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Short communication

Stepwise gradient of buffer concentration for capillary electrochromatography of peptides on sulfonated naphthalimido-modified silyl silica gel

Kaname Ohyama, Mitsuhiro Wada, Naoya Kishikawa, Yoshihito Ohba, Kenichiro Nakashima, Naotaka Kuroda*

Graduate School of Biomedical Sciences, Course of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

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Abstract

The advantage of using a stepwise gradient of buffer concentration in CEC was demonstrated with the mixed-mode stationary phase, 3-(4-sulfo-1,8-naphthalimido)propyl-modified silyl silica gel (SNAIP). Before the application of a stepwise gradient, the effect of buffer concentration on the separations of six peptides and tryptic digests was investigated. Bubble formation caused by Joule heating at currents up to 95 μA was successfully suppressed by using SNAIP column even without pressurization, which contributed to a stepwise gradient of buffer concentration. Utilizing the stepwise gradient improved and shortened the separation of six peptides as compared to the separation under an isocratic elution.

Keywords: Capillary electrochromatography; Peptides; Stepwise gradient; 3-(4-Sulfo-1,8-naphthalimido)propyl-modified silyl silica gel

1. Introduction

Capillary electrochromatography (CEC) attracts the increasing attention as a separation technique for charged biomolecules, such as peptides and proteins, because it provides high efficiency and selectivity by the combination of liquid chromatography and electrophoresis [1,2]. In CEC of charged analytes, mixed-mode stationary phases have become a valuable alternative to the conventional octadecyl silica (ODS) which hardly retains charged analytes [1,3,4]. To further explore the potential of mixed-mode stationary phases for the separation of a wide variety of charged analytes, it is important to utilize the gradient elution.

In order to improve the separation of complex samples with CEC, several methods of gradient elution of mobile phase (e.g. stepwise, pressurized, electrically assisted), voltage and temperature were suggested [5]. Among them, the stepwise gradient of mobile phase on a commercial CE instrument was proved to be easily realized with high accuracy and has been successfully applied to the analyses of drugs [6,7], DNA additives [8] and aromatic compounds [9]. Using a mixed-mode stationary phase for the separation of charged analytes, the gradients of not only hydro-organic composition but also buffer concentration in the mobile phase must be useful. Especially when the electrostatic interaction is dominant in the retention by mixed mode stationary phase, the gradient elution of buffer concentration will be more effective. However, the gradient of buffer concentration has never been explored in CEC while that of mobile phase composition has been practically used in CEC with different gradient methods [6,7,10–13]. This is mainly because the major focus of previous CEC studies was on the separation of neutral compounds by reversed-phase mode. In addition, when the buffer concentration is too high, the bubbles can be generated within a capillary column by Joule heating, which hinders the possibility of gradient of buffer concentration.

* Corresponding author. Tel.: +81 95 819 2894; fax: +81 95 819 2444.
E-mail address: n-kuro@net.nagasaki-u.ac.jp (N. Kuroda).

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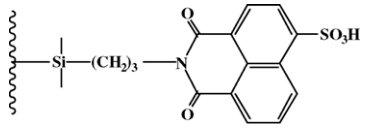

Fig. 1. Structure of 3-(4-sulfo-1,8-naphthalimido)propyl-modified silyl silica gel.

We recently developed a novel mixed-mode stationary phase for CEC, 3-(4-sulfo-1,8-naphthalimido)propyl-modified silyl silica gel (SNAIP, Fig. 1), that was synthesized with the idea to use the naphthalimido moiety and sulfonic acid groups as the chromatographic retentive sites [14,15]. Also, the sulfonic acid groups work as an EOF generator and actually provided high EOF even at low pH. The separation mechanism in CEC with SNAIP was a hybrid of electrophoretic migration and chromatographic retention involving hydrophobic, electrostatic as well as π–π interactions [14]. Under an isocratic elution, the SNAIP column has already been proved to be better for the electrochromatographic separation of charged peptides than other mixed mode columns [15]. In addition, no bubble was observed within SNAIP column even when the current reached 40 μA. This remarkable characteristic will expand the range of buffer concentration in a mobile phase and make the gradient of it effective.

In this study, to apply the stepwise gradient of buffer concentration for CEC with SNAIP, the effect of buffer concentration on the CEC separations of peptides as well as protein digests was investigated. Finally, we described the introduction of the stepwise gradient of buffer concentration to the separation of peptides by CEC on SNAIP.

2. Experimental

2.1. Materials and instrumentation

All chemicals were of analytical grade. 4-Sulfo-1,8-naphthalic anhydride potassium salt was purchased from Aldrich (Milwaukee, WI, USA). The sources of model peptides were as follows: Gly–Val, Gly–Ile and Gly–Phe from Tokyo Kasei (Tokyo, Japan), Gly–His, Gly–Lys and Lys–Lys from Sigma (St Louis, MO, USA). The stock solutions of model peptides were prepared by dissolving 1 mg of each compound in 1 mL of water. The stocked peptide mixture or digests were diluted to appropriate concentrations (model peptides: 12.8–60 mg/L) with a mobile phase prior to injection. Horse heart cytochrome *c* from Wako (Osaka, Japan) was dissolved in a buffer solution containing 50 mM ammonium bicarbonate and 2 mM calcium chloride (pH 7.8) at a concentration of 10 mg/mL. [(L-Tosylamido-2-phenyl)ethylchloromethylketone] (TPCK)-treated trypsin (Sigma) was prepared in water at a concentration of 0.2 mg/mL. The digestion of cytochrome *c* was carried out by mixing 150 μL of each solution for 12 h at 37 °C. The enzymatic digestion was stopped at 80 °C for 10 min and the digests were stored at −20 °C.

All the CEC experiments were performed on a CAPI-3200 system equipped with a photodiode array detector (Otsuka Electronics, Osaka). Fused-silica capillaries (375 μm o.d. × 75 μm i.d.) were obtained from Polymicro Technologies (Phoenix, AZ, USA). SNAIP stationary phase (particle size, 3 or 5 μm) and CEC columns (packed length, 9 cm; total length, 37 cm) packed with SNAIP were prepared as reported in our previous literature [16]. The modification ratio of 3-aminopropyl silyl silica gel with 4-sulfo-1,8-naphthalic anhydride were 0.523 mmol/g for 5 μm particle and 0.471 mmol/g for 3 μm particle which were estimated from the percentage of carbon by elemental analysis. Phosphate buffers (100 mM) were prepared by dissolving appropriate amount of KH_2PO_4 in water, then adjusting to desired pH by H_3PO_4. The mobile phase, prepared by mixing methanol, water and the phosphate buffer, was degassed thoroughly prior to use. Instead of pressuring at both ends of the capillary column, the CEC system was thermostatically maintained at 18 °C throughout the analysis in order to avoid bubble formation within the capillary column. The detection wavelength was set at 205 nm.

3. Results and discussion

The applicability of the stepwise gradient of buffer concentration in a mobile phase for the separation of peptides in CEC with SNAIP was investigated. Before the application, it was needed to understand the effect of buffer concentration on the separation. In an attempt to describe the retention of charged analytes in CEC, Rathore and Horvath [17,18] have defined a CEC retention factor, k^*, as:

$$k^* = \frac{t_m(1 + k^*) - t_0}{t_0}$$

where t_m and t_0 denote the retention time of the analyte and that of an inert and neutral tracer (EOF marker), respectively. Thiourea was chosen as an EOF marker in this study. k_e^* is the velocity factor, indicating the contribution of electrophoretic mobility to the separation of charged analytes in CEC, and is given by

$$k_e^* = \frac{\mu_p}{\mu_0}$$

where, μ_p is the electrophoretic mobility of the analyte which is obtained from CZE measurements under the same conditions as the CEC separation. The interstitial EOF mobility in the CEC column, μ_0, is equal to the apparent EOF mobility within the CEC column multiplied by the ratio of current in open tube to that in packed column [17,18]. The peak locator, k_c^*, can be expressed in a similar manner to HPLC as follows:

$$k_e^* = \frac{t_m - t_0}{t_0}$$

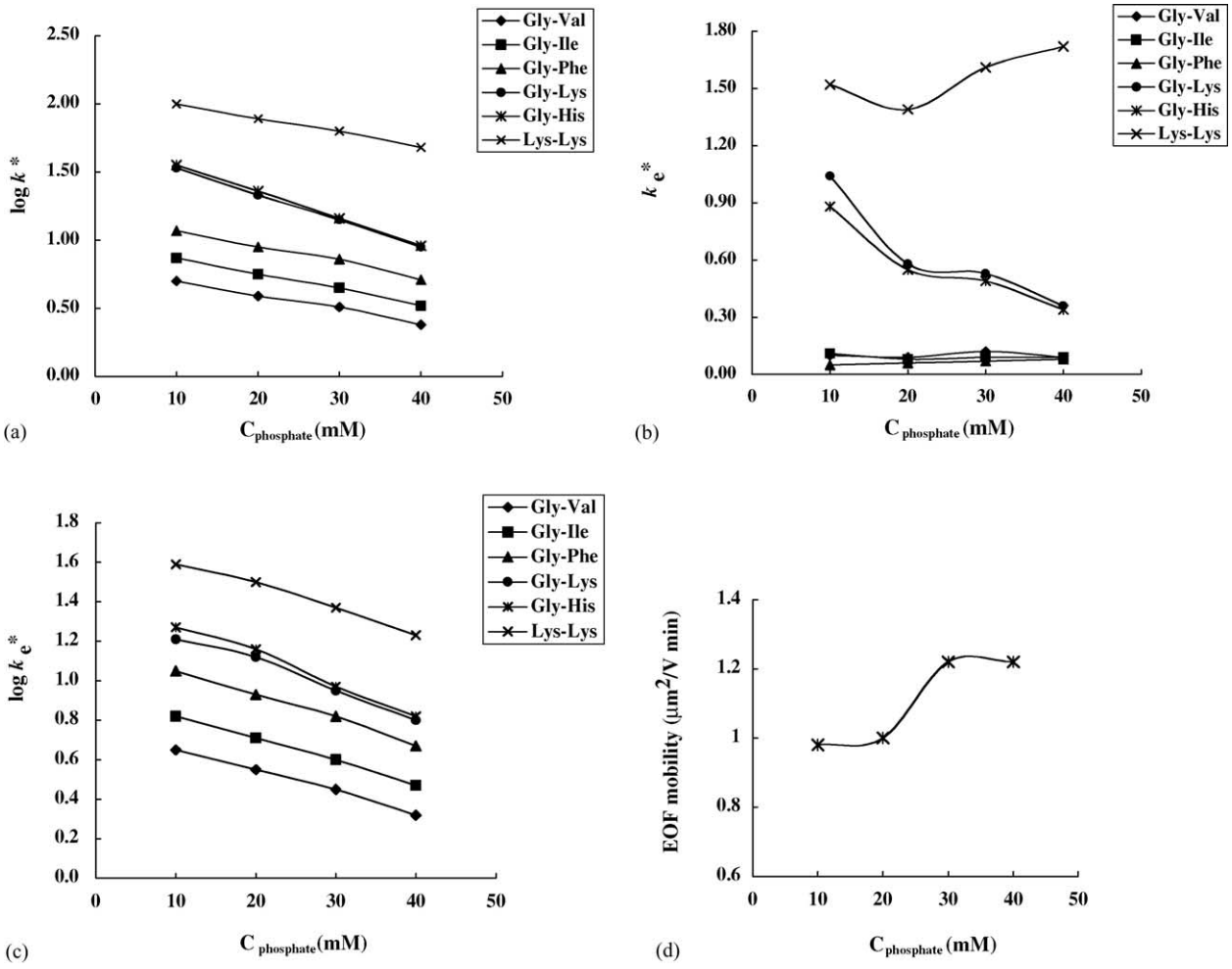

Fig. 2. Plots of logarithmic parameters ($\log k^*$, $\log k_c^*$) and velocity factor (k_e^*) of model peptides and EOF mobility vs. buffer concentration in the mobile phase. CEC conditions: 37 cm × 75 μm i.d. packed capillaries with SNAIP, 5 μm; mobile phase, phosphate buffer (pH 3.8)/methanol: 60/40 (v/v, %) at different buffer concentrations; applied voltage, 20 kV; electrokinetic injection, 20 kV for 8 s.

The effects of buffer concentration on the parameters and EOF mobility were studied in the range from 10 to 40 mM with a mobile phase of phosphate buffer (pH 3.8) and 40% methanol (Fig. 2). Increasing the buffer concentration from 10 to 40 mM resulted in a decrease in electrochromatographic retention, k^*, as shown in Fig. 2. This manifestly reflected the attenuation of electrostatic interaction between the dissociated amino group of peptides and the fixed negative charge on the stationary phase. The electrophoretic mobilities of the peptides increased as the buffer concentration increased. An increase in both the apparent EOF mobility and the ratio of current was observed as buffer concentration rose, which in turn led to the decrease (Gly–Lys and Gly–His), slight increase (Lys–Lys) and constant (Gly–Val, Gly–Ile and Gly–Phe) in the value of velocity factor k_e^*. The reduction in electrostatic interaction and the increase in the EOF mobility when increasing the buffer concentration contributed to the decrease in the peak locator k_c^* and separation time.

Fig. 3 illustrates the effect of buffer concentration on the separation of the tryptic digests of cytochrome *c* with the SNAIP column. All of the mobile phases provided a sufficient separation and, as expected, increasing buffer concentration reduced the separation time from 130 min (20 mM, Fig. 3a) to 22 min (50 mM, Fig. 3b) and sharpened all peaks, especially later peaks. Using the mobile phase with 50 mM phosphate buffer, the resolution of tryptic digests on SNAIP column was superior or comparable to the pressurized gradient CEC in previous works [13,19].

As described above, CEC with SNAIP was proved to be useful for the separation of peptides and the buffer concentration in a mobile phase largely affected the separations of charged analytes. Thus, the gradient elution of buffer concentration in CEC of peptides was examined. The separation patterns in Fig. 4a and b were obtained under the isocratic elution of 30 and 50 mM phosphate buffer (pH 3.8) containing 40% methanol, respectively. The peptide mixture contained the pair of Gly–Lys and Gly–His which required a moderate buffer concentration to achieve acceptable resolution and Lys–Lys which required a relatively high buffer concentration for its earlier elution. It can be seen from Fig. 4a and b

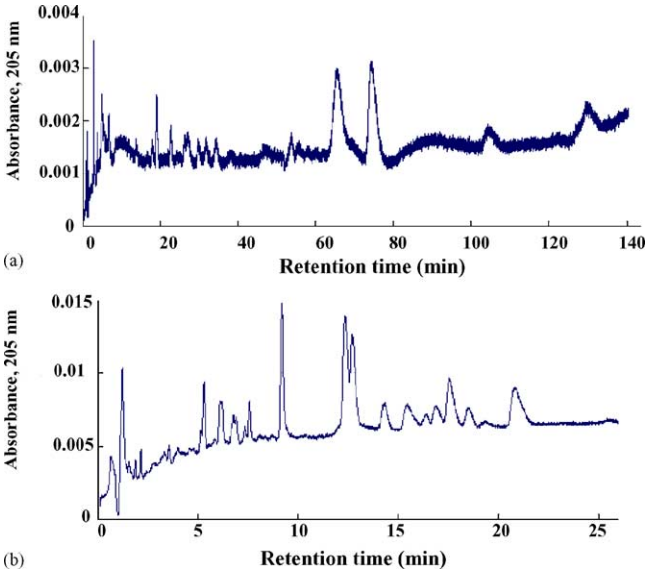

Fig. 3. Effect of buffer concentration on the separation of tryptic digests of cytochrome *c*. CEC conditions: 37 cm × 75 μm i.d. packed capillaries with SNAIP, 3 μm; mobile phase, phosphate buffer (pH 3.8)/methanol: 60/40 (v/v, %) at different buffer concentrations: (a) 20 mM (27 μA); (b) 50 mM (95 μA); applied voltage, 20 kV; electrokinetic injection, 20 kV for 10 s.

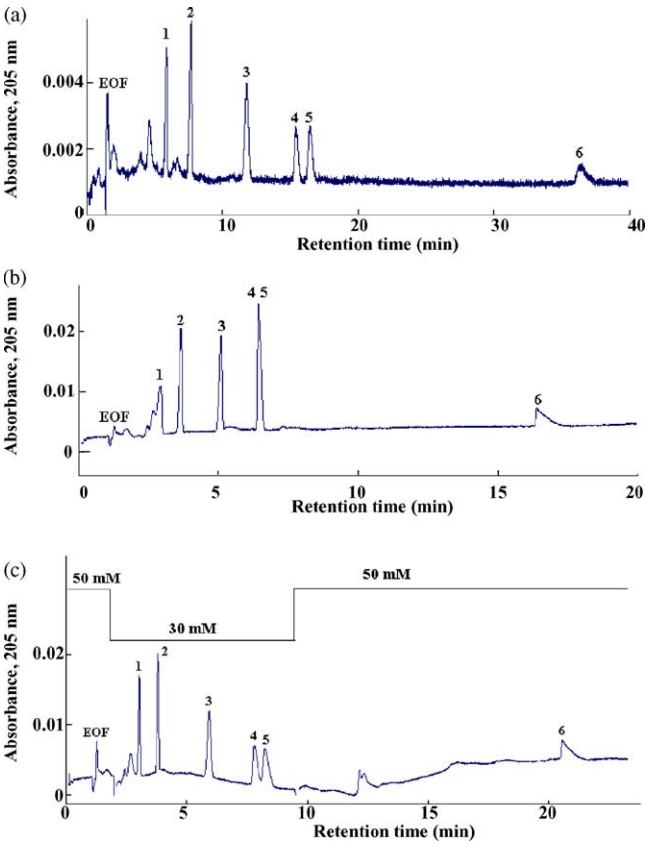

Fig. 4. CEC of model peptides on SNAIP column. CEC conditions: mobile phase: (a) 30 mM phosphate buffer (pH 3.8)/methanol: 60/40 (v/v, %); (b) 50 mM phosphate buffer (pH 3.8)/methanol: 60/40 (v/v, %); (c) phosphate buffer (pH 3.8)/methanol, 60/40 (v/v, %) (initial 50 mM for 2 min, intermediate 30 mM for 7.5 min and final 50 mM). Other conditions as in Fig. 2. Peaks: 1, Gly–Val; 2, Gly–Ile; 3, Gly–Phe; 4, Gly–Lys; 5, Gly–His; 6, Lys–Lys.

that multi-stepwise gradient of buffer concentration should be useful in order to separate all six peptides within a shorter separation time. The initial condition, 50 mM phosphate buffer (pH 3.8) and 40% methanol, was held for 2 min, after which the voltage was removed and the buffer vials were replaced with that including 30 mM phosphate buffer with the same composition as the initial one and the voltage was re-applied. After 7.5 min, the voltage was removed, and the initial mobile phase vials were replaced, and the voltage was re-applied; these conditions were held for 14 min to accelerate the elution of Lys–Lys and re-establish the initial condition prior to the next injection. As can been seen in Fig. 4c, Gly–Lys and Gly–His were fully separated and the use of this stepwise gradient resulted in a reduction of the separation time from 38 to 22 min compared with the isocratic elution of the mobile phase including 30 mM phosphate buffer and 40% methanol. A baseline disturbance in the UV signal at approximately 12 min appeared due to the border between the two mobile phases. Three consecutive runs of the CEC with stepwise gradient of buffer concentration gave reproducible electrochromatography with relative standard deviations for the retention times ranging from 1.28 to 1.93%. The performance of SNAIP column was maintained for more than 6 months. Specifically, after 81 injections, the relative changes of EOF and retention times for Gly–Val, Gly–Ile and Gly–Phe were from 1.10 to 3.25%.

In conclusion, using SNAIP column even without the pressurization, the application range of electrochromatography could be extended to separation with high current (∼95 μA) which is unavoidable to employ high buffer concentration for earlier separation of peptides. Indeed, a stepwise gradient of buffer concentration in CEC with SNAIP juggled the better resolution of peptides and the accelerated elution of strongly retained peptide. Simple peptide mixtures are preferably separated under an isocratic condition where significant changes in the separation are achieved by small changes of the parameters (i.e. organic modifier content, temperature and applied voltage) [20,21]. On the other hand, complex peptide mixtures such as digests often requires gradient elution [12,19], and therefore, the proposed CEC method will work as a complement for separation of complex mixtures. Gradient of buffer concentration will be effective for combination of the better resolution of closely related peptides and the early elution of multiply charged peptides when using mixed-mode stationary phase in CEC. Furthermore, the knowledge about stepwise gradient of buffer concentration described here can be transferred to other gradient methods.

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