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Size separation of proteins by capillary zone electrophoresis with cationic hitchhiking (CZECH)

Vladislav Dolnik^{*} and William A. Gurske

Alcor BioSeparations LLC, Palo Alto, CA 94303, USA

Abstract

The paper describes a method of size separation of proteins by capillary sieving electrophoresis with cationic surfactant. Proteins are separated within 12 minutes with repeatability of migration times better than 0.2%. Some proteins achieve the separation efficiency of 200,000 theoretical plates. The method can be used for determination of protein relative molecular masses. The accuracy of the determined relative molecular masses and the limitation of the method were investigated by the analysis of more than 60 proteins. The method also allows separation of protein oligomers. Proteins can be quantitated after the electrokinetic injection in the concentration range 0.07–0.43 g/L. The average detection limit is about 2 mg/L.

Keywords

Capillary sieving electrophoresis; Capillary zone electrophoresis with cationic hitchhiking; CZECH; Protein size separation; SDS capillary electrophoresis

1 Introduction

Capillary electrophoresis is a powerful tool for separation of proteins and polypeptides [1–13]. Electrophoresis in the presence of ionic surfactants has a long history. The influence of SDS and cetylpyridinium bromide on the electrophoretic behavior of bacteria was studied as early as in 1946 [14]. A significant effect of ionic surfactants on the migration behavior of proteins in paper electrophoresis was described a few years later [15,16]. SDS polyacrylamide gel electrophoresis (PAGE) [17–19] became a popular method [20] for separation of protein according to their relative molecular masses (M_r). It was found that 1 g of protein binds 1.4 g of SDS, independently of ionic strength [21]. SDS-protein complexes were described as rod-like particles, the length of which varies with the molecular mass of the protein [21]. Later protein-surfactant complexes were modeled as pearl-necklace structures with spherical micelles on a protein fiber [22].

Some proteins migrate anomalously in SDS PAGE [17,23]. The migration deviation in SDS PAGE may be an inherent property of acidic proteins because the esterification of carboxyl groups normalized their migration [23]. It was found that some proteins including pepsin, papain, and glucose oxidase did not bind measurable amount of SDS [24].

SDS PAGE has been adapted into capillary format with a crosslinked polyacrylamide gel as a sieving matrix [25,26], later the crosslinked gel was replaced with sieving polymer

Correspondence: Dr. Vladislav Dolnik, Alcor BioSeparations LLC, 1031 Moffett Circle, Palo Alto, CA. 94303, USA, ladia@dolnik.net.

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solutions [27–43] and the method was also modified for separations on microchip [44,45]. In capillary SDS electrophoresis, the anomalous migration of some proteins has been known for years. Post-translational modifications of proteins, such as glycosylation, phosphorylation, and sulfonation were identified as a significant source of errors and cause of discernable deviations from idealized migration of SDS-protein complexes [39]. To understand the incidence of the M_r errors, 65 proteins were analyzed by SDS-PAGE and SDS capillary sieving electrophoresis (CSE) and the obtained results were compared with the values obtained by other techniques. Significant deviations from the literature data were observed for a number of proteins [46]. To improve the accuracy of the M_r determination, it was suggested to use the Ferguson method [33,34]. It improved the accuracy of M_r for some proteins but, e.g., for IgG L-chain, where SDS CSE originally showed 87% error, the Ferguson method did not get below 17% error [34].

Shortly after the introduction of SDS PAGE, a method to separate proteins by PAGE in the presence of cationic surfactants was developed [23]. Nevertheless, the ability of cationic detergents to properly bind proteins for electrophoretic size separations was questioned soon, claiming the cationic surfactants interacted with proteins in a cooperative mode at a concentration close to their critical micelle concentration (CMC) whereas the corresponding process for SDS occurred well below the CMC [47]. In the meantime various formulations were proposed to size separate proteins by PAGE with cetylpyridinium chloride [48] and cetyltrimethylammonium bromide (CTAB) [49–53]. Various protocols were also developed for sample preparation, including one without heating the proteins after the addition of CTAB [50].

We have developed a fast, high-resolution, and high-repeatability method of capillary electrophoresis for size separation of proteins in the presence of a cationic surfactant and called it capillary zone electrophoresis with cationic hitchhiking (CZECH).

2 Materials and Methods

2.1. Materials

CZECH Gel™ [57] was obtained from Alcor BioSeparations LLC (Palo Alto, CA, USA). Tris(carboxyethyl)phosphine hydrochloride (TCEP) was a product of Gold Biotechnology (St. Louis, MO, USA). 12-2-12 gemini surfactant was synthesized by Marcela Lísková (Institute of Analytical Chemistry of the Czech Academy of Sciences, Brno, Czech Republic). Ovomucoid, uricase, urease, butyrylcholine esterase, κ -casein, and proteinase K were obtained from Worthington (Lakewood, NJ, USA). All other chemicals were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

2.2 Sample preparation

Protein samples were prepared by dissolving 5 g/L protein in 100 mM KCl, 2 g/L cetyltrimethylammonium chloride (CTAC), and 10–50 mM TCEP or 100 mM dithiothreitol (DTT) and incubated at 70 °C for 10 min.

2.3 Separation

The CE experiments were performed on a 3D CE capillary electrophoresis instrument (Agilent, Waldbronn, Germany) with UV detection at 214 nm. Bare fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with 75 μ m id and 360 μ m od (the total capillary length of 335 mm and effective length of 250 mm) were used to perform separations. Between the runs, the capillary was flushed with 0.1 M citric acid, 0.1 M 2-hydroxyisobutyric acid (2-HIBA), or 0.1 M HCl for 4 min and then the capillary was filled with the separation medium for 4 min.

3 Results and Discussion

3.1 Size separation of model proteins

Seven model proteins (lysozyme, β -lactoglobulin, ovalbumin, glutamate dehydrogenase, BSA monomer, phosphorylase b, and BSA dimer) with M_r from 14,200 to 134,000 were separated by CZECH in less than 12 minutes (Fig. 1). To use these proteins as standards for determination of M_r , their mobilities were calculated and plotted against their logarithmic M_r (Fig. 2). Linear and quadratic fits of the data were compared: the experimental data fit better a quadratic equation rather than a simple linear fit. The squared correlation coefficient of 0.97 validates this protein mixture for the calibration of M_r in CZECH. The excellent repeatability and separation efficiency (see below) indicate the electroosmotic flow is significantly suppressed.

The sieving matrix contained a cationic surfactant at a concentration above its CMC. The presence of a cationic surfactant typically reverses the direction of the electroosmotic flow. How much is the electroosmotic flow eliminated, we can only conjecture from indirect observations since there is not any method available that would allow measuring low electroosmotic mobility in viscous solutions.

Figure 1 actually shows 10 overlaid electropherograms from 10 consecutive runs rather than a single electrophoretic run. The repeatability of the migration times from these 10 runs is calculated and summarized in Table 1. The values of relative standard deviation of migration times fall below 0.2% for all 7 proteins.

Further we investigated the separation efficiency of CZECH. Table 1 shows the separation efficiencies of the model proteins, expressed in a number of theoretical plates. The fastest proteins, lysozyme and β -lactoglobulin, exhibit about 200,000 theoretical plates and all proteins show more than 100,000 theoretical plates, with the exception of ovalbumin and BSA. These two proteins obviously do not follow the empirical rule that the separation efficiency declines in a monotonic way with increasing migration times. This anomaly is most likely caused by microheterogeneity of these proteins, i.e., by the presence of closely related forms of these proteins with slightly differing mobilities in a single peak. Ovalbumin is well known to contain at least 9 glycoprotein isoforms [54]. These isoforms are likely to slightly differ in mobilities and this leads to a band broadening in CZECH. Similarly the BSA monomer exists in several isoforms with different arrangements of disulfidic bridges [55]. These isoforms are also likely to bind various amounts of ionic surfactants and migrate as a single broad band in CZECH. It should be noted that this interpretation is not consistent with the idealized model of sieving electrophoresis with ionic surfactants: Ideally BSA as well as all other proteins should be completely unfolded and reduced prior the separation, so it should occur as just a single polypeptide chain rather than several isoforms. As will be shown and discussed later, BSA monomer migrates properly even without any heat denaturation and reduction. Broader BSA peaks are not specific for CZECH but were also previously observed in SDS CSE [36,37]. This supports the hypothesis of microheterogeneity in the BSA monomer peak. The textbook description of electrophoresis with ionic surfactants probably needs some update.

3.2 Ferguson plot

The Ferguson plot of protein mobilities was frequently used in PAGE in absence of ionic surfactants [56], nevertheless its use was proposed also for SDS CSE [33,34]. It shows a relationship between the protein mobility and the concentration of a sieving medium. Thus, each protein is represented here by a straight line, the slope of which is called the retardation coefficient K_R and which reveals how strongly the protein migration is retarded by the presence of the sieving polymer. The Ferguson plot can be used to determine M_r of proteins

when the square root of the absolute value of the retardation coefficient is plotted vs. the logarithm of M_r of the protein.

The model proteins were separated by CZECH at 5 concentrations of the sieving polymer and the calculated mobilities were plotted in the corresponding Ferguson plot (Fig. 3). A deviation from linearity is discernable, particularly for large proteins. They migrate more slowly than predicted at all polymer concentrations. This also holds when considering the values extrapolated into free solution electrolyte without any polymer. The square root of the retardation coefficient for 7 model proteins was plotted vs. $\log M_r$ and provided a linear relationship that could be described by the equation $\log M_r = 1.4244 (-K_R)^{-0.5} - 0.1718$. The squared correlation coefficient of 0.98 validated this procedure as an alternative for the determination of relative molecular masses of proteins in CZECH.

3.3 Accuracy of M_r

To ascertain reliability of CZECH as a method for determination of M_r of proteins, we measured M_r of over 60 proteins and compared them with the data obtained by other methods. The results are summarized in Table 2. Because the calibration standard proteins may hypothetically exhibit some anomaly in migration, we constructed an overall calibration curve (Fig. 4) with the corresponding equation $\mu = 59.7 - 8.71 \log M_r$. The calculated mobility and M_r values were entered in Table 2. Then we gradually eliminated proteins that exhibited errors in M_r over 15%. With 30 remaining proteins, a new calibration curve was constructed described by the equation $\mu = 63.04 - 9.413 \log M_r$. Squared correlation coefficient of 0.985 was obtained for this plot.

The proteins listed in Table 2 can be divided into 3 categories: proteins with correct M_r , proteins with erroneous M_r , and proteins not generating any discernable peak. Approximately half of the tested proteins showed errors in M_r lower than 10 – 15%. We consider the success with approximately half of the proteins tested to justify CZECH as a method to measure M_r of proteins. However, similarly as in SDS CSE, it is necessary to cautiously evaluate all the generated data.

There were several proteins that showed an unacceptable error of M_r . The largest error was found for cytochrome c. It migrated as a narrow symmetrical peak in CZECH, indicating that slower migration was caused by factors other than wall adsorption. The electrostatic repulsion between basic protein and cationic surfactant was the simplest explanation of this behavior. Cytochrome c binds less cationic surfactant than other proteins and this causes an error in M_r of over 500%. (This hypothesis was confirmed by a development of a procedure normalizing the cytochrome c migration that will be described in a greater detail in a paper that follows.)

We were rather surprised by the deviation from the expected migration of soybean trypsin inhibitor. This protein migrates properly in SDS CSE and frequently is used as one of the calibration proteins. In CZECH its apparent mobility is, however, too low and the apparent M_r too high. We tried several procedures to normalize its migration including an addition of urea, replacement of quaternary ammonium surfactants with primary amines and with 12-2-12 gemini surfactant, varying the pH for the denaturing reaction. We also chemically modified the protein to reduce its native effective charge. None of these procedures was successful and we could not elucidate the enigmatic migration of soybean trypsin inhibitor.

The proteins that do not provide a distinct electrophoretic peak in CZECH simply do not show any convincingly sharp peak, independently of the injection method (electrokinetic injection and pressure injection). Some of these proteins do not properly migrate in SDS

CSE either [46,58–60]. Probably, excessive β -sheet structure is the cause of the anomalous behavior and denaturation [58–61].

3.4. Oligomer separations

Hypothetically, there should not be any oligomers remaining in a protein sample after its heating with an ionic surfactant. Nevertheless, the presence of BSA oligomers was already observed in early work on SDS PAGE [19]. CZECH separates BSA into 8–9 oligomers (Fig. 5). To obtain that high number of oligomers, it is essential to avoid any denaturation during the sample preparation and also any reduction by DTT or TCEP. It is also necessary to increase the amount of the injected BSA as much as possible, making almost a saturated solution of BSA: At lower starting concentration of BSA, the bigger oligomers become undetectable. The higher concentration is also favorable for generation of oligomers. However, this conclusion is based on our empirical observations rather than on measurements of association equilibria. If the mobility of BSA oligomers is plotted vs. logarithmic M_r a straight line is obtained that can be used to calibrate M_r in the range 60,000 – 500,000 (Fig 6). The squared correlation coefficient of 0.996 corroborates its use for M_r determination. This is a competitive advantage of CZECH, because SDS CSE and SDS PAGE typically do not determine M_r over 250,000. However, it should be emphasized that the conditions appropriate for the calibration of M_r with BSA oligomers are suitable for qualitative analysis only. For quantitation, potassium chloride or another salt comprising high-mobility ions has to be added to the sample to make the calibration lines linear (see section 3.5). The addition of KCl, however, diminishes the electrokinetic injection of the BSA oligomers so that the BSA oligomers larger than tetramer are typically not detected. At constant voltage, the elevated conductivity of the KCl zones also affects the electric field strength in the protein zones. As the result, the migration times of proteins from KCl-free samples differ from those of proteins prepared in the presence of KCl. This explains the difference between the calibration curves for the determination of M_r in Figs. 2 and 6.

CZECH can separate oligomers of other proteins too, including ovalbumin and phosphorylase b. Nevertheless, some proteins do not denature on the capillary and require a heat denaturation prior the analysis. In this respect, the method does not differ significantly from SDS electrophoresis [59,60].

When the mobilities calculated for BSA oligomers are plotted in a log-log format (Fig. 7), a curve is obtained that is reminiscent to the log-log mobility curves of DNA [62]. Those log-log curves of DNA were used to describe and distinguish various migration modes of DNA in electrophoresis. By a direct comparison of the mobility curves of BSA and DNA, it is apparent that BSA oligomers migrate in a reptation mode. The reptation mode was already proposed for SDS CSE [39]. However, Fig. 7 suggests the reptation mode to dominate at the M_r above approx. 100,000. While it is not difficult to imagine DNA molecules migrating like a snake through a sieving matrix, it is not easy to picture BSA oligomers as a single long fiber in the form of a pearl necklace.

3.5. Quantitation

In capillary electrophoresis, pressure injection is a simple and effective way to introduce samples into the capillary. In capillary sieving electrophoresis, the increased viscosity may, however, compromise the accuracy of the injection. That is why electrokinetic injection is preferred in CSE. However, there is a trap that is not fully realized by the CE users: the effect of the sample conductivity on the injected amount of analytes. Fig. 8a shows the calibration lines for proteins dissolved in 11 g/L CTAB only. The calibrations are strongly non-linear and, at a higher concentration, they do not respond to the changes in the protein concentration in the sample at all. (In the extreme case of a sample containing just one

analyte, its injected amount depends on the applied injection electric charge only and is independent of its concentration in the sample; see Appendix.) The conditions of Fig. 8a are obviously inappropriate for quantitative analysis. To overcome this predicament, the samples for electrokinetic injection have to exhibit high conductivity, preferably by an addition of chloride for anionic separations and potassium or ammonium for cationic analyses. Calibration lines of proteins dissolved in 100 mM KCl and 11 g/L CTAB and separated by CZECH are shown in Fig. 8b. They are linear in the concentration range 0.16 – 0.83 g/L and can be used to quantitate these proteins. The addition of potassium chloride, however, results in a lower sensitivity (less protein is injected at the same injection charge) and the larger the protein, the more pronounced is this effect. To increase the overall sensitivity of the method, higher injection current and/or injection time can be applied. It translates, however, in a considerable potassium zone with conductivity and electric field different from the rest of the capillary. At constant voltage, it affects electric field in the protein zones and thus migration times and also peak area of the analyzed proteins. It is essential to inject both samples and calibration standards under the same conditions. For a quantitative analysis of real-world samples (serum, urine, etc.) the samples have to be prepared accordingly, removing all low-molecular compounds and replacing them with the components of the sample denaturing solution, e.g., by a repeated washing on Microcon centrifugal filters. The upper injection limit depends also on the total length of the capillary. In a 335-mm long capillary, the samples can be injected for up to 30–60 s at 10 kV. 30-s injection at 10 kV translates into a lower limit of detection (2 mg/L) and lower limit of quantitation (70 mg/L, here defined as the concentration allowing run-to-run repeatability better than 5%) but also into a narrower range of linearity (2–430 mg/L). The calibration lines in Fig. 8b are to demonstrate their linearity rather than calibration constants. (Protein oligomers, such as, e.g., BSA monomer and dimer, cannot be quantitated from one calibration standard.)

4 Concluding remarks

Capillary zone electrophoresis with cationic hitchhiking can be used for repeatable high-speed, high-resolution size separations of proteins. The method allows quantitation of proteins in the range 0.07–0.43 g/L. Protein oligomers can be also separated by this method.

Acknowledgments

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Abbreviations

CSE	capillary sieving electrophoresis
CTAC	cetyltrimethylammonium chloride
CZECH	capillary zone electrophoresis with cationic hitchhiking
G3PDH	glyceraldehyde-3-phosphate dehydrogenase
GDH	glutamate dehydrogenase
2-HIBA	2-hydroxyisobutyric acid
TCEP	tris(carboxyethyl)phosphine hydrochloride

References

1. Dolnik V. Electrophoresis. 1997; 18:2353–2361. [PubMed: 9456050]

2. Dolnik V. Electrophoresis. 1999; 20:3106–3115. [PubMed: 10596818]
3. Dolnik V, Hutterer KM. Electrophoresis. 2001; 22:4163–4178. [PubMed: 11824635]
4. Hutterer K, Dolnik V. Electrophoresis. 2003; 24:3998–4012. [PubMed: 14661231]
5. Dolnik V. Electrophoresis. 2006; 26:126–141. [PubMed: 16307430]
6. Dolnik V. Electrophoresis. 2008; 29:143–156. [PubMed: 18058764]
7. El Rassi Z. Electrophoresis. 2010; 31:174–191. [PubMed: 20039288]
8. Kaši ka V. Electrophoresis. 1999; 20:3084–3105. [PubMed: 10596817]
9. Kaši ka V. Electrophoresis. 2001; 22:4139–4162. [PubMed: 11824634]
10. Kaši ka V. Electrophoresis. 2003; 24:4013–4046. [PubMed: 14661232]
11. Kaši ka V. Electrophoresis. 2006; 27:142–175. [PubMed: 16307429]
12. Kaši ka V. Electrophoresis. 2008; 29:179–206. [PubMed: 18046694]
13. Kaši ka V. Electrophoresis. 2010; 31:122–146. [PubMed: 19950358]
14. Dyar MT, Ordal EJ. J Bacteriol. 1946; 51:149–167.
15. Putnam FW. Adv Protein Chem. 1948; 6:79–122. [PubMed: 18884346]
16. Jerchel D, Scheuer H. Z Naturforsch. 1953; 8b:541–547.
17. Shapiro AL, Vinuela E, Maizel JV. Biochem Biophys Res Commun. 1967; 28:815–820. [PubMed: 4861258]
18. Shapiro AL, Maizel JV. Anal Biochem. 1969; 29:505–514. [PubMed: 4183017]
19. Dunker AK, Rueckert RR. J Biol Chem. 1969; 244:5074–5080. [PubMed: 5824577]
20. Kresge N, Simoni RD, Hill RL. J Biol Chem. 2006; 281:e19–e21.
21. Reynolds JA, Tanford C. Proc Natl Acad Sci USA. 1970; 66:1002–1007. [PubMed: 5269225]
22. Ospinal-Jiménez MPD. Langmuir. 2011; 27:928–935. [PubMed: 21182321]
23. Williams JG, Gratzer WB. J Chromatogr. 1971; 57:121–125. [PubMed: 4102682]
24. Nelson CA. J Biol Chem. 1971; 246:3895–3901. [PubMed: 4327191]
25. Cohen AS, Karger BL. J Chromatogr. 1987; 397:409–417. [PubMed: 3654832]
26. Dolnik V, Cobb KA, Novotny M. J Microcol Sep. 1991; 3:155–159.
27. Liu, Y.; Redy, P.; Ratnayake, C.; Koh, EV. U.S. Patent Application 20040050702.
28. Liu, Y.; Redy, P.; Ratnayake, C.; Koh, EV. U.S. Patent Application 20090314638.
29. Ganzler K, Greve KS, Cohen AS, Karger BL, Guttman A, Cooke NC. Anal Chem. 1992; 64:2665–2671. [PubMed: 1284102]
30. Wu D, Regnier FE. J Chromatogr. 1992; 608:349–356. [PubMed: 1430034]
31. Guttman A, Horvath J, Cooke N. Anal Chem. 1993; 65:199–203.
32. Werner WE, Demorest DM, Stevens J, Wiktorowicz JE. Anal Biochem. 1993; 212:253–258. [PubMed: 8368499]
33. Werner WE, Demorest DM, Wiktorowicz JE. Electrophoresis. 1993; 14:759–763. [PubMed: 7691594]
34. Guttman A, Shieh P, Lindahl J, Cooke N. J Chromatogr A. 1994; 676:227–231.
35. Nakatani M, Shibukawa A, Nakagawa T. J Chromatogr A. 1994; 672:213–218. [PubMed: 8069394]
36. Nakatani M, Shibukawa A, Nakagawa T. Electrophoresis. 1996; 17:1210–1213. [PubMed: 8855406]
37. Karim MR, Janson JC, Takagi T. Electrophoresis. 1994; 15:1531–1534. [PubMed: 7536670]
38. Tsuji K. J Chromatogr B. 1994; 662:291–299.
39. Guttman A. Electrophoresis. 1995; 16:611–616. [PubMed: 7588533]
40. Hu S, Ye YL, Surh G, Clark JJ, Dovichi NJ. LC GC (Europe). 2002; 15:166–169.
41. Craig DB, Polakowski PM, Arriaga E, Wong JCY, Ahmadzadeh H, Stathakis C, Dovichi NJ. Electrophoresis. 1998; 19:2175–2178. [PubMed: 9761200]
42. Hunt G, Nashabeh W. Anal Chem. 1999; 71:2390–2397. [PubMed: 10405607]
43. Salas-Solano O, Tomlinson B, Du S, Parker M, Strahan A, Ma S. Anal Chem. 2006; 78:6583–6594. [PubMed: 16970337]

44. Yao S, Anex DS, Caldwell WB, Arnold DW, Smith KB, Schultz PG. Proc Natl Acad Sci USA. 1999; 96:5372–5377. [PubMed: 10318890]
45. Bousse L, Mouradian S, Minalla A, Yee H, Williams K, Dubrow R. Anal Chem. 2001; 73:1207–1212. [PubMed: 11305653]
46. Guttman A, Nolan J. Anal Biochem. 1994; 221:285–289. [PubMed: 7810868]
47. Nozaki Y, Reynolds JA, Tanford C. J Biol Chem. 1974; 249:4452–4459. [PubMed: 4210504]
48. Schick M. Anal Biochem. 1975; 63:345–349. [PubMed: 235854]
49. Akins RE, Tuan RS. Mol Biotechnol. 1994; 1:211–228. [PubMed: 7859162]
50. Akins RE, Levin PM, Tuan RS. Anal Biochem. 1992; 202:172–178. [PubMed: 1377887]
51. Akin DT, Shapira R, Kinkade JM Jr. Anal Biochem. 1985; 145:170–176. [PubMed: 4003759]
52. Eley MH, Burns PC, Kannapell CC, Campbell PS. Anal Biochem. 1979; 92:411–419. [PubMed: 443541]
53. Panyim S, Thitipongpanich R, Supatimusro D. Anal Biochem. 1977; 81:320–327. [PubMed: 907096]
54. Landers JP, Oda RP, Madden BJ, Spelsberg TC. Anal Biochem. 1992; 205:115–124. [PubMed: 1443548]
55. Gabaldon M. Int J Biol Macromol. 2002; 30:259–267. [PubMed: 12297233]
56. Ferguson KA. Metabolism. 1964; 13:985–995. [PubMed: 14228777]
57. Dolnik, V. U.S. Patent Application 20100187113.
58. Manning M, Colon W. Biochemistry. 2004; 43:11248–11254. [PubMed: 15366934]
59. Gudiksen KL, Gitlin I, Whitesides GM. Proc Natl Acad Sci USA. 2006; 103:7968–7972. [PubMed: 16698920]
60. Zhang S, Xia K, Chung WK, Kramer SM, Colon W. Protein Sci. 2010; 19:888–892. [PubMed: 20091769]
61. Nielsen MM, Andersen KK, Westh P, Otzen DE. Biophys J. 2007; 92:3674–3685. [PubMed: 17351005]
62. Noolandi J, Slater GW, Lim HA, Viovy JL. Science. 1989; 243:1456–1458. [PubMed: 2928779]

5 Appendix

Consider a model with electrokinetic injection of a sample containing a single analyte A and its counter ion R, with mobilities μ_A and μ_R , respectively. For the sake of simplification, A is a strong univalent base and R is a strong univalent acid. During the injection, the electric charge Q passes through the capillary

$$Q = I t \quad (1)$$

where I is electric current and t time. Combining it with the modified Ohm's law

$$E = I / \kappa S \quad (2)$$

where E is the electric field in the sample zone, κ specific conductivity of the sample zone, and S capillary cross section, the injection charge is obtained

$$Q = E \kappa S t \quad (3)$$

The migration velocity v_A of analyte A equals

$$v_A = \mu_A E = I_A / t \quad (4)$$

where l_A is the path ion A migrates within time t . If the mobility of the analyte A does not significantly differ from the mobility of the buffer co-ion and the electric field in the injected sample zone is about the same as in any place of the capillary then the injection charge Q is

$$Q = V_A \kappa_A / \mu_A \quad (5)$$

where V_A is the volume of the sample zone (diffusion neglected). Using the relationship between specific conductivity and mobilities, it follows that

$$Q = V_A c_A F (\mu_A - \mu_R) / \mu_A \quad (6)$$

Where F is the Faraday constant and c_A is the concentration of analyte A (and counter ion R) in the sample zone, and

$$Q = F n_A (\mu_A - \mu_R) / \mu_A \quad (7)$$

where n_A is the molar amount of the injected analyte A. It can be expressed as

$$n_A = I t \mu_A / F (\mu_A - \mu_R) \quad (8)$$

The injected amount of analyte n_A is independent of its concentration in the sample and this deeply simplified example shows that simple electrokinetic injection cannot be used to quantitate analytes that significantly contribute to the sample conductivity.

Figure 1. Separation of standard proteins in 10 consecutive runs

Sample: 5 g/L proteins, 100 mM KCl, 2 g/L CTAC, 10 mM TCEP, incubation at 70 °C for 10 min, electrokinetic injection 10 s at +10 kV. Capillary: bare fused silica, 75 μ m id, 360 μ m od, total length: 335 mm, effective length: 250 mm. Separation medium: CZECH Gel^{TM}. Separation voltage: +10 kV. Detection by UV absorption at 214 nm.

Figure 2. Electrophoretic mobility of standard proteins vs. logarithm M_r
Experimental electrophoretic mobilities μ expressed in $10^{-9} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$, experimental conditions as in Fig. 1.

Figure 3. Ferguson plot for standard proteins

Proteins: + - lysozyme, ○ - β -lactoglobulin, ⋈ - ovalbumin, ◇ - glutamate dehydrogenase, Δ - BSA (monomer), × - phosphorylase b, □ - BSA dimer. Experimental electrophoretic mobilities μ expressed in $10^{-9} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$, experimental conditions as in Fig. 1.

Figure 4. Electrophoretic mobility of selected proteins vs. logarithm M_r
 Experimental electrophoretic mobility μ expressed in $10^{-9} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$. The diagram includes the proteins from Table 1 with errors in calculated M_r lower than 15%. Experimental conditions as in Fig. 1.

Figure 5. Separation of BSA oligomers

Sample: 20 g/L BSA in 2 g/L CTAC, no heating denaturation. Pressure injection, 5 s at 50 mbar. Other experimental conditions as in Fig. 1.

Figure 6. Electrophoretic mobilities of BSA oligomers vs. relative molecular masses M_r
 Experimental electrophoretic mobilities μ expressed in $10^{-9} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$, experimental conditions as in Fig. 5.

Figure 7. Log-log plot of electrophoretic mobility of BSA oligomers vs. M_r
 Experimental electrophoretic mobilities μ expressed in $10^{-9} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$, experimental conditions as in Fig. 5.

Figure 8a.

Figure 8b.

Figure 8. Calibration curves for quantitation of proteins

A) Low-conductivity samples: electrokinetic injection 4 s at 2 kV. (Sample: 0.16- 0.83 g/L protein in 11 g/L CTAB, 10 g/L DTT, incubation at 100°C for 2 min.). b) high-conductivity samples: electrokinetic injection 4 s at 2 kV. (Sample: 0.16- 0.83 g/L protein in 11 g/L CTAB, 100 mM KCl, 10 g/L DTT, incubation at 100°C for 2 min.)

Table 1
Repeatability of migration times and separation efficiency of proteins in CZECH

Protein	Average t_m [min]	SD	RSD [%]	Average number of theoretical plates	RSD [%]	Number of theoretical plates/m
lysozyme	6.35	0.0083	0.13	197,000	2.4	788,000
β -lactoglobulin	6.68	0.0060	0.09	278,000	1.8	1,112,000
ovalbumin	7.37	0.0022	0.03	33,000	1.2	132,000
glutamate DH	8.04	0.0137	0.17	147,000	1.7	588,000
BSA (monomer)	8.54	0.0026	0.03	46,000	4.6	184,000
phosphorylase b	9.37	0.0178	0.19	119,000	2.2	476,000
BSA (dimer)	10.23	0.0123	0.12	17,000	9.6	68,000

Table 2

Accuracy of M_r by CZECH

Protein	$M_{r, lit}$ 1)	μ_{exp} 2)	μ_{calc} 2,3)	μ error 4)	M_r 5)	M_r error 6)
cytochrome c	12,400	17.1	24.5	-30.2%	75,900	512%
lysozyme	14,200	23.9	24.0	0.0%	14,200	0.0%
α -lactalbumin	14,200	24.0	24.0	0.2%	14,000	-1.4%
hemoglobin α , porcine	15,100	23.8	23.7	0.4%	14,700	-2.6%
hemoglobin α , rabbit	15,100	23.9	23.7	1.0%	14,300	-5.3%
hemoglobin α , equine	15,100	23.7	23.7	-0.1%	15,100	0.0%
hemoglobin α , bovine	15,100	24.0	23.7	1.2%	14,100	-6.6%
hemoglobin α , human	15,100	24.0	23.7	1.2%	14,100	-6.6%
histone	15,300	22.6	23.6	-4.6%	19,900	30.1%
hemoglobin β , porcine	15,900	22.9	23.5	-2.7%	18,500	16.4%
hemoglobin β , rabbit	15,900	23.5	23.5	0.3%	15,700	-1.3%
hemoglobin β , equine	15,900	23.4	23.5	-0.5%	16,300	2.5%
hemoglobin β , bovine	15,900	23.4	23.5	-0.3%	16,200	1.9%
hemoglobin β , human	15,900	23.2	23.5	-1.2%	17,000	6.9%
superoxide dismutase	16,300					
myoglobin, equine, muscle	17,200	22.3	23.2	-3.8%	21,300	23.8%
myoglobin, equine, heart	17,200	22.2	23.2	-4.2%	21,800	26.7%
β -lactoglobulin	18,200	23.2	22.9	1.2%	17,000	-6.6%
κ -casein	19,000	20.8	22.8	-8.6%	30,600	61.1%
trypsin inhibitor, soybean	20,100	19.8	22.5	-12.2%	39,400	96.0%
papain	21,000	20.3	22.4	-9.0%	34,300	63.3%
α -casein	23,000	21.9	22.0	-0.3%	23,400	1.7%
α -casein, dephosphorylated	23,000	21.9	22.0	-0.2%	23,300	1.3%
β -casein	23,800	21.7	21.8	-0.7%	24,700	3.8%
trypsin	23,800	21.6	21.8	-1.2%	25,400	6.7%
trypsinogen	24,000	22.6	21.8	3.7%	19,700	-17.9%

Protein	$M_{r, \text{lit}}$	$\mu_{\text{exp}}^{2)}$	$\mu_{\text{calc}}^{2,3)}$	$\mu \text{ error}^{4)}$	$M_r^{5)}$	$M_r \text{ error}^{6)}$
α -chymotrypsin	25,000	22.4	21.6	3.7%	20,600	-17.6%
α -chymotrypsinogen A	25,700	22.2	21.5	3.0%	21,900	-14.8%
concanavalin A	27,000	21.7	21.3	1.5%	24,900	-7.8%
proteinase K	27,000					
ovomucoid	28,000					
carbonic anhydrase	29,000	20.5	21.0	-2.4%	32,900	13.4%
uricase	32,000	19.4	20.6	-5.8%	42,800	33.8%
pepsin	34,700	20.5	20.3	1.1%	32,800	-5.5%
alcohol dehydrogenase	35,000	20.1	20.3	-0.8%	36,500	4.3%
G3PDH	36,000	20.1	20.1	-0.2%	36,400	1.1%
aldolase	39,500	19.7	19.8	-0.2%	39,900	1.0%
peroxidase	40,000					
enolase	42,500	19.7	19.5	1.4%	39,800	-6.4%
ovalbumin	44,000	21.3	19.3	10.0%	27,400	-37.7%
glutamate dehydrogenase	55,000	19.6	18.4	6.2%	41,600	-24.4%
acid phosphatase	55,000					
α -amylase	57,000	19.2	18.3	5.0%	45,700	-19.8%
cellulase	60,000	15.3	18.1	-15.5%	118,900	98.2%
catalase	62,500					
BSA monomer	67,000	18.2	17.6	3.5%	57,500	-14.2%
lactoperoxidase	77,500	16.4	17.0	-3.8%	90,700	17.0%
transferrin, human	80,000	16.8	16.9	-0.5%	81,600	2.0%
apotransferrin, bovine	80,000	16.6	16.9	-1.9%	86,600	8.3%
ovalbumin dimer	88,000	18.1	16.5	9.7%	59,400	-32.5%
urease	90,770	16.0	16.4	-2.2%	99,000	9.1%
phosphorylase b	97,000	16.9	16.1	4.9%	80,100	-17.4%
amylglucosidase	97,000	15.7	16.1	-2.6%	107,600	10.9%
butyrylcholin esterase	110,000					
β -galactosidase	116,000	16.3	15.4	6.4%	91,300	-21.3%

Protein	$M_{r, \text{lit}}^1)$	$\mu_{\text{exp}}^{2)}$	$\mu_{\text{calc}}^{2,3)}$	$\mu \text{ error}^4)$	$M_r^5)$	$M_r \text{ error}^6)$
glutathione reductase	118,000					
polyphenol oxidase	128,000					
BSA dimer	134,000	14.9	14.8	0.9%	129,700	-3.2%
glucose oxidase	150,000					
BSA trimer	201,000	13.2	13.1	0.8%	195,700	-2.6%
BSA tetramer	268,000	12.2	11.9	2.2%	251,300	-6.2%

1) Values of protein relative molecular mass ($M_{r, \text{lit}}$) were taken from the literature provided by Sigma-Aldrich and Worthington.

2) Mobilities expressed as $10^{-9} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$.

3) Mobilities μ_{calc} calculated from the equation $\mu_{\text{calc}} = 59.7 - 8.71 \log M_{r, \text{lit}}$

4) The mobility errors calculated as $100 \times (\mu_{\text{exp}} - \mu_{\text{calc}}) / \mu_{\text{calc}}$.

5) Relative molecular masses M_r calculated from the equation $M_r = 10^{-0.1148\mu} + 6.85855$

6) The M_r errors calculated as $100 \times (M_r - M_{r, \text{lit}}) / M_{r, \text{lit}}$.