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Journal of Chromatography B, 729 (1999) 355–359

JOURNAL OF CHROMATOGRAPHY B

Short communication

Effect of salt concentration on separation patterns in static capillary isoelectric focusing with imaging detection☆

Qinglu Mao, Janusz Pawliszyn*

Department of Chemistry, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada

Received 23 December 1998; received in revised form 18 March 1999; accepted 22 March 1999

Abstract

Salts introduced into protein samples have an impact on the pH gradient in free solution in isoelectric focusing (IEF), which is reflected by the separation pattern. In this study, samples containing different concentrations of phosphate-buffered saline (PBS) were focused in capillary format and detected in a real-time mode using an imaged capillary isoelectric focusing (CIEF) system at 280 nm. It was observed that salt compressed the pH gradient with a degree of 4.3% at a PBS concentration interval of 10 m*M*. As a result, the same sample components, therefore, were focused at different positions inside the capillary. Using two p*I* markers as the internal standards, the separation patterns in the presence of salts were corrected to the salt-free matrix by simply stretching the electropherograms. The stretched electropherograms of model samples, p*I* markers and myoglobin, demonstrated the feasibility of this correction. This simple method is promising for identifying proteins, which may exhibit different p*I* values after their mutation and stability process, when salt is present in the sample.

Keywords: Imaging detection; Salt concentration; Proteins

1. Introduction

Capillary isoelectric focusing (CIEF), which was first reported by Hjerten and Zhu [1], provides excellent resolution of proteins with the advantage that separations are carried out in a capillary format with on-tube detection and automated analysis [2]. Recently, imaged CIEF, where IEF is performed in the absence of flow, has been developed [3]. Due to the elimination of the mobilization step, imaged CIEF offers several advantages over the single-point detection CIEF, such as reduced protein precipitation, short analysis time, stable pH gradient and uniform resolution [4]. Also, sample introduction can be accomplished as for HPLC, eliminating any unpredictable influences caused by different sample loading methods in the single-point detection CIEF mode [5]. Moreover, peak widths are not broadened by the detection, unlike single-point detection where degree of peak broadening is related to the migration time [5]. In general, effects of external factors, such as sample loading and detection method, on the separation pattern can be neglected in imaged CIEF. The fundamental aspects of imaged CIEF for protein analysis are of interest. The effect of sample salt

☆Presented at the 9th Annual Frederick Conference on Capillary Electrophoresis, Frederick, MD, October 19–21 1998.

*Corresponding author. Tel.: +1-519-888-4641; fax: +1-519-746-0435.

E-mail address: janusz@sciborg.uwaterloo.ca (J. Pawliszyn)

0378-4347/99/$ – see front matter
PII: S0378-4347(99)00129-2

concentration on the separation pattern is discussed in this communication.

Protein samples are prepared by several procedures, many of which introduce salts. These salts can affect the pH gradient in capillary isoelectric focusing. Through mutation or stability processes, proteins may change into one or more new variants with different p*I* values [6]. In the presence of different salt concentrations, the small change of p*I* values often cannot be properly identified because of the effect of salt concentration on the separation pattern. For this reason, it is necessary to correct the separation pattern. Using the CIEF system with focusing and mobilization, Rodriguez-Diaz et al. [7] reported that salt content can have a significant adverse effect on reproducibility. The use of internal p*I* markers facilitates peak identification by p*I* values, and also corrects variations due to the multiple parameters that can influence the migration times of the protein zones. However, it represents migration time, and is an indirect observation for the pH gradient compression. In this study, the effect of salts on the separation pattern are investigated using imaged CIEF. The separation patterns under different sample salt concentrations are corrected by simply stretching the electropherograms by reference to the two p*I* marker internal standards.

2. Experimental

2.1. Apparatus

The experiments were performed using the iCE280 imaged CIEF system (Convergent Bioscience, Toronto, Canada). The detector is operated in absorption mode at 280 nm. A 5-cm long capillary internally coated with fluorocarbon was assembled in a cartridge format [8]. The coated capillary (I.D. 100 μm, O.D. 170 μm) was obtained from J&W Scientific (Folsom, CA, USA). The cartridge is connected with an eight-port, two-position valve for sample introduction. The loop volume is 2.5 μl, while the amount of the sample injected into the injection port is 10 μl [3,4]. A syringe pump (Model A-90, Razel Scientific Instruments, Stamford, CT, USA) was used to deliver the washing solution. The sample injection and the imaged CIEF instrument are fully controlled by a PC computer.

2.2. Reagents and chemicals

All chemicals were of analytical-reagent grade, and solutions were prepared using deionized and distilled water. Methylcellulose (4000 cP for 2% soln.) was purchased from Aldrich (Milwaukee, WI, USA). Phosphate-buffered saline (PBS) was obtained from Sigma (St. Louis, MO, USA). Low molecular mass p*I* makers (substituted monomethylphenols), 10.1, 8.6, 7.4, 6.6 and 5.3, were purchased from Bio-Rad (Missisauga, Canada). *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED), used as blocking reagent, was obtained from Sigma. Myoglobin was purchased from Sigma. Samples were prepared by mixing p*I* markers, final concentration, 5 μg/ml each, or/and myoglobin, 100 μg/ml, with 2% carrier ampholyte solution (Pharmalyte, pH 2–11, Sigma), 0.15% methylcellulose, and 0.1% TEMED.

2.3. Procedures

The detailed procedure has been described in elsewhere [3,4]. To substantially reduce the electroosmotic flow (EOF), the capillary was conditioned with 0.15% methylcellulose solution for 20 min before focusing. To match this treatment, all the sample solutions and the electrolytes also contained 0.15% methylcellulose. To perform imaged CIEF, the two electrolyte reservoirs were first filled with anolyte (100 m*M* H_3PO_4) and catholyte (40 m*M* NaOH), respectively. Samples were injected to the 2.5-μl loop fixed on the valve and then delivered into the separation capillary by a microsyringe pump. After the sample solution inside the capillary is stabilized for 20 s, a high voltage of 1.5 or 3 kV is applied to start the isoelectric focusing. The focusing time was set at 3.5 min, and the dynamic focusing process can be monitored every 20 s using the imaging detection system.

3. Results and discussion

3.1. isoelectric focusing performed in free solution in the absence of flow

The imaged CIEF technique, employing whole column imaging detection, allows isoelectric focus-

ing to take place in the absence of flow. The internal coating of capillary is very important to ensure that the focused zones remain static after focusing. If EOF in a capillary is significant, the advantages of imaged CIEF will be lost. Both static and dynamic coating have proven effective for reducing the EOF. Several polymer coatings provide pH stability and are suitable for CIEF [6]. In our experiments, fluorocarbon-coated separation capillary was adopted, combining with the conditioning procedure, where 0.15% methylcellulose was used to condition the capillary for 20 min. After this treatment, EOF is substantially reduced, and hundreds of runs have been observed to be reproducible [3]. Fig. 1 shows three consecutive runs for one sample containing five p*I* markers. For 10 replicate runs, standard deviation of p*I* marker 8.6 is 0.05 pH units for the pH 2–11 carrier ampholytes, which corresponds to a RSD of 0.6%. Using the relative position (set one p*I* marker as internal standard), the standard deviations are less than 0.01 pH units providing RSD of about 0.1%.

One of the significant advantages of imaged CIEF is that a dynamic focusing process can be displayed in a real-time mode, which provides a direct observation of the fundamental aspects of this equilibrium method and eases the control to the optimization process. The additional information obtained from the dynamic focusing process is helpful for the identification due to the different focusing patterns for individual proteins. As shown in Fig. 2, the dynamic focusing process is well displayed. The last four images (120, 160, 200 and 240 s) show that the focused zones remain static. This demonstrates that the EOF has been substantially reduced. As observed in the electropherograms, the optimum focusing time for the samples is about 3.5 min.

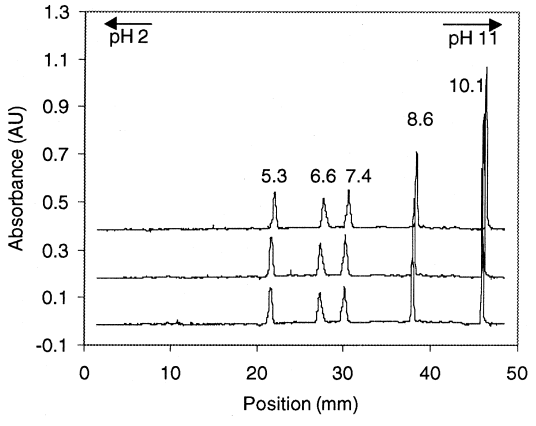

Fig. 1. Electropherograms for three consecutive runs of five p*I* markers separated by absorption imaged CIEF. The p*I* marker concentrations were 5 μg/ml each. All samples contained 2% pH 2–11 ampholytes, 0.5% TEMED and 0.15% methylcellulose.

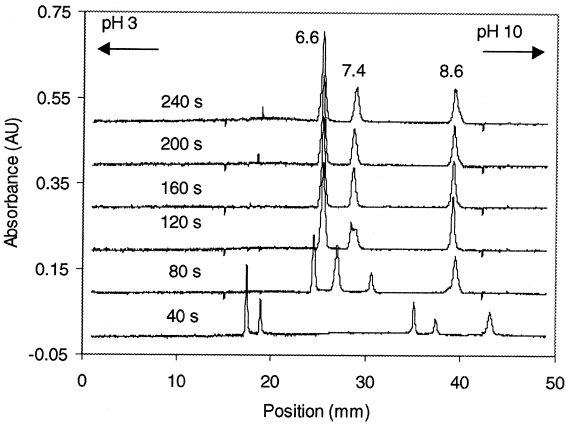

Fig. 2. Dynamic focusing process of three p*I* markers displayed by imaged CIEF. The concentrations of the three p*I* markers were 5 μg/ml each; 0.15% methylcellulose and 2% pH 3–10 Pharmalytes were added to the sample, and 3.0 kV voltage was applied. Images were taken at a time interval of 40 s.

3.2. Effect of salt concentration on the separation pattern

The impact of salts on the separation pattern was investigated using model compounds. Five p*I* markers, 10.1, 8.6, 7.4, 6.6, and 5.3, are mixed with different amount of PBS. The CIEF was then performed for 3.5 min to obtain the separation patterns. Fig. 3 shows a set of separation patterns of five p*I* markers in the presence of five different salt concentrations. It is obvious that salt compressed the pH gradient as concentration increased. The degree of the compression is observed as 0.2 pH unit in the range of 5.3 to 10.1 (4.8 pH unit), i.e. 4.3%, at a PBS concentration interval of 10 m*M*. In another example, shown in Fig. 4, the separation pattern for myoglobin mixed with two p*I* markers, 10.1 and 5.3, was also compressed. In particular, the minor variant of myoglobin, p*I* 6.8 (*), disappeared with the increase in salt concentration. This demonstrates that salt has a large influence on the proteins and their focusing pattern.

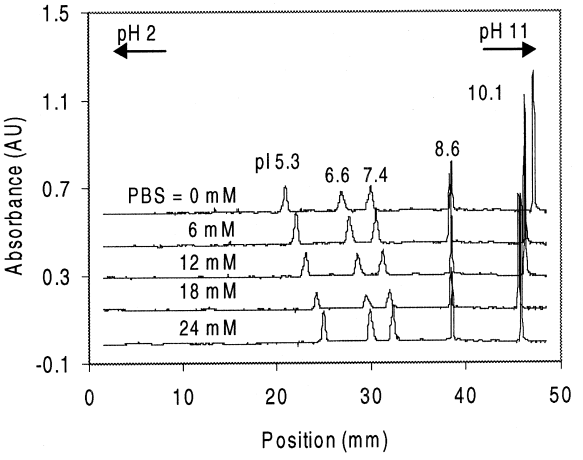

Fig. 3. Electropherograms for a mixture of five p*I* markers with five different PBS concentrations, separated by absorption imaged CIEF. The p*I* marker concentrations were 5 μg/ml each. All samples contained 2% pH 2–11 ampholytes, 0.5% TEMED and 0.15% methylcellulose. The applied voltage was 3 kV.

The focusing current is also an index for this effect. The higher the salt concentration, the higher the starting focusing current is. In general, the mechanism of salt effect is similar to that of the blocking reagent, TEMED. During the focusing process, salt is stripped out of the sample solution in the presence of an applied electric field. It migrates towards the two ends of the separation capillary and finally forms two zones and, therefore, squeezes the pH gradient inside the capillary. In addition, as mentioned above, salt compressed the protein focusing, as a result, the minor variant of myoglobin disappeared at high salt concentration.

3.3. Correction of separation pattern

As observed in Figs. 3 and 4, the compression extent of the pH gradient is closely related to the concentrations of salts present in the samples. For this reason, we proposed to stretch the electropherograms, using the two p*I* markers bracketing the peaks of interest as references. The positions of the two p*I* markers in different samples are aligned to the same position. Fig. 5 shows the results of stretching the electropherograms of the five p*I* markers (originally shown in Fig. 3). The figures shows that the positions of the three p*I* markers located between the outermost ones are in good agreement. The RSD for the peak positions for the three p*I* markers, 8.6, 7.4 and 6.6 in the five samples is 0.2%. The separation patterns of myoglobin in the presence of different salt concentrations also demonstrated the feasibility of this correction method, as shown in Fig. 6. The RSD of the peak positions for myoglobin 7.2 in the nine runs is 0.1%. The proposed method is promising

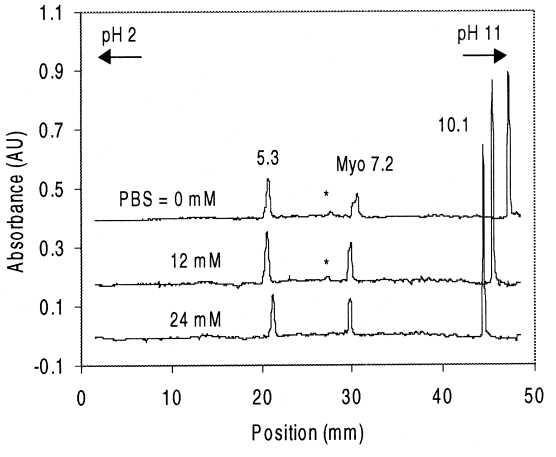

Fig. 4. Electropherograms for myoglobin under three different PBS concentrations separated by absorption imaged CIEF. p*I* makers 5.3 and 10.1, with concentrations of 5 μg/ml each, were used as internal standards. All samples contained 2% pH 2–11 ampholytes, 0.5% TEMED and 0.15% methylcellulose. The applied voltage was 3 kV.

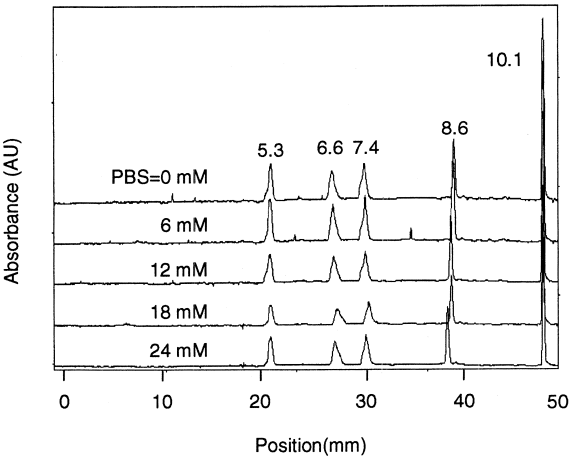

Fig. 5. Correction of the electropherograms of five p*I* marker mixture samples with five different PBS concentrations originally shown in Fig. 3.

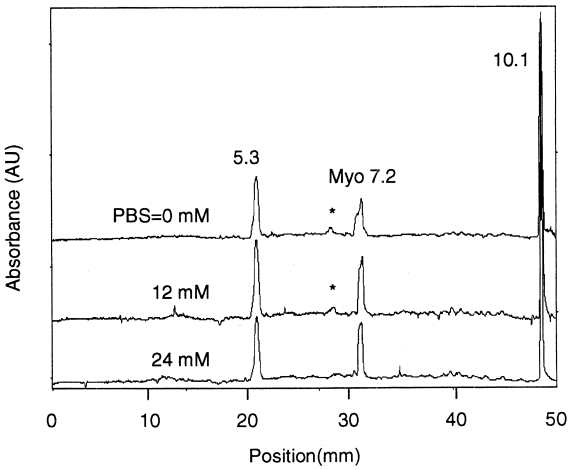

Fig. 6. Correction of the electropherograms of myoglobin mixed with two p*I* markers. The experimental conditions are the same as in Fig. 4. The focusing for each sample was repeated three times.

not only for the determination of mutation and stabilization effects but also for the situation where the concentration of salt is under the salt tolerance level (less than 30 m*M*). In this case, no desalting is necessary as only the correction of peak positions is needed.

Acknowledgements

This work was supported by the Natural Sciences and Engineering Research Council of Canada, and the Convergent Bioscience, Ltd. We would like to thank Dr Jiaqi Wu and Arthur Watson, of Covergent Bioscence, Ltd., for their valuable discussion and help, and Heather Lord for her assistance in preparing the manuscript.

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