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Free-solution electrophoresis of proteins in an improved density gradient column and by capillary electrophoresis

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Abstract

The electrophoretic mobilities of bovine serum albumin, β-lactoglobulin A and B, α-lactalbumin and myoglobin were measured in free solution using an improved version of the Boltz-Todd vertical density-gradient electrophoresis column. Dialysis membranes were used for the isolation of the side-arm electrodes from the column and large-volume electrode containers were connected to each other by a circulating buffer loop. The improvements increased reliability, facilitated removal of electrode gas, prevented proteins from contacting electrodes and allowed the use of low conductivity buffers without ion depletion. A low conductivity buffer (Tris–glycine) allows the use of high fields for rapid separations. The apparatus is modular and allows easy modification of column dimensions. We have also measured the electrophoretic mobility of these proteins in a coated capillary in the absence of significant electroosmotic flow.

1. Introduction

The electrophoretic separation of proteins in free solution is important for several reasons. An obvious reason is to use differences in free-solution electrophoretic mobility as a preparative method for the isolation of proteins. A second reason is to measure electrophoretic mobilities of proteins for use in mathematical modeling of electrophoretic and electrokinetic separation (electrochromatography) processes to gain a better understanding of the separation methods. Predictive electrophoretic models are necessary for the efficient design and operation of industrial separation processes.

The use of density gradients to stabilize electrophoretic separations has a long history [1–3]. Various configurations have been used to separate cells, organelles and proteins. Density gradient electrophoretic separations are simple in principle, however, in operation they can be cumbersome and unreliable. We have adapted a vertical column previously used for cell separations [4,5] for use in protein separations. This column is modular, flexible in dimensions, improved in reliability and ease of operation, simple to construct, and of low cost.

Capillary zone electrophoreis (CZE) is known

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for its high resolution and rapid separations. The use of open tube capillaries with coated walls allows the measurement of free-solution mobilities with low electroosmotic flow, although interactions and adsorption of the proteins with the wall coating is possible [6,7]. We have measured the mobilities of the model proteins using CZE to confirm the mobilities measured by density gradient electrophoresis.

2. Experimental

2.1. Solutions and buffer

The buffer used in this study was made up as a concentrated stock of 39 m*M* tris(hydroxymethyl)aminomethane (Tris) and 470 m*M* glycine (10 × buffer) with the properties shown in Table 1. Sucrose solutions were made by weighing the desired sucrose and adding one-tenth the final volume of 10 × buffer and diluting to the final volume.

2.2. Viscosity and conductivity measurements

Fractions were collected from the density gradient and the viscosities were measured using a cone and plate viscometer (Brookfield Model DV-II, Stoughton, MA, USA) at 25°C. The conductivities of the fractions were measured using a dip probe and conductivity meter (YSI Model 35, Yellow Springs, OH, USA) with the samples at 25°C.

Table 1
Physical properties of buffers at 25°C

Solution	Conductivity (μS/cm)	pH	Viscosity (P·s)
1 × Buffer	102	8.3	0.00090
1 × Buffer, 1% Sucrose	96	8.3	0.00094
1 × Buffer, 35% Sucrose	40	8.3	0.00300
1 × Buffer, 40% Sucrose	34	8.3	0.00370
10 × Buffer	875	8.3	0.00090

Composition of 1 × buffer is 3.9 m*M* Tris and 47 m*M* glycine. Values of average of two or more measurements.

2.3. Density gradient electrophoresis

The glass water-jacketed columns had inside and outside diameters of 22 and 25 mm, respectively. Dialysis membranes with a molecular mass cutoff of 6000 to 8000 (Spectrapor 1, Spectrum Medical Industries, Los Angeles, CA, USA) were bonded to circular acrylic adapters (Fig. 1) using cyanoacrylate ester glue. These adapters were connected to side-arm reservoirs that contained a coil of platinum wire that was 17 cm from the axis of the column. The short column was 21.5 cm long (total current path length of 55.5 cm) and the long column was 38 cm long (total current path length of 72 cm). A linear gradient from 1 to 35% (w/v) sucrose containing 1 × buffer was formed by the use of a two-chambered acrylic gradient former (Hoefer Scientific Instruments, San Francisco, CA, USA). Solutions were pumped in and out of the column using a peristaltic pump (Minipuls 3,

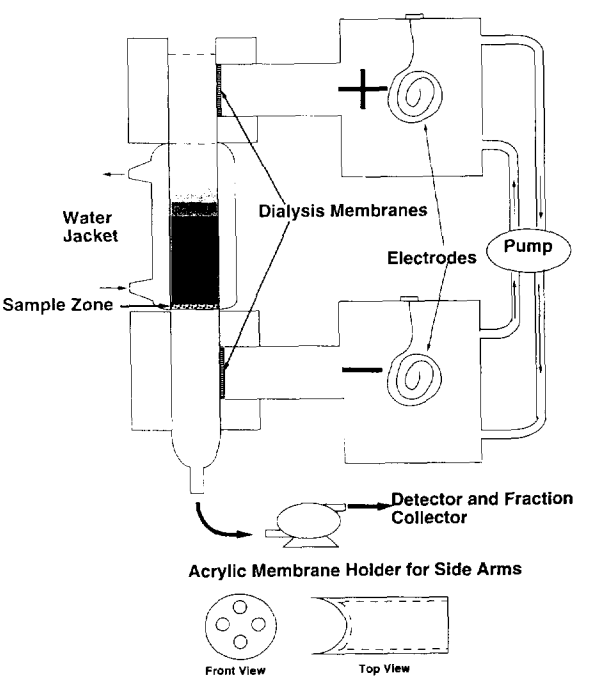

Fig. 1. Diagram of density gradient column and acrylic membrane adaptor used, showing polycarbonate blocks, modular column, electrode buffers and circulation pump, and acrylic membrane holders.

Gilson Medical Electronics, Middleton, WI, USA). A total volume of 50 ml (pumped in at 2 ml/min) and 100 ml (pumped in at 4 ml/min) was used for the short and long columns, respectively. The resulting gradients were 13.1 and 26.3 cm long, respectively. The 1 ml sample (made up in 1 × buffer, bromophenol blue 0.01 mg/ml and 37% sucrose) was pumped in at 0.4 ml/min followed by a solution of 40% (w/v) sucrose containing 1 × buffer pumped in at 4 ml/min until the blue sample zone was at the bottom of the water-jacked region of the column, which was held at 25 ± 0.2°C. The electrode containers each contained approximately 1.8 l of 1 × buffer. At the end of the electrophoresis run the field was switched off, the pump flow was reversed, and the column contents were pumped out at either 4.0 ml/min (long column) or 2.0 ml/min (short column). The column effluent was monitored at 280 nm (Holochrome Detector equipped with 0.05 ml flow cell, Gilson Medical Electronics, Middleton, WI, USA) and went to a fraction collector.

2.4. Capillary zone electrophoresis

Mobilities were measured using a BioFocus 3000 CZE instrument (BioRad, Richmond, CA, USA) with a polyacrylamide coated capillary, 24 cm × 25 μm (Biofocus No. F148-3031, BioRad). The capillary was 19.5 cm long from the negative electrode to the detector. The column was maintained at 25°C, and 10 × buffer was used. Samples were dissolved in 1 × buffer at a concentration of 0.1 mg/ml and injected electrophoretically at −10 000 V for 5 s. Detection was at 214 nm. Electroosmotic flow velocity was measured by adding 0.02 ml of mesityl oxide to 0.75 ml of positive electrode buffer and measuring the time required for the neutral marker front to reach the detector (4.5 cm from electrode).

2.5. Gel electrophoresis

A Tris–glycine polyacrylamide gel electrophoresis system [8] was used with modifications to confirm the separation of the proteins on the column. The electrode buffer was 5 m*M* Tris and 38.7 m*M* glycine, pH 8.3. Polyacrylamide slab gels (9.4% T, 2.6% C) of 1.5 mm thickness containing 0.38 *M* Tris pH 8.9 were run for 800 V h. Samples were loaded in electrode buffer containing 7.5% sucrose and a trace of bromophenol blue. Gels were stained colloidal Coomassie brilliant blue [9,10].

2.6. Reversed-phase high-performance liquid chromatography

Protein samples were analyzed by reversed-phase high performance liquid chromatography (RP-HPLC) using a C_{18} protein and peptide column (250 × 4.6 mm I.D., No. 218TP1104, Vydac, Hesperia, CA, USA) using a mobile phase composed of a gradient of 0.1% trifluoroacetic acid (pump A) and acetonitrile (pump B). The flow-rate was 1.0 ml/min at ambient temperature. A linear gradient was run to 40% B in 2 min after injection from initial conditions of 20% B. A second gradient was immediately run to 50% B in 25 min. Detection was at 280 nm.

2.7. Size exclusion HPLC

Size exclusion chromatography for the separation of bovine serum albumin monomer and dimer was performed using a BioSep SEC-S3000 column (300 × 7.8 mm I.D.; Phenomenex, Torrance, CA, USA). The mobile phase was 0.1 *M* sodium phosphate pH 7.15 with a flow-rate of 1.0 ml/min. Detection was at 280 nm.

2.8. Materials

Myoglobin (horse heart), bovine serum albumin (fraction V), α-lactalbumin (bovine milk), β-lactoglobulin A and B (bovine milk), glycine and Tris were obtained from Sigma. Blue Dextran 2000 was obtained from Pharmacia (Uppsala, Sweden). The properties of the proteins are shown in Table 2.

Table 2
Properties of the model proteins used

Protein	Isoelectric point	Molecular mass	Ref.
Bovine serum albumin	4.9, 4.7 (25°C)	68 000	14,15
β-Lactoglobulin A	5.2 (15°C)	36 700 (dimer)	16,17
β-Lactoglobulin B	5.3 (15°C)	36 700 (dimer)	16,17
α-Lactalbumin	4.8 (by precipitation)	14 400	15,18
Myoglobin	7.4 (15°C)	17 500	16

3. Results and discussion

3.1. Density gradient electrophoresis experiments

The configuration of the columns is shown in Fig. 1. The short and long columns had gradient volumes of 50 and 100 ml with the same final and initial sucrose concentrations in the gradient. The slope of the resulting density gradients were therefore one half as large in the long column as in the short column. The conductivity and viscosity gradients of the long column are shown in Fig. 2 and Fig. 3, respectively. After a run the top region in the column showed increased conductivity and the bottom region in the column (towards the negative electrode) had a decreased conductivity. These are the regions in the column between the gradient and the membrane that are isolated from the buffer containers by the membranes. This indicates that the composition of the buffer in these regions has changed during the run. The gradient region of the column did not significantly change during the run (Fig. 2). The use of the external circulating loops did not influence the current or voltage, owing to the small diameter of the tubing used (1.6 mm I.D.) and the squeezing of the tubing by the peristaltic pump used for recirculation, so that the current through this path was insignificant. The runs were 2 h in average duration and during this period the buffer pH and conductivity in the large reservoirs did not significantly change with or without the recirculation feature probably due to the large volumes used. The columns were run at constant voltage and the current did not significantly change during the run. Typical currents with the short column at 1000 V was 4.0 mA and with the long column at 2000 V was 6.4 mA. Fig. 4 shows the results of a separation of a mixture of proteins on the short column (13.1 cm gradient). The same mixture of proteins was separated on the long column (26.3 cm) with very similar results (Fig. 5). We were able to increase the physical distance between the separated protein zones by the use of the longer column. Bovine serum

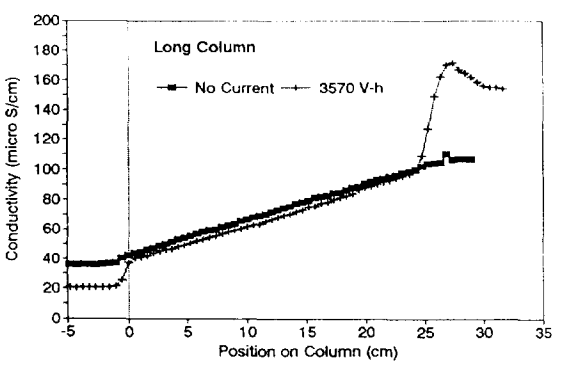

Fig. 2. Conductivity gradient present in long column before and after an electrophoresis run.

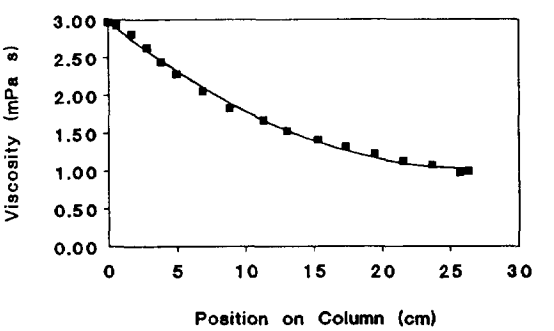

Fig. 3. Viscosity measurements (closed squares) of fraction of the long column sucrose density gradient. The solid line is the fit of the quadratic equation used (Eq. 4).

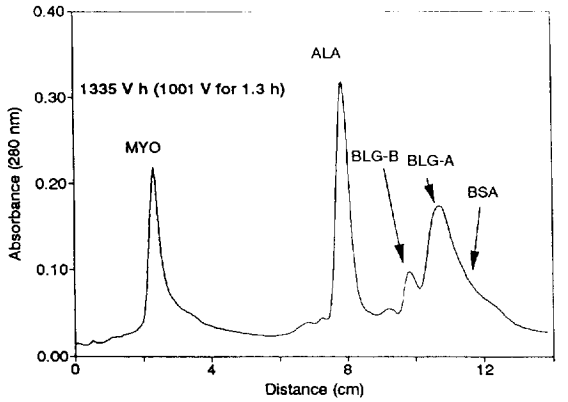

Fig. 4. Separation of the protein mixture (Table 2) on the short column. The identity of the proteins are myoglobin (MYO), α-lactalbumin (ALA), β-lactoglobulin A (BLG-A), β-lactoglobulin B (BLG-B), and bovine serum albumin (BSA).

albumin appeared as a broad peak and overlapped with β-lactoglobulin A. When the column fractions were analyzed by electrophoresis on the native gel system [8] the leading front of the BSA peak (highest mobility) was enriched in the dimer form. The BSA preparation used in this study had monomer, dimer, and higher mass components of 77.4 ± 2.1%, 16.6 ± 1.1%, and 6.1 ± 1.0%, respectively (three determinations) as measured by size exclusion chromatography.

The effect of increased protein loading on

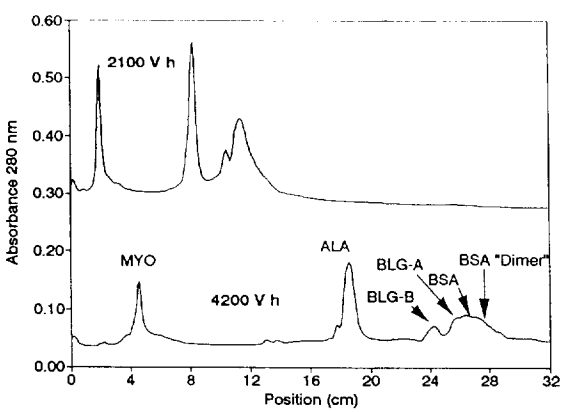

Fig. 5. Separation of the protein mixture on the long column. The mixture was run for either 2100 V h (1500 V for 1.4 h) or 4200 V h (2001 V for 2 h). The identities of the peaks were confirmed by gel electrophoresis or size-exclusion HPLC of the isolated fractions as described in text.

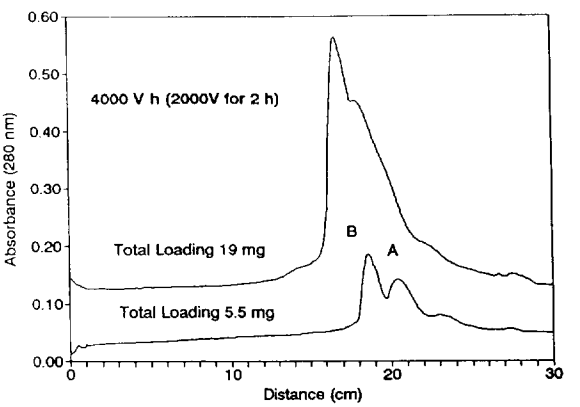

Fig. 6. Separation of β-lactoglobulin A and B. The indicated amount of β-lactoglobulin dissolved in 1 ml of 37% sucrose in 1 × buffer was loaded on the column and run at 2000 V for 2 h.

resolution on the performance of this column was determined by loading varying amounts of β-lactoglobulin. Fig. 6 shows that under these conditions with a constant sample size of 1 ml, a satisfactory separation could be obtained between β-lactoglobulin A and B when approximately 5 mg was loaded.

3.2. Calculation of electrophoretic mobilities from density gradient column

The physical properties of the buffers and sucrose solutions used are shown in Table 1. The gradients of conductivity (Fig. 2) and viscosity (Fig. 3) that are present in the column complicate calculation of electrophoretic mobilities from the protein migration data. Procedures for calculating electrophoretic mobilities from density gradient electrophoresis have been discussed previously [5,11]. The standard electrophoretic mobility μ_o, defined as the velocity per unit field in 1 × buffer at 25°C, can be calculated from the instaneous velocity of the protein through the sucrose gradient:

$$\frac{dx}{dt} = \mu(x)E(x) = \mu(x)\frac{I}{Ak(x)} \quad (1)$$

Where A is the cross sectional area of column (3.801 cm^2), I is the applied current, $k(x)$ is the local conductivity, and the instaneous mobility

$\mu(x)$ depends on the standard mobility according to:

$$\mu(x) = \frac{\mu_o \eta_o}{\eta(x)} \quad (2)$$

Where η_o = viscosity of water at 25°C (0.890 Pa s) and $\eta(x)$ is the local viscosity. Experimentally, the distance migrated x is measured at time t so the solution of the integral of Eq. 1 for μ_o with Eq. 2 replacing instaneous mobility is:

$$\mu_o = \frac{\int_0^x \frac{A\eta(x)k(x)}{I\eta_0} dx}{\int_0^t dt} \quad (3)$$

The viscosity profile in the 26.3 cm column (Fig. 3) was measured (units of mPa s) and found to be:

$$\eta(x) = 2.99 - 0.147x + 0.00276x^2 \text{ mPa s} \quad (4)$$

The conductivity profile in the 26.3 cm column (Fig. 2) was measured (units of μS/cm) and found to be:

$$k(x) = 42.0 + 2.40x \ \mu\text{S/cm} \quad (5)$$

These equations give the following the empirical function:

$$\mu_o = \frac{A}{I\eta_o t} \int_0^x (42.0 + 2.40x)(2.99 - 0.147x + 0.00276x^2)dx \quad (6)$$

The units of A are in cm^2, I in μA, η_o in mPa s, x in cm, and t in s. The integration gives a closed form for calculating the standard mobility:

$$\mu_o = \frac{A}{I\eta_o t}(125.16x + 0.555x^2 - 0.079x^3 + 0.00165x^4) \quad (7)$$

Eq. 7 determines standard mobility in a 26.3 cm long gradient of sucrose concentration from 1–35% (w/v). If the same gradient is compressed over half the distance the relationship is

$$\mu_o = \frac{A}{I\eta_o t}(125.16x + 0.995x^2 - 0.3143x^3 + 0.01325x^4) \quad (8)$$

The electroosmotic flow in the column was not measured but it is expected not to be significant [4] because blue dextran and colored protein (myoglobin) maintained a flat zone during electrophoresis. Likewise, the effect of heat dissipation in the column could be neglected, as indicated by the flatness of colored bands and indicated by heat transfer calculations. A repeat of the calculations reported in Ref. [11] using currents and conductivities typical of these experiments predicts a 1.8°C higher temperature at the center of the column than at the wall where heat is removed at constant temperature. This calculation was confirmed experimentally by inserting a mercury thermometer in the center of the column into the middle of the gradient. An increase of 1.4°C was measured in the long column with an applied field (2000 V and 6.0 mA). Over this temperature range, any modification in conductivity is counteracted by reduced viscosity, and migration velocity (hence mobility) will be unchanged, as observed.

3.3. Calculation of electrophoretic mobilities using capillary zone electrophoresis

We used a polyacrylamide coated capillary to measure electrophoretic mobility of the model proteins. The electrophoretic mobilities of the proteins were measured using the 10 × buffer because the 1 × buffer did not resolve any of the proteins, probably due to adsorption of all the proteins to the capillary in this low ionic strength buffer. The ionic strength of the 1 × buffer was calculated to be 1.5 mM [12,13]. The 10 × buffer gave satisfactory peak shapes for the proteins except for the more basic protein, myoglobin which did not resolve. This indicates that adsorption can still be a problem with basic proteins at higher pH, even with a coated capillary. The 10 × buffer had a current of only 2 μA at 10 000 V with this capillary. A typical separation is shown in Fig. 7. The relative peak widths are narrower than the corresponding peaks in Fig. 4, for example due to the thinner sample bands and shorter diffusion times in CZE compared with density gradient electrophoresis. The pumping of

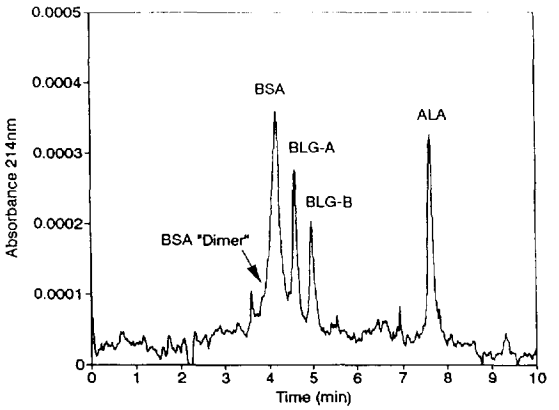

Fig. 7. Capillary zone electrophoresis of the protein mixture. The samples were injected electrophoretically (−10 000 V for 5 s) and run at 10 000 V and monitored at 214 nm.

the sample out of the density gradient column should not contribute significantly to the increased peak width in density gradient electrophoresis [11]. The leading edge of the bovine serum albumin contained the dimer (determined by purified dimer) but was not fully resolved from the monomer. The mobilities of the model proteins measured by density gradient zone electrophoresis and CZE 10 000 V (corrected for electroosmotic velocity) are shown in Tables 3 and 4, respectively. The electrophoretic mobility was calculated by dividing the migration time (in s) by the length to the detector (19.5 cm) by the field. The field was calculated by dividing the total voltage by the total length of the capillary

Table 3
Electrophoretic mobility of proteins measured by density gradient zone electrophoresis

Protein	Mobility $(cm^2/V \cdot s) \times 10^{-5} \pm$ S.D. (n)
Bovine serum albumin	28.9 ± 1.3 (5)
β-Lactoglobulin A	27.2 ± 1.3 (9)
β-Lactoglobulin B	25.3 ± 1.3 (9)
α-Lactalbumin	19.4 ± 2.6 (8)
Myoglobin	5.8 ± 0.7 (5)

S.D. is the standard deviation of the mean of the number of measurements (n).

Table 4
Electrophoretic mobility of proteins measured by capillary zone electrophoresis

Protein	Mobility $(cm^2/V \cdot s) \times 10^{-5} \pm$ S.D. (n)
Bovine serum albumin	20.5 ± 0.3 (5)
β-Lactoglobulin A	18.9 ± 0.1 (5)
β-Lactoglobulin B	17.3 ± 0.2 (5)
α-Lactalbumin	12.0 ± 0.1 (5)

S.D. is the standard deviation of the mean of the number of measurements (n). Values have been corrected for electroosmotic velocity of the column, which was $0.69 \pm 0.20 \times 10^{-5}$ $cm^2/V \cdot s$ ($n = 5$).

(24 cm). We measured the velocity of the proteins in fields of 5000, 10 000 and 15 000 V to determine if heating of the capillary was significantly affecting velocity or denaturation of the proteins occurred. Table 5 shows that the measured electrophoretic mobilities was constant for the three fields.

A very systematic difference between the mobilities measured by the two methods is seen in Tables 3 and 4. The mobility of BSA in 10 × buffer can be calculated from the mobility in 1 × buffer, assuming the ionic strength is 1.5 mM [12] at pH 8.3 and that the radius (a) for BSA molecule is 2.76 nm. In this case, by referring to the standard plot of $1/\kappa$ (Debye length) vs. ionic strength [19] and Wiersema's [20,21] tabulation of mobilities as a function of κa, it is found that $\kappa a = 0.35$ in 1 × buffer and 1.25 in 10 × buffer, and the calculated mobility of BSA in 10 × buffer is $25.9 \cdot 10^{-5}$ $cm^2/V \cdot s$. From Wiersema's table, we also infer a zeta potential of 50 mV for BSA. By repeating this exercise for the four test proteins, it is found that the calculated mobilities in 10 × buffer all differ from the observed mobilities in 10 × buffer using CZE by $5.5 \pm 0.1 \cdot 10^{-5}$ $cm^2/V \cdot s$ (observed CZE mobilities slower). This difference between the two methods is not accounted for. Based on the results that myoglobin did not resolve on CZE, presumably due to adsorption of the protein to the capillary, the difference could be due to adsorption we are unable to measure.

Table 5
The effect of field on electrophoretic mobility of proteins measured by capillary zone electrophoresis

Protein	Mobility ($cm^2/V \cdot s$) $\times 10^{-5} \pm$ S.D. (n)		
	5000 V	10 000 V	15 000 V
Bovine serum albumin	20.0 ± 0.0 (3)	19.9 ± 0.3 (5)	20.0 ± 0.2 (3)
β-Lactoglobulin A	17.7 ± 0.2 (3)	18.0 ± 0.1 (5)	17.9 ± 0.1 (3)
β-Lactoglobulin B	16.1 ± 0.2 (3)	16.6 ± 0.2 (5)	16.3 ± 0.1 (3)
α-Lactalbumin	11.0 ± 0.2 (3)	11.3 ± 0.1 (5)	11.1 ± 0.3 (3)

Values were not corrected for electroosomotic velocity. S.D. is the standard deviation of the mean of the number of measurements (n).

4. Conclusions

The electrophoretic mobility measurements made in this density gradient apparatus offer a complete uniform data set for the model proteins studied. The use of this low conductivity (low ionic strength) buffer allowed the use of high fields for rapid separations. The electrophoretic mobility measurements of these proteins are also relatively high thereby facilitating rapid separations. The rapid separation and low heating characteristics of this buffer should facilitate studies using more labile proteins. The modular nature of the column allows the easy changing of column dimensions and gradient length. The use of membranes in the side arms avoids the use of acrylamide plugs which leak and can leach compounds. The membranes last for months if they are kept wet.

Measurements of electrophoretic mobility using CZE are rapid and easy, but are not without potential for error. We were not able to use the low conductivity buffer (1 × buffer) with the CZE. This buffer has a very low ionic strength (1 × = 1.5 mM) [12,13]. We were not able to resolve myoglobin by CZE even using the 10 × buffer. Adsorption to the capillary coating may be the cause of myoglobin not being detected. Adsorption to the coating and the proteins may also affect the accuracy of the measured mobilities and this may be the source of the difference between the CZE and density gradient electrophoresis (DGE) measurements. The mobilities measured by the CZE method had lower standard deviations compared to the values measured by DGE. This is most likely due to the automated nature of CZE. The DGE method involves the preparation of a number of sucrose solutions and pumping the solutions in and out of the column. The DGE method involves a lot of manual steps when compared to the relatively simple automated CZE method.

The density gradient column used in this research allows the rapid and easy separation of moderate amounts of proteins based on differences in electrophoretic mobility. This method would allow milligram quantities of proteins to be purified. With care reliable measurements of the electrophoretic mobility can be done on mixtures as well as purified proteins. The calculation of electrophoretic mobilities is more complicated with the density gradient, but the technique does not result in adsorption. The effects of protein–protein interactions and protein–solute interactions can be studied using this improved column.

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References

[1] H. Svensson, L. Hagdahl and K.-D. Lerner, Science Tools, 4 (1957) 1–10.
[2] H. Svensson, in P. Alexander and R.R. Block (Editors), Analytical Methods of Protein Chemistry, Volume 1, Pergamon Press, Oxford, 1960, pp. 195–244.

[3] M. Bier, in M. Bier (Editor), Electrophoresis: Theory, Methods, and Applications, Academic Press, Orlando, FL, 1959, pp. 263–316.
[4] R.C. Boltz, Jr. and P. Todd, in P.G. Righetti, C.J. van Oss, and J. Vanderhoff (Editors), Electrokinetic Separation Methods, Elsevier/North-Holland Biomedical Press, Amsterdam, 1978. pp. 229–250.
[5] L.D. Plank, W.C. Hymer, M.E. Kunze, G.M. Marks, J.W. Lanham and P. Todd, J. Biochem. Biophys. Meth., 8 (1983) 275–289.
[6] M.M. Bushey and J.W. Jorgenson, J. Chromatogr., 480 (1989) 301–310.
[7] M. Zhu, D.L. Hansen, S. Burd and F. Gannon, J. Chromatogr., 480 (1989) 311–319.
[8] R.M. Hillier, J. Dairy Res., 43 (1976) 259–265.
[9] V. Neuhoff, N. Arold, D. Taube and W. Ehrhardt, Electrophoresis, 9 (1988) 255–262.
[10] L. Anderson, 2D News, 3 (1989) 3.
[11] D.T.L. Hawker, R.H. Davis, P. Todd, S.R. Rudge and R. Lawson, Biotech. Progress, 8 (1992) 429–436.
[12] S.R. Rudge, S.K. Basak and M.R. Ladish, AIChE J., 39 (1993) 797–808.
[13] S.K. Basak, A Velayudhan and M.R. Ladish, Appl. Biochem. Biotech., 44 (1994) 243.
[14] B.J. Radola, Biochim. Biophys. Acta, 295 (1973) 412–428.
[15] D.J. Reeder, in J.W. Jorgenson and M. Phillips (Editors), New Directions in Electrophoretic Methods, American Chemical Society, Washington, DC, 1987, pp. 102–116.
[16] FMC Bioproducts, Rockland, Maine.
[17] H.E. Swaisgood, in P.F. Fox (Editor), Developments in Dairy Chemistry-1, Elsevier, London, 1982, pp. 1–59.
[18] C.A. Zittle, Arch. Biochem. Biophys., 64 (1956) 144–151.
[19] S.R. Rudge and P. Todd, in M.R. Ladish, R.C. Wilson, C.-d.C. Painton and S.E. Builder (Editors), Protein Purification, From Molecular Mechanisms to Large-Scale Processes, American Chemical Society, Washington, DC, 1990, pp. 224–270.
[20] P.H. Wiersema, A.L. Loeb and J.T.G. Overbeek, J. Coll. Interface Sci., 22 (1966) 77–99.
[21] C.J. van Oss, Sep. Purif. Methods, 4 (1975) 167–188.