using mass spectrometry detection in order to study differences of the system with cIEF with respect to, e.g., detection limits and ionisation efficiencies. The study of the respective distributions of the carrier ampholytes in both systems would also

provide more insight into the parameters governing this distribution and thereby permit a better understanding of the separation process.

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