Isoelectric Focusing Sample Injection for Capillary Zone Electrophoresis in a Fused Silica Capillary

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An improvement has been made to couple isoelectric focusing (IEF) sample injection and capillary zone electrophoresis in an untreated fused silica capillary. Electroosmotic flow is efficiently prevented by simply using a rubber block at the outlet end of the capillary during IEF sample injection. The experimental conditions that affect the concentration effect are discussed. A concentration enhancement factor of over 100-fold can be easily obtained for two model proteins: lysozyme and ribonuclease A.

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Introduction

Capillary electrophoresis (CE) has been developed into one of the most powerful techniques for the separation and detection of proteins, owing to its high separation efficiency and short analysis time.^{1,2} However, conventional CE is limited to a relatively poor concentration detection limit due to the low volume loadability of the capillary;^{3,4} also, sample preconcentration is always considered before CE separation.⁵⁻¹⁵ Among various concentration methods, isoelectric focusing (IEF) has been demonstrated to be an effective approach for the concentration of proteins.^{14,15} In a conventional IEF, carrier ampholytes are used to establish a stable pH gradient.¹⁶ Recently, IEF without carrier ampholytes¹⁷ and carrier ampholyte free-IEF¹⁸ are also being developed, and have shown the feature of a high concentration factor. To couple IEF and other CE modes, two capillaries were used in a traditional manner,¹⁸ but a dead volume and sample loss were generally inevitable in the coupling of two different capillaries. Recently, we have proposed a method to combine IEF with CE in a single deactivation capillary.¹⁹ By our method, a short section of a porous capillary wall was made near the injection end of the capillary. The inlet end was immersed into protein samples with pH 3.0 buffer, while the porous section was immersed into a sodium hydroxide solution. When H^+ from the inlet end electromigrated toward the porous section, and OH^- electromigrated toward the inlet end from the porous section, the H^+ met OH^- , and a neutralization reaction occurred. A pH gradient formed at the boundary of the neutralization reaction in the capillary (Fig. 1A). When positively charged proteins electromigrated to the boundary, they were isoelectrically focused at a point corresponding to their respective isoelectric point (pI). This means that the proteins would concentrate at the neutralization reaction boundary. After a certain time, the sample was replaced by a buffer, and a separation voltage was

applied across the two ends of the capillary for carrying out conventional CE in the whole capillary. The focused proteins were recharged positively by combining with H^+ and migrated towards the detector in the CE process. A concentration enhancement factor of over 100-fold could be easily obtained for proteins, such as bovine serum albumin (BSA), lysozyme and ribonuclease A. The method possesses the advantage of no existence of a dead volume and no sample loss, as well as no need for coupling components.

However, this method is difficult to apply in an untreated capillary. For an untreated fused silica capillary, the electroosmotic flow (EOF) drove the neutralization reaction boundary to migrate along the capillary, as illustrated in Fig. 1B. This resulted in a broadening of the boundary, which in turn induced a broadening of the focused proteins. Therefore, the protein peaks were very broad, and the concentration factor decreased greatly. Although EOF can be greatly eliminated by

Fig. 1 Illustration of IEF sample injection for proteins in a deactivated capillary (A) and untreated fused silica capillary (B).

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Fig. 2 Experimental setups for IEF sample injection in an untreated fused silica capillary with a rubber block attached on the outlet end (A) and the following CE (B).

coating the capillary wall,²⁰ the coating method can not completely eliminate the EOF. In the present work, an improvement was made to couple the IEF sample injection and capillary zone electrophoresis (CZE) in an untreated fused silica capillary. EOF was efficiently prevented by simply using a rubber block at the outlet end of the capillary during IEF sample injection (Fig. 2A). Lysozyme and ribonuclease A were used as two model proteins to investigate the experimental conditions affecting the concentration effect (BSA used in our previous work was not used here because of its severe adsorption problem).

Experimental

All reagents were commercially available and of analytical grade. All solutions were prepared with distilled deionized water. The CE system consisted of a high-voltage power supply (Matsusada Precision Inc., Osaka, Japan), an untreated fused silica capillary (i.d., 50 μ m; o.d., 375 μ m; GL Sciences Inc., Tokyo, Japan), and an UV absorbance detector (CE-2070, Japan Spectroscopic Co. Ltd., Tokyo, Japan). The detection wavelength was set at 220 nm. The total length of the capillary was 50 cm, and the length from the inlet end to detector was 40 cm. Before use, the capillary was rinsed with a diluted sodium hydroxide solution, water and a buffer solution (pH 3.0) in turn.

A short section of the capillary wall at about 2 cm from the inlet end was made to be porous by the HF etching method.¹⁹ Only small molecules and ions could selectively pass through the porous wall.

Figure 2 shows the setup for IEF sample injection and CE performed in an untreated fused silica capillary. Firstly, the capillary and vial 3 were filled with a tris-phosphoric acid running buffer (pH 3.0). Secondly, proteins dissolved in the buffer and 0.1 M sodium hydroxide solutions were added to vials 1 and 2, respectively. The position of the capillary was adjusted to make the outlet end tightly contact with a rubber block in the bottom of vial 3. Thirdly, an electric voltage of 800 V was applied across vials 1 and 2 for a certain time (Fig. 2A). Fourthly, the protein sample and sodium hydroxide solutions in vials 1 and 2 were replaced with a pH 3.0 buffer solution, and the outlet end of the capillary was lifted away from the rubber block. Finally, a voltage of 20 kV was applied across the two ends of the capillary (Fig. 2B).

Fig. 3 Electropherograms for 1.5×10^{-6} g/mL of the model proteins with electric voltages of 400 V (a) and 800 V (b) across vials 1 and 2. Other conditions for IEF sample injection were as follows: injection time, 40 min; pH in vials 1 and 2, 3.0 and 13.0, respectively. The conditions for CE were: pH of three vials, 3.0; the electric voltage was 20 kV across the whole capillary. Peaks 1 and 2 were lysozyme and ribonuclease A, respectively.

Results and Discussion

When IEF-CE was performed in an untreated fused silica capillary, the sample focusing and CE separation were significantly influenced by EOF. The migration times and peak heights for the two model proteins were unrepeatable. Moreover, all of the obtained peaks were broad. This might be related to migration of the neutralization reaction boundary, as shown in Fig. 1B, driven by EOF, along the separation capillary during IEF sample injection. Although a dynamic coating with hydroxypropylmethylcellulose (0.5%, m/m) was tried to eliminate the EOF, the problems described above still existed because EOF could not be completely prevented. A piece of rubber was then closely attached to the outlet end of the capillary in the IEF sample-injection process, as illustrated in Fig. 2A. It was found that the EOF was efficiently eliminated. As a result, the IEF of proteins occurred in the narrow boundary of the neutralization reaction during sample injection.

As can be expected, the concentration factor increased with a decrease in the width of the focused zone. In our previous work, the length of capillary between vials 1 and 2 was 5 cm. Here, it was shortened to 2 cm. The corresponding focused zone became narrower, and the obtained peaks were sharper.

A constant injection voltage of 400–1000 V was applied across vials 1 and 2 during IEF sample injection. As Fig. 3 shows, the larger was the injection voltage, the higher were the protein peaks. Because a larger injection voltage means a higher electrophoretic velocity of the analytes, under the condition of a constant sample injection time a larger concentration factor could be obtained. However, if the injection voltage was larger than 800 V, it was observed that bubbles were easily produced in the focusing section of the capillary. Hence, the electrical voltage applied between vials 1 and 2 was below 800 V during the injection process.

The effect of the injection time on the concentration of proteins was also investigated. Figures 4a, 4b and 4c illustrate electropherograms of 5.0×10^{-6} g/mL model proteins with injection times of 20, 30 and 40 min, respectively. The peak heights of two model proteins increased with the injection time. A comparison of the IEF-CE method with a conventional CE was made. Figure 5 shows electropherograms obtained from a conventional CE method for 5.0×10^{-5} g/mL (a) and the present method for 5.0×10^{-7} g/mL (b) of the model proteins. The concentration enhancement factor was calculated to be 150 for

Fig. 4 Electropherograms for 5.0×10^{-6} g/mL of the model proteins with injection times of 20 (a), 30 (b) and 40 (c) min, respectively. Other conditions were the same as those in Fig. 3b. Peaks 1 and 2 were lysozyme and ribonuclease A, respectively.

lysozyme and 140 for ribonuclease A when the injection time was 50 min. Compared to the previous work,¹⁹ the present method shows an improved peak shape and better separation efficiency. The plate number was greatly increased for either of the two model proteins, especially for ribonuclease A. In previous work, the plate number was only 1.9×10^4 for lysozyme and 2.4×10^3 for ribonuclease A, while in the present method it was increased to 3.1×10^4 and 4.6×10^4 , respectively. Also, good repeatability of the migration time and the peak height was observed. In all cases, the relative standard deviations (RSDs) for either of the two proteins were below 1% and 5% ($n = 5$) for the migration time and the peak height, respectively. The peak heights were proportional to the concentration of the two model proteins in the range of 5.0×10^{-7} – 5.0×10^{-5} g/mL. The detection limits for the two model proteins were 1.5×10^{-7} g/mL when the injection time was 50 min.

In summary, IEF sample injection and CZE were successfully coupled in an untreated fused silica capillary. By simply using a rubber block at the outlet end of the capillary, EOF was efficiently eliminated during IEF sample injection. Applications of the method will be further reported.

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Fig. 5 Electropherograms obtained with a conventional CE method for 5.0×10^{-5} g/mL (a) and the present method for 5.0×10^{-7} g/mL (b) of the model proteins. In the conventional CE method, the electrokinetic injection voltage was 20 kV across the whole capillary and the injection time was 10 s. In the present methods, the injection time was 50 min and all other conditions were the same as those given in Fig. 3b. Peaks 1 and 2 were lysozyme and ribonuclease A, respectively.

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