Chromatographia (2011) 73:1145–1153
DOI 10.1007/s10337-011-2003-9

ORIGINAL

CZE with On-line Micellar Sample Stacking for Determination of Protein Concentration of Biopharmaceuticals

Medhat A. Al-Ghobashy · Martin A. K. Williams · Götz Laible · David R. K. Harding

Received: 5 December 2010 / Revised: 3 February 2011 / Accepted: 8 March 2011 / Published online: 22 March 2011

Abstract A capillary zone electrophoresis total protein assay was developed and validated in polyethylene oxide (PEO) dynamically coated capillaries. On-line large-volume sample stacking was employed. Protein samples were denatured using SDS and then injected into PEO-filled capillaries. Such treatment enabled injection of a sample volume of ≈8% of the total capillary volume and stacking of protein-SDS molecules at the interface between the sample plug and the PEO plug. Results showed that SDS enhanced the sensitivity not only by protein denaturation but also by forming micelles, in which protein-SDS partitioned. Sensitivity of the method was further enhanced through using capillaries with (tenfold) extended detection path-length. Such strategies resulted in a limit of detection of 0.26 μg mL^{-1} (3.64 nM BSA). A linear relationship between protein concentration and integrated peak area was obtained over a wide concentration range (8.49–135.87 μg mL^{-1}—$R^2 = 0.995$). The method is particularly useful for determination of total protein concentration in chromatography fractions. It overcomes low UV absorptivity of proteins, presence of UV absorbing additives and high salt content. Contrary to conventional methods for determination of protein concentration, this method does not involve an interaction with a dye. Thus, variations due to differences in surface properties among proteins or due to differences in posttranslational modifications of the same protein are eliminated. The protocol was successfully applied for the determination of the concentration of a biopharmaceutical protein rhMBP in chromatography fractions. This protein has been previously produced in milk of transgenic cows and several charge isoforms were detected.

Keywords Capillary zone electrophoresis · Large-volume sample stacking · Polyethylene oxide · Biopharmaceuticals · Human myelin basic protein

Introduction

Production of biopharmaceuticals is a complex process and a tremendous effort and cost is involved in the development of every new product. A well-defined analysis strategy that addresses the key features of the recombinant product has to be developed and validated [1, 2]. In previous work, recombinant human myelin basic protein (rhMBP) was produced in milk of transgenic cows [3, 4]. This protein is a prospective biopharmaceutical for treatment of multiple sclerosis [5]. Similar to its human counterpart, several charge isoforms were detected. This was attributed to differences in the pattern of posttranslational modifications (PTM) [6].

Commonly used procedures for determination of protein concentration involve an interaction between the protein

Electronic supplementary material The online version of this article (doi:10.1007/s10337-011-2003-9) contains supplementary material, which is available to authorized users.

M. A. Al-Ghobashy (✉)
Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Cairo 11562, Egypt
e-mail: medhat.alghobashy@cu.edu.eg

M. A. Al-Ghobashy
Biotechnology Centre, Faculty of Pharmacy, Cairo University, Cairo, Egypt

M. A. K. Williams · D. R. K. Harding
Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand

G. Laible
Agresearch, Ruakura Research Centre, Hamilton, New Zealand

and a reagent. An extensive list of these procedures is covered in literature [7]. Previous investigations showed that rhMBP isoforms interact differently with conventional in-gel protein stains such as Coomassie Blue, Deep Purple fluorescent stains and selective stains for His-rich proteins [3]. Moreover, ligand-binding assays using surface plasmon resonance revealed differences between the PTM patterns of rhMBP when compared to its human counterpart. Such differences were reflected in the binding strength and kinetics of interaction of either protein with monoclonal anti-hMBP [4]. These findings raised a concern about the reliability of conventional procedures for determination of rhMBP concentration that relies on interaction between a protein and a reagent. The same concern applies for other proteins with a complex pattern of PTM, especially if present in complex mixtures with other proteins of different nature.

With all the advances in the field of protein capillary electrophoresis (CE), no reports have been published to investigate the applicability of CE in the determination of total protein concentration. This could be attributed to the poor detection limits obtained using UV absorption detection commonly employed in CE. The poor light absorptivity of protein and the short detection path length, inherent to CE capillaries contributed to this limitation [8, 9]. Several strategies have been proposed to improve detection limits such as using high-sensitivity detectors. Such detectors are expensive or require modifications of the CE system. On the other hand, on-line pre-concentration via sample stacking represents a novel, economic tool for improvement of UV detection sensitivity. In sample stacking with hydrodynamic injection, long injections of sample solutions (large-volume sample stacking, LVSS) are utilized in order to maximize sensitivity enhancement [9, 10].

Adsorption of proteins to capillary wall is another limiting factor in protein determination using CE [11]. Dynamic coating of CE capillaries is a widely used, economic approach for combating this problem [12]. Polyethylene oxide (PEO) is a linear, neutral, hydrophilic polymer, which has surface coating properties. PEO is available in a series of molecular weights and the viscosity of PEO solutions depends on both the concentration and the molecular weight of the PEO polymer employed. High molecular weight PEO polymers and co-polymers have been in use as sieving matrices in capillary gel electrophoresis of proteins and nucleotides. While preconditioning of fused silica capillaries with low molecular weight PEO polymers was used for dynamic coating of the capillary wall. The dynamic coat suppresses the electroosmotic flow (EOF) and prevents adsorption of protein molecules to the capillary wall [12–17].

Under capillary zone electrophoresis (CZE) conditions, proteins migrate according to their charge and frictional forces. The apparent net charge carried out by a protein molecule depends on the isoelectric point (pI) of the protein and pH of the BGE employed [11]. The use of SDS in conjunction with a reducing agent is a commonly used procedure for preparation of protein samples for both conventional and capillary gel electrophoresis [18, 19]. This pre-treatment effectively imparts a negative charge on protein molecules proportional to the mass of the protein ($\approx$1.4 g SDS/g protein). Protein–SDS complexes migrate at the same speed under the effect of an applied electric field [18–21].

Here, we used protein denaturation using SDS to cancel differences between protein molecules. In particular, those due to different patterns of PTM of the same protein that result in charge isoforms. The development of a quantitative CZE protocol for determination of total protein concentration that does not involve an interaction with a dye is described. Commonly encountered limitations of protein CE such as adsorption to the capillary wall and limited UV detection sensitivity were tackled. The robustness of the method was evaluated in the presence of salts commonly employed in downstream purification and known of their deleterious effect on electrophoresis techniques.

Experimental

Chemicals and Samples

A BSA stock solution (10.0 mg mL^{-1}) was prepared in 50 mM HEPES buffer (pH 7.0). Denatured BSA samples (1.0 mg mL^{-1}) were prepared by incubation of suitable volumes of BSA stock solution with 35.0 mM SDS (or as specified) and 5% *v*/*v* β-mercaptoethanol at 95 °C for 5 min. The entire milk from consecutive afternoon and morning milking was collected and pooled to form a representative 1-day sample from transgenic cows (TGmilk). All milk samples used in this study were prepared from defatted, freeze-dried milk powder by dissolving suitable amounts in MilliQ water to 10% *w*/*v* concentration. The rhMBP was prepared from TGmilk samples as previously described [3]. Briefly, direct capture of the rhMBP from the TGmilk was achieved using cation exchange chromatography (SP Sepharose BB, GE Healthcare, Uppsala, Sweden). The rhMBP was eluted from the column using 50 mM HEPES (pH 7.0) containing 0.5 M NaCl. Fractions containing the rhMBP were further purified using immobilized metal affinity chromatography (IMAC, Ni^{2+} Sepharose HP, GE Healthcare). A gradient elution was employed (50–500 mM imidazole) and fractions containing the rhMBP were pooled together. The identity, purity, and integrity of the rhMBP in the final preparation were confirmed using SDS-PAGE followed by Western blotting.

CE-grade NaOH (1.0 M) and H_3PO_4 (1.0 M) were obtained from Agilent Technologies, Waldbronn, Germany. Polyethylene oxide (PEO, 100 kDa) and all other chemicals were of analytical grade and were obtained from Sigma (New York, USA).

Instruments

All CE experiments were carried out using an Agilent $HP^{3D}CE$ with a diode array detector (DAD), Agilent Technologies. Electrophoregrams and data analysis were all carried out using 3D-CE Chemstation software (ver. A.10.02), Agilent Technologies. All capillaries used were bare fused silica capillaries (325 μm O.D. and 75 μm I.D.) of total length of 72 cm and effective length to the detection window of 63.5 cm (Agilent Technologies). A special alignment interface, high-sensitivity detection cell that increased the detection path length from 75 to 1,200 μm was purchased from Agilent Technologies. Sample plug volume calculations were carried out using CE Expert software v.1.0 (Beckman Coulter, California, USA).

Analysis Conditions

A background electrolyte (BGE) of 50 mM HEPES (pH 7.0) was used for all electrophoretic runs and sample preparation. Capillaries were pre-flushed with 0.1 M NaOH, 0.1 M H_3PO_4 for 2 min each, MilliQ water for 5 min, and then 0.10% PEO in 50 mM HEPES buffer (pH 7.0). Injection was carried out at 50 mbar for 40 s ($\approx$8% of capillary volume. Separation was carried out at 25 °C under negative polarity conditions (-30 kV $\approx$ -417 V cm^{-1}) and detection was carried out at 214 nm with the detection window at the anodic end of the capillary.

Establishment of the Dynamic Coat

Dynamic coating of the capillaries was achieved using 0.1% PEO in 50 mM HEPES (pH 7.0) as described above. The functionality of the dynamic coat was investigated using a negatively charged marker, Orange G (OG). The repeatability and reproducibility of the dynamic coat were evaluated by analysis of the marker sample (4% OG *v*/*v* in 50 mM HEPES buffer, pH 7.0) over five consecutive runs within the same day and over 2 days.

Effect of SDS Concentration and Sample Volume

A set of denatured BSA samples (1.0 mg mL^{-1}) was prepared in the presence of different SDS concentrations (4.4–35.0 mM). A non-denatured BSA sample was prepared and analyzed under the same conditions for comparative purposes. A set of OG control samples was also prepared using the same SDS concentration range in order to investigate the stacking mechanism. Optimized conditions were used to analyze BSA samples employing an injection time of 10–320 s.

Effect of Salt

The effect of the presence of NaCl in chromatography fractions on the stacking efficiency was investigated. A series of BSA standard solutions covering a wide BSA concentration range was prepared in the presence of (0–200 mM NaCl). Samples were analyzed and results were studied.

Calibration, Validation and Application

A series of BSA standard solutions (0.02–300.00 μg mL^{-1}) was prepared and analyzed. Migration time-corrected integrated peak area (PA) was plotted versus BSA concentration and various validation parameters were derived from the calibration curve. The predictability of the calibration curve was evaluated via analysis of a validation set of BSA standard solutions. Predicted concentrations were compared to the nominal ones and those obtained using Bradford method [22]. Samples of the chromatographic fractions were either desalted or diluted with 50 mM HEPES buffer (pH 7.0) to a salt concentration less than 50 mM before analysis. Results were compared in order to investigate the effect of any NaCl and imidazole traces.

Results and Discussion

In this CZE protocol, protein samples were incubated with SDS and a reducing agent. Denatured protein samples were then injected into a capillary preconditioned and pre-filled with a PEO polymer solution. In the following sections, we investigated the functionality of the preconditioning protocol and the efficiency of sample stacking. Emphasis has been given to understanding of the mechanism of sample stacking and evaluation of the robustness of the method in the presence of salt. Optimized conditions were employed to construct the calibration curve, validate the method and analyze samples of rhMBP.

Establishment of the Dynamic Coat

Optimization of the capillary preconditioning steps and selection of the optimum PEO polymer type has been investigated previously [23]. In this study, 50 mM HEPES buffer (pH 7.0), in which samples were obtained was selected as the BGE in all electrophoretic runs. In addition to the beneficial characteristics of this organic buffer in CE

applications [24–26], it helped minimize the sample preparation time.

The efficiency of the preconditioning procedure was evaluated on the basis of its ability to suppress the EOF and form a reproducible dynamic coat. Orange G is a negatively charged marker commonly used in capillary gel electrophoresis applications for determination of protein molecular weight (MW). It has a small MW and migrates faster than protein–SDS molecules through sieving matrices [18]. In this study, OG was chosen in order to simulate the behavior of the negatively charged protein–SDS molecules. A short plug of OG was analyzed under different conditions (as described above), electrophoregrams and current traces were inspected. The migration of OG towards the anode indicated the suppression of the EOF by the dynamic coat. While in the absence of the dynamic coat, the marker was found to migrate in the opposite direction, under the effect of EOF. A stable current trace of a constant value along the run time (30 min) indicated the stability of the dynamic coat (Fig. 1). The negatively charged marker was analyzed over five consecutive runs within the same day and over 2 days. No significant difference (P 0.05) in the migration time of the marker peak indicated good repeatability and reproducibility of the dynamic coat. Results indicated that using PEO polymer only in preconditioning of the capillary was sufficient to establish a reliable dynamic coat.

Effect of SDS Concentration

Protein–SDS complexes carry a negative charge that is proportional to the mass of the protein as discussed earlier. However, differences exist in the electromigration patterns of these complexes due to the large amount of protein secondary structures that exist in the presence of SDS [21]. It has been reported also that protein unfolding typically occurs above the critical micelle concentration (CMC) of SDS that is less than 4.0 mM in HEPES buffer [20, 27].

When a denatured BSA sample (1.0 mg mL^{-1}) was analyzed, a single, well-resolved peak was detected. This indicated that the BSA–SDS complexes were stacked at the interface between the PEO plug and the sample plug. The effect of SDS concentration on the performance of our method was investigated in more detail. A series of denatured and non-denatured BSA samples (1.0 mg mL^{-1} each) was prepared in the absence of SDS and using a range of SDS concentration (4.4–35.0 mM). In the absence of SDS, a broad peak representing the heterogeneity of the native BSA molecules was detected (Fig. 2e). Notable differences in the BSA peak shapes and migration patterns were noted upon using different SDS concentrations (Fig. 2a–d). These results showed clearly that protein denaturation using SDS was not the only mechanism for obtaining good stacking of protein–SDS complexes. Excess SDS was critical in order to obtain narrow and intense peaks that indicate efficient stacking.

Results showed that protein-SDS complexes migrated much slower than expected, despite negative charges. As SDS concentration decreased, a gradual forward shift in migration time, a decrease in peak height and an increase in peak width was noted. The change in peak shape was considered as an evidence for reduction in the stacking efficiency as the concentration of SDS decreases (Fig. 2). Shifts in t_m upon using different SDS concentration were not easily explained. At such high SDS concentrations, the CMC of SDS in HEPES buffer has been already exceeded [27]. It could be postulated that protein–SDS complexes interacted with excess SDS and formed micelles that migrated slower than free protein–SDS complexes and helped in the stacking process. In future experiments, SDS

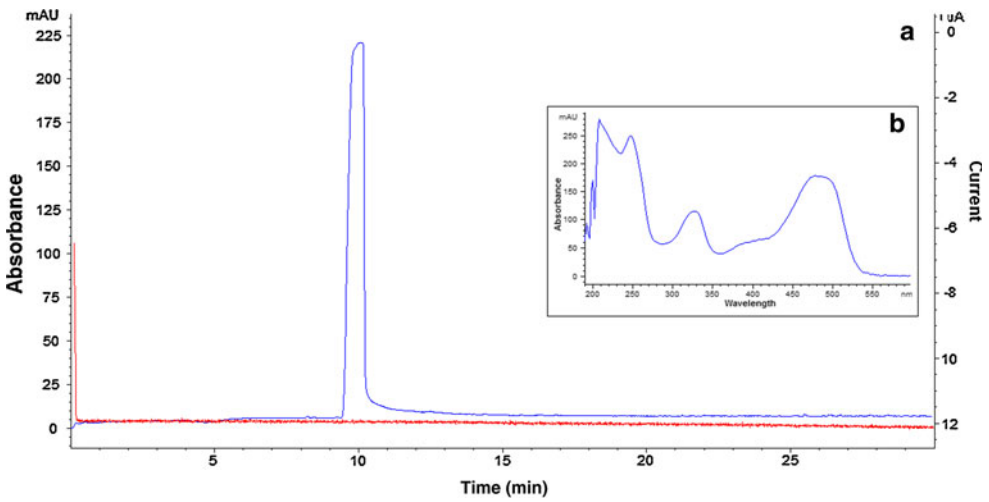

Fig. 1 **a** Electrophoregram and the current trace showing the migration behavior of the negatively charged orange G marker. **b** The DAD absorption spectrum confirming the identity of the orange G marker. Fused silica capillary total/effective length: 72 cm/63.5 cm × 75 µm I.D., voltage: −30 kV, temperature: 25 °C, detection: UV at 214 nm, hydrodynamic injection: 50 mbar—40 s and sample: 4% *v/v* OG in 50 mM HEPES buffer (pH 7.0)

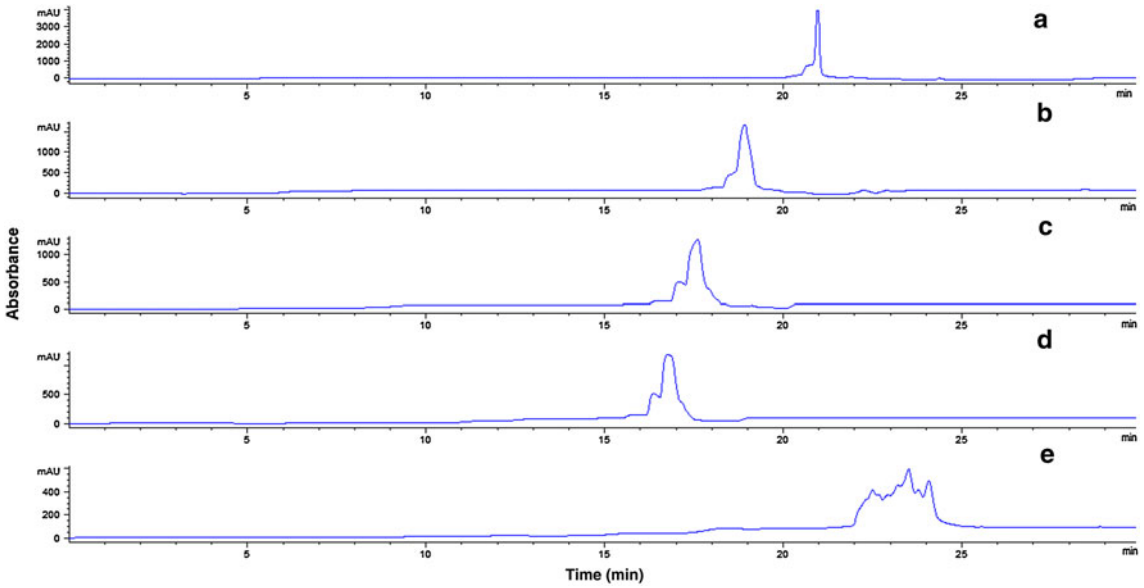

Fig. 2 Electrophoregrams showing the UV traces obtained by analysis of denatured BSA samples using different SDS concentrations: (**a** 35.0, **b** 17.5, **c** 8.8, **d** 4.4 mM) and a non-denatured BSA sample (E). Fused silica capillary total/effective length: 72 cm/63.5 cm × 75 μm I.D., voltage: −30 kV, temperature: 25 °C, detection: UV at 214 nm, hydrodynamic injection: 50 mbar—40 s and sample: 1.0 mg mL^{-1} BSA in the presence of different SDS concentrations

concentration of 35.0 mM was employed for sample preparation in order to facilitate protein quantitation.

Stacking Mechanism

In order to understand the effect of SDS concentration, a set of OG control samples was prepared using a wide range of SDS concentration. The migration behavior of the OG marker was found to change significantly in the presence of SDS. It followed a trend similar to that noted with protein–SDS complexes. Such an agreement confirmed our hypothesis about micelle formation. In the case of OG samples, the SDS concentration range was kept narrow in order to monitor closely the effect of SDS (Fig. 3). Two peaks were detected for OG, as confirmed from the spectra obtained using the DAD. Figure 3 shows a small peak at 10–11.5 min, and a larger peak that pertained to the migration behavior reported for BSA.

The migration behavior of OG marker demonstrated clearly the effect of excess SDS present in the sample plug. Above the CMC, excess SDS formed micelles. Protein–SDS complexes and OG partitioned between the aqueous and micellar phases. This explained the shift in t_m for both of them. Micellar OG migrated faster than micellar protein–SDS complexes, which could be attributed to the difference in molecular weight between OG and BSA. Upon decreasing the SDS concentration, the number of micelles available for the analyte (OG or BSA) to partition into decreased, leading to more analyte in the aqueous phase. As analytes migrated through the capillary, a dynamic equilibrium was established that was detected as a moving peak. This conclusion agrees with what has been reported in literature. The gradual change in migration time is an indication of a dynamic equilibrium. The t_m recorded under these conditions can be utilized to extract valuable information about binding/dissociation kinetics [28, 29].

Effect of Sample Volume

Previous investigations showed that injection of the protein–SDS complexes into a capillary pre-filled with a PEO solution resulted in sample stacking. This approach resulted in a significant improvement in the peak shape and the detection sensitivity. In this experiment, the effect of the sample plug volume relative to the capillary volume was investigated. A set of BSA samples (1.0 mg mL^{-1} each) was analyzed employing injection times of 10–320 s (≈1.8–47.0% of the capillary volume). A peak for BSA was recorded even when the longest injection time was employed, which indicated successful stacking of the protein–SDS complexes. An injection time of 40 s (7.6% of capillary volume) was selected since broad peaks with non-reproducible migration time were noted for injection times longer than that (results not shown).

Effect of Salt

Salts are common additives in chromatography owing to their role in the differential screening of the interactions between protein molecules and the active sites on the

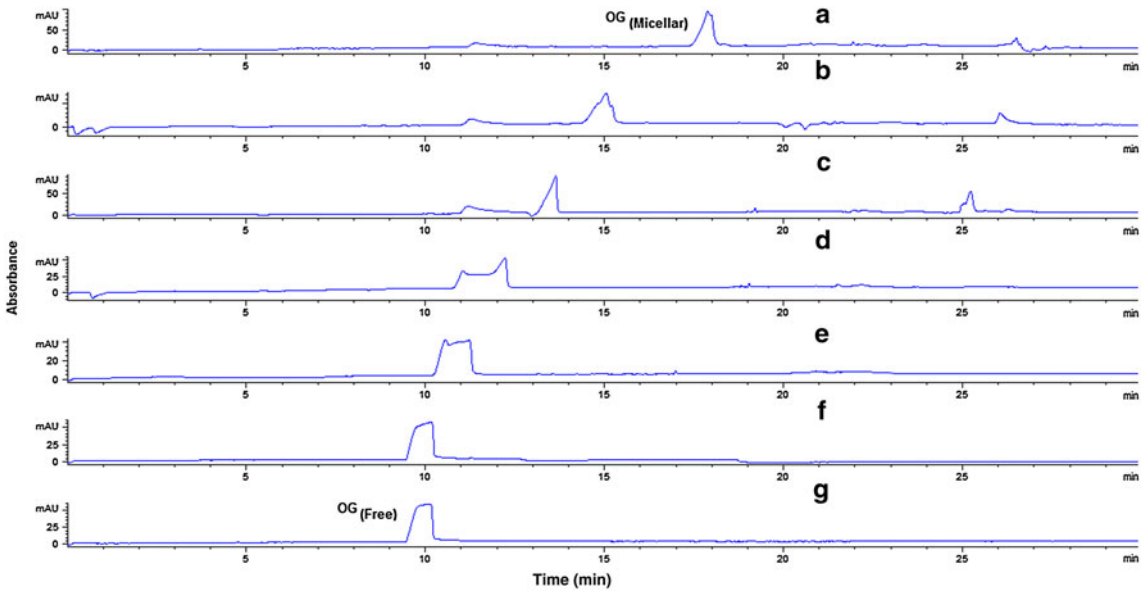

Fig. 3 Electrophoregrams showing the migration behavior of the negatively charged orange G marker using different SDS concentration: (**a** 35.0, **b** 28.0, **c** 22.4, **d** 17.9, **e** 14.3, **f** 11.5 and **g**: 0.0 mM). Fused silica capillary total/effective length: 72 cm/63.5 cm × 75 µm I.D., voltage: −30 kV, temperature: 25 °C, detection: UV at 214 nm, hydrodynamic injection: 50 mbar—40 s and sample: 4% *v*/*v* OG in 50 mM HEPES buffer (pH 7.0). These results confirmed the existence of two different populations of OG (free and micellar) in the capillary lumen. The shift in the migration time of the OG second peak indicated equilibrium between OG(Free) and OG(Micellar)

chromatographic resins. However, salts are known to have deleterious effects on the performance of electrophoretic analytical methods. Desalting is a common practice for samples intended for CE analysis. In addition, changes in the sample ionic strength has been reported to affect protein–SDS interaction [20].

The effect of salt was studied in more detail in order to ensure the robustness of the method and to determine the limit for the concentration of salt in the samples. A series of BSA samples over a wide range of concentration (0.5–10.0 mg mL^{-1}) was prepared in the presence of 0.5 M NaCl. Samples were analyzed before and after a twofold dilution with the BGE. The effect of dilution was found to be critical to obtaining a well-resolved single peak for the protein–SDS complexes, especially at the lower protein concentration range (Fig. 4). These results indicated that the loss of good peak shape was mainly due to disruption of protein–SDS interaction under the effect of salt. An in-depth study of the tolerable range of salt concentration is described below over the linear range of the assay.

Calibration and Validation

In this study, a protocol for the determination of total protein concentration was developed and validated according to ICH guidelines [30]. Selectivity of the method was assessed by analyzing BSA standard samples, milk and chromatography fractions obtained during the downstream purification of rhMBP. A well-resolved peak for protein–SDS, regardless of the type of sample was obtained. This indicated the freedom from interference by matrix components such as salt and imidazole, as will be discussed in detail below. The repeatability and reproducibility of the method was investigated based on the stability of the dynamic coat as discussed above using OG marker. A series of BSA standards (0.02–300 µg mL^{-1}) was analyzed employing an injection time of 40 s. The mean migration time was found to be (21.8 ± 0.5 min) which indicated good reproducibility for the migration time. The PA was utilized in order to improve the reproducibility of the response [31]. The PA values were plotted versus BSA concentration and a linear relationship was obtained over a BSA concentration range of 8.49–135.87 µg mL^{-1}. The regression equation was calculated ($Y = 2.8737X - 6.026$); where Y is the PA and X is the BSA concentration. A correlation coefficient of 0.995 and a random distribution of the residuals showed a good linearity of the calibration curve over this concentration range. The SD of the intercept and the slope of the calibration curve were used to calculate the limit of detection (LOD) and lower limit of quantitation (LLOQ). The calculated LOD (0.26 µg mL^{-1}) was found in good agreement to the visually determined one. However, the predictability of the calibration curve at the calculated LLOQ (0.97 µg/mL) was found to be highly variable. Thus, the lower limit of the linear range of the

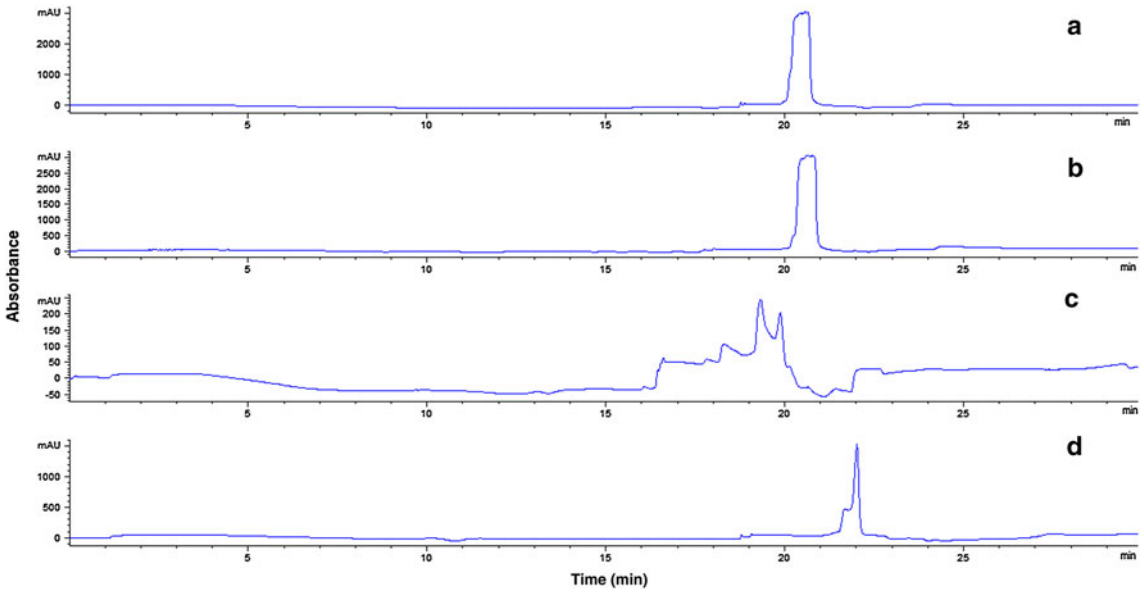

Fig. 4 Electrophoregrams showing the UV traces obtained by analysis of two BSA standard samples containing 0.5 M NaCl before and after twofold dilution with 50 mM HEPES buffer (pH 7.0). **a** and **b** 10.0 mg mL^{-1} BSA before and after dilution, respectively, **c** and **d** 0.5 mg mL^{-1} BSA before and after dilution, respectively. Fused silica capillary total/effective length: 72 cm/63.5 cm × 75 µm I.D., voltage: −30 kV, temperature: 25 °C, detection: UV at 214 nm and hydrodynamic injection: 50 mbar—40 s

calibration range (8.49 µg mL^{-1}) was considered as the LLOQ. The accuracy of the method was evaluated by analysis of a series of BSA standard samples over five concentration levels covering the linear range of the calibration curve. The predicted concentrations were compared to the actual values and to those obtained using Bradford assay. No significant difference (P 0.05) using two-way ANOVA indicated accuracy of the method. The same set of standards was analyzed within the same day and over 2 days, no significant difference in PA proved repeatability and reproducibility. A summary of the data obtained is reported in Table 1.

In order to accurately evaluate the effect of salt on the robustness of the developed method, three BSA standard solutions covering the calibration range of the method were prepared. Each standard sample was prepared in triplicate containing variable amounts of NaCl (0–200 mM). All samples were analyzed using the optimized method. The BSA concentrations were then determined from the calibration curve and compared with their nominal values. No significant difference between the actual and predicted protein concentrations (P 0.05) was noted over salt concentration up to 50 mM (results not shown).

Application to Chromatographic Fractions

Chromatographic fractions (SPBB and IMAC fractions) containing rhMBP were obtained using the described downstream purification protocol. Isoform profiling was carried out using a previously developed capillary isoelectric focusing (CIEF) protocol [23]. Briefly, CIEF experiments were carried out in PEO dynamically coated capillaries employing a mixture of carrier ampholytes covering a pH range of 3–11. Experimental conditions were optimized accordingly in order to stretch the pH gradient over the pI range of rhMBP to reveal the minor differences between rhMBP isoforms [23]. Several bands for rhMBP of pI ranging from 6.0 to 9.0 were detected (Fig. 5) which was attributed to different patterns of PTM. For determination of total protein concentration, samples were diluted with HEPES buffer, denatured, and then

Table 1 Summary of the validation data obtained for the CZE method using BSA standard samples

Parameter	
Regression equation	
Slope	2.8737
Intercept	6.026
Correlation coefficient (R^2)	0.995
Linear range (µg mL^{-1})	8.49–135.87
LOD (µg mL^{-1})	0.26 µg mL^{-1}
Accuracy ($n = 5$)	
CZE assay (% Recovery ± RSD, %)	99.71 ± 0.72
Bradford assay (% Recovery ± RSD, %)	100.48 ± 1.62
Precision ($n = 5$)	
Repeatability (RSD, %)	0.68
Reproducibility (RSD, %)	1.15

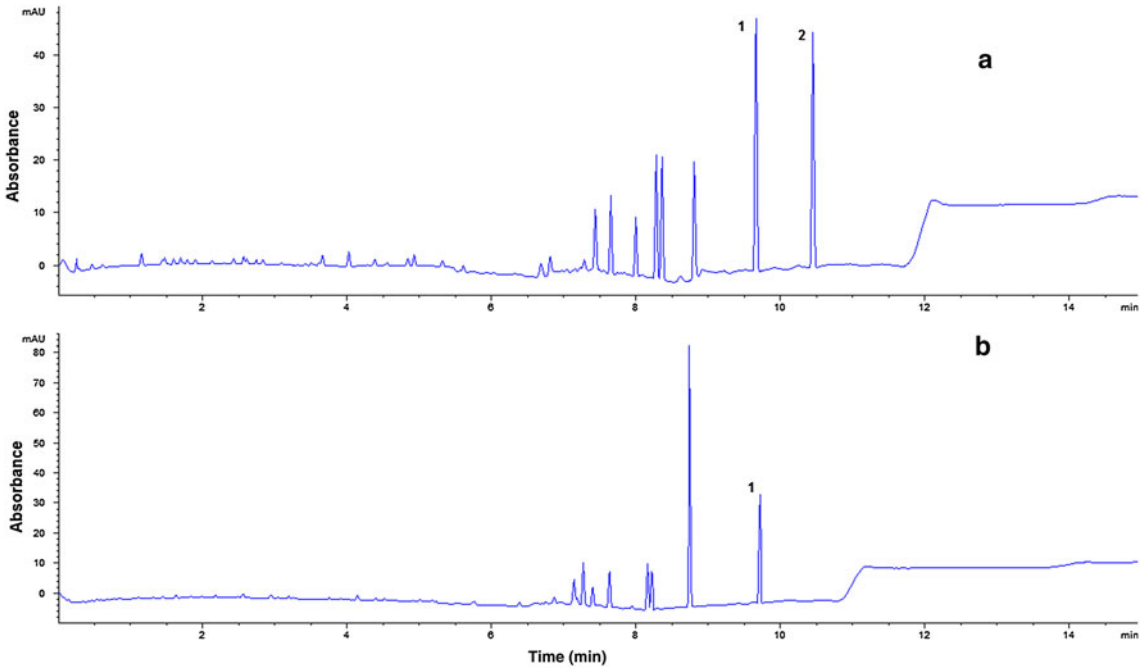

Fig. 5 Electrophoregrams showing the protein composition of TGmilk chromatographic fractions: SPBB (**a**) and IMAC (**b**) using capillary isoelectric focusing. Dynamically coated fused silica capillary total/effective length: 33 cm/24.5 cm × 50 µm I.D., anolyte: 100 mM H_3PO_4, catholyte: 20 mM NaOH, focusing and mobilization: 15 kV—35 mbar, temperature: 25 °C, hydrodynamic injection: 950 mbar—2 min and detection: 280 nm. (1: β-CN, 2: α_s-CN)

Table 2 Summary of the analysis results (recovery and purity) of rhMBP samples obtained during the downstream purification ($n = 3$)

Sample	Volume (mL)	rhMBP (mg)[a]	Recovery (*w/w* %)[b]	Total protein (mg)[c]	Purity (*w/w* %)[d]
TGmilk[e]	25.6	2.60	100	896.25	0.29
Cation exchange chromatography	50.0	2.05	78.85	2.56	80.08
Affinity purification (IMAC)	20.0	2.00	76.92	2.18	91.74

[a] Determined using the dot blotting assay (TGmilk) and ELISA assay (chromatography fractions)

[b] Cumulative recovery %, relative to rhMBP amount in TGmilk

[c] Determined using the CZE assay

[d] rhMBP (mg)/total protein (mg) × 100

[e] 10% defatted freeze-dried transgenic milk

analyzed as described above. Similar to BSA, results showed that dilution was essential in obtaining good peak shape (Fig. S1, Electronic supplementary material). All proteins in the sample were found to elute as one band with a reproducible t_m similar to that of BSA (Fig. S1 and Fig. S2, Electronic supplementary material). This observation confirmed that the suggested pre-treatment eliminated differences between proteins in the sample. No interference was detected from imidazole as shown in Fig. S1, Electronic supplementary material. Total protein concentration was determined from the calibration curve developed using BSA standard. Results were used along with those obtained using ELISA immunoassay for rhMBP in order to determine the recovery and purity of rhMBP (Table 2).

Conclusion

In this study, a CZE protocol with on-line micellar sample stacking was developed in order to determine the total protein concentration in chromatography fractions. The CZE assay provided a good alternative for determination of total protein concentration without the need of color reagents. The sensitivity of detection was enhanced via sample stacking of denatured protein molecules. Detection sensitivity was physically enhanced by using an extended path length detection cell. The protocol was considered valid for determination of the total protein concentration in mixture using a calibration curve developed using BSA standard. Stacking of the protein in one band eliminated

the possibility of interference by other UV absorbing additives. The performance of the method proved to be robust in the presence residual salts from chromatography fractions.

References

1. Zhang C, Van Cott KE (2007) In: Shukla AA, Etzel MR, Gadam S (eds) Process scale bioseparations for the biopharmaceutical industry. Taylor and Francis Group, pp 367
2. Kelner DN, Bhalgat MK (2007) In: Shukla AA, Etzel MR, Gadam S (eds) Process scale bioseparations for the biopharmaceutical industry. Taylor and Francis Group, pp 395–418
3. Al-Ghobashy MA, Williams MAK, Brophy B, Laible G, Harding DRK (2009) J Chrom B 877:1667–1677
4. Al-Ghobashy MA, Williams MAK, Laible G, Harding DRK (2010) J Mol Recognit 23:84–92
5. Lutton JD, Winston R, Rodman TC (2004) Exp Biol Med 229:12–20
6. Harauz G, Ishiyama N, Hill CMD, Bates IR, Libich DS, Fares C (2004) Micron 35:503–542
7. Planning S (2007) Curr Protoc Protein Sci Unit 3.4:1–29
8. Dolnik V (2008) Electrophoresis 29:143–156
9. Chun M-S, Kang D, Kim Y, Chung D (2001) Microchem J 70:247–253
10. Chang P-L, Chiu T-C, Chang H-T (2006) Electrophoresis 27:1922–1931
11. Huang Y-F, Huang C-C, Hu C-C, Chang H-T (2006) Electrophoresis 27:3503–3522
12. Horvath J, Dolnik V (2001) Electrophoresis 22:644–655
13. Miksik I, Sedlakova P, Mikulikova K, Eckhardt A, Cserhati T, Horvath T (2006) Biomed Chromatogr 20:458–465
14. Huang Y-F, Hsieh M-M, Tseng W-L, Chang H-T (2006) J Proteome Res 5:429–436
15. Horka M, Ruzicka F, Horky J, Hola V, Slais K (2006) J Chromatogr B 841:152–159
16. Kuo C-Y, Chiou S-S, Wu S-M (2006) Electrophoresis 27:2905–2909
17. Lucy CA, MacDonald AM, Gulcev MD (2008) J Chromatogr A 1184:81–105
18. Hu S, Jiang J, Cook LM, Richards DP, Horlick L, Wong B, Dovichi NJ (2002) Electrophoresis 23:3136–3142
19. Laemmli UK (1970) Nature 227:680–685
20. Otzen DE (2002) Biophys J 83:2219–2230
21. Parker W, Song PS (1992) Biophys J 61:1435–1439
22. Zor T, Selinger Z (1996) Anal Biochem 236:302–308
23. Al-Ghobashy MA, Williams MAK, Harding DRK (2008) Anal Lett 41:1914–1931
24. Varenne A, Descroix S (2008) Anal Chem Acta 628:9–23
25. Stoyanov AV, Pawliszyn J (2004) Analyst 129:979–982
26. Bouškova E, Presutti C, Gebauer P, Fanali S, Beckers JL, Bocek P (2004) Electrophoresis 25:355–359
27. Thorsteinsson MV, Richter J, Lee AL, DePhillips P (2005) Anal Biochem 340:220–225
28. Liu Y, Ling X, Fan H, Zhong Y, Li D, Wang Y (2007) J Chromatogr A 1143:284–287
29. Jing P, Kaneta T, Imasaka T (2005) Anal Sci 21:37–42
30. ICH (2005) Validation of analytical procedures: test and methodology. In: International conference on harmonization of technical requirements for registration of pharmaceticals for human use.
31. Goodall DM, Williams SJ, Lloyd DK (1991) Trends Anal Chem 10:272–279