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Study of flow rate in pressurized gradient capillary electrochromatography using splitter and separation of peptides using an Amide stationary phase

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Abstract

A pressurized gradient capillary electrochromatograph using a splitter was constructed. The variation in flow rate during gradient elution was investigated and separations of peptides using an Amide stationary phase were demonstrated. The flow rate, which is one of the important factors to control chromatographic behavior, was increased during the gradient elution, and the mismatching of mobile phase between the column and the resistance tubing derived three variation patterns in the flow rate. The electrophoretic migration in electrochromatography could enhance in separation of peptides. The separated peak number of tryptic digest of bovine serum albumin was increased from 30 to 40 by the application of +5 kV.

Keywords: Electrochromatography; Flow rate; Hydrophilic interaction chromatography; Peptides

1. Introduction

Capillary electrochromatography (CEC) is a separation method in which sample solutes are separated both chromatographically and electrophoretically [1–13]. Capillary electrochromatography can be categorized into two modes, i.e., electroosmotic flow-driven CEC and pressurized flow-driven CEC (pCEC). Though the pCEC mode has a disadvantage in the theoretical plate height, it has no risk of bubble formation or dry-out problems. In addition, the enhancement in separation using electrophoretic migration is expected in pCEC [6,7]. Therefore, both CEC with and without pressurized flow has been well studied in recent years.

The gradient elution method is often used in conventional liquid chromatography, and it is a powerful technique for achieving high-performance separation. Therefore, gradient elution techniques for CEC with and without pressurized flow have also been developed [8]. A delay volume is a serious problem in capillary liquid chromatography (cLC) and capillary electrochromatography. A delay volume of 10 μL is not a problem in liquid chromatograph when using a conventional column, e.g, i.d. 4.6 mm. However, a 10 μL delay volume can bring about destructive problem in both cLC and CEC, because of their small flow rate, e.g., 1 μL/min. When gradient elution is performed in cLC or CEC, the delay volume in microliters order provides a delay of a few tens of minutes in mobile phase variation. Therefore, the wasteful period is in the initial stage of the separation procedure. To avoid this weak point, a split flow is often used in apparatuses for cLC and pCEC.

Various physicochemical parameters are affected by the composition of the mobile phase. Viscosity is one of the most important parameters to the flow rate, and the flow rate is one of the important factors to control chromatographic behavior. The increase in viscosity results in an increase in flow resistance in a column, and it will reduce both velocity of pressurized and electroosmotic flows. The increase in viscosity can often affect the pressure at the column inlet, which is important factor to the flow rate. Since the variation

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of permittivity of the mobile phase changes zeta potential, the electroosmotic flow velocity is affected by the composition of the mobile phase. The variation of conductivity of the column changes column temperature under application of voltage, and almost all physicochemical parameters depend on temperature. Therefore, it is difficult to predict the flow rate in a gradient elution in capillary electrochromatography. In this study, the variation of the flow rate during a gradient elution has been investigated.

Electrochromatography is often used for the separation of peptides [9–13]. In this study, separations of peptides using pressurized flow-driven electrochromatography with TSKgel Amide-80 stationary phase were performed. The Amide-80 is a silica gel particle modified with carbamoyl groups, and is used for hydrophilic interaction chromatography (HILIC) [14,15]. Hydrophilic peptides, which cannot be retained in a reversed-phase mode, are retained in the hydrophilic interaction mode using TSKgel Amide-80. The water content in the mobile phase is increased in a gradient elution using the TSKgel Amide-80 to accelerate elution of peptides with high retention. The effect of application of high voltage on separation of peptides in pressurized gradient capillary electrochromatography was investigated.

2. Experiment

2.1. Apparatus

A schematic diagram of an apparatus used in this experiment is shown in Fig. 1. The apparatus was comprised of two pumps (LC-10AD, Shimadzu, Kyoto, Japan), a pump controller (SCL-10A, Shimadzu), a mixer (volume: 0.1 mL, P/N228-35830-91, Shimadzu), a laboratory-made splitter, resistance tubing (fused-silica capillary, 50 cm × 50 μm i.d., supplied by GL Science, Tokyo), a UV detector (CE-1575, Jasco, Tokyo, Japan), a nano-liters injector (Nano-Injector, injection volume: 20 nL, Chemco, Osaka, Japan), a high voltage power supply (HCZE-30PN, Matsusada Precision, Shiga, Japan), and a laboratory-made capillary column (i.d. 0.15 mm). The packed and whole lengths of the capillary column are described in each figure caption. The capillary column was packed with carbamoyl groups bonded silica gel particles (particle diameter: 5 μm, TSKgel Amide-80, Tosoh, Tokyo) using a slurry-packing method. Signals of UV absorbance, electric current, and pump pressure (pressure at column inlet) were stored in a PC simultaneously using a 20 Hz A/D converter (K8DL-G16, Omron, Kyoto, Japan). The splitter and injector were connected by a low delay volume connector, with a volume of 0.2 μL (fused-silica tubing, 45 mm × 75 μm i.d.).

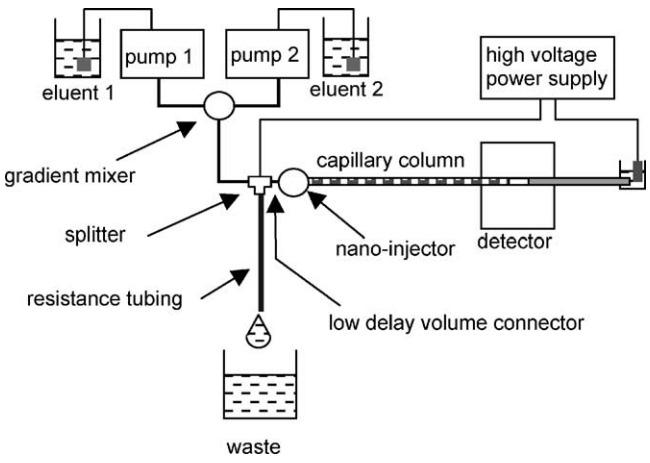

Fig. 1. Schematic diagram of apparatus for pressurized flow-driven gradient capillary electrochromatograph.

2.2. Conditions

A mixture of distilled water and acetonitrile (Wako, Kyoto, Japan) containing trifluoroacetic acid (TFA, Wako) was used as a mobile phase. The total flow rate of two pumps was set at 0.2 mL/min for this experiment. The split ratio ranged from 1000 to 2000 in this experiment. The high voltage was applied to the outlet reservoir, and the body of the nano-injector and splitter were grounded for safe operation. Details of the gradient program and other experimental conditions are described in each figure caption.

3. Results and discussion

3.1. Variation of flow rate in column during gradient elution

The flow rate is one of the important factors to control chromatographic behavior such as elution time, peak form, theoretical plate number and etc., so the knowledge about the flow rate in gradient elution is necessary to achieve fine separation. We studied the variation in the flow rate in our gradient capillary electrochromatograph using a splitter.

The variation of flow rate during gradient elution with and without application of voltage is shown in Fig. 2A. The open circle and closed rectangle marks indicate flow rates in the capillary column without and with application of +5 kV, respectively. The lines in Fig. 2A and B are the variation of water content in the mobile phase (right axis). The flow rate in Fig. 2 was calculated by the retention time of thiourea (t_0 marker). Thiourea was injected into the column each minute, and the elution time ranged from 1.2 to 2.0 min. The flow rate plotted at X min was calculated from the retention time of thiourea injected at X min. As shown in Fig. 2A, the flow rate in the column increased during the gradient elution, and the variation of the flow rate precedes the variation in mobile phase composition. This precedence is given by the difference between the injection (plotted) and elution time of thiourea.

The viscosity of the mixture of water and acetonitrile is higher than that of acetonitrile, and is almost proportional

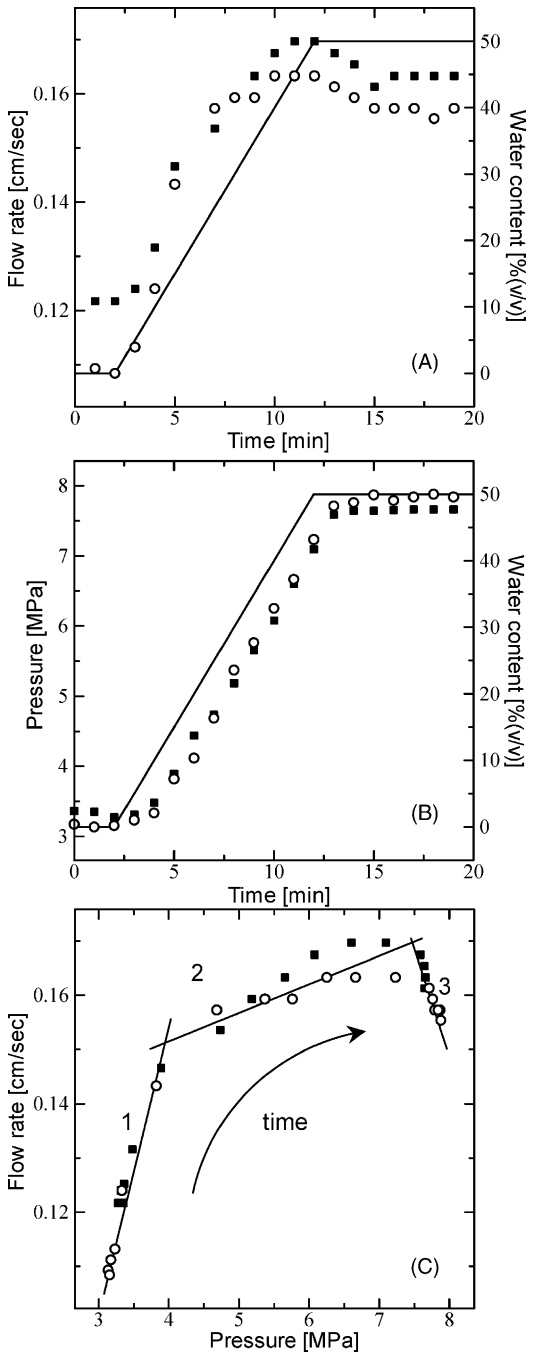

Fig. 2. Variation in flow rate during gradient elution (A), variation of the pressure at the column inlet (B), and relation between the pressure at the column inlet and flow rate (C). Open circle and closed rectangular marks indicate applications of 0 kV and +5 kV, respectively. Column: i.d. 0.15 mm, packed length 129 mm, whole length 211 mm, packed with TSK gel Amide-80; gradient elution: (a) acetonitrile containing 0.01% TFA, (b) 50% acetonitrile containing 0.01% TFA, (a) in 2 min, 0–100% (v/v) (b) in 10 min, (b) in 8 min; sample: thiourea (t_0 marker); detection: absorbance of 210 nm.

to the water content within the range of 0–50% (v/v) [16]. Almost all mobile phase flows to the resistance tubing in this apparatus, i.e., split ratio ranges from 1000 to 2000. The pressure drop, Δp, in the resistance tubing (fused-silica capillary) is given by the Hargen–Poiseuille equation:

$$\Delta p = \frac{32ul\eta}{r^2}$$

where u, l, η, and r are mean flow velocity, length of resistance tubing, viscosity, and inner diameter of resistance tubing, respectively. The parameters of l and r are constants. In this experiment, constant flow mode was used, so u can be regarded as a constant in this experimental condition. Therefore, the viscosity of the mobile phase controls the pressure at the column inlet. The variation of the pressure at the column inlet is shown in Fig. 2B. The pressure was increased during the gradient elution and the variation pattern in the pressure is well correlated with the gradient program. In the other words, it became clear that the pressure is almost proportional to the viscosity of the mobile phase. However, there is a delay of about 1.5 min between variations in the pressure and mobile phase composition. The delay volume from the mixer and the splitter may result in this delay.

The relation between the pressure at the column inlet and the flow rate in the column is shown in Fig. 2C. The flow rate was varied with three parts during gradient elution, as shown in Fig. 2C. The flow rate was accelerated quickly at the initial state (step 1), then increased moderately (step 2). Finally, the flow rate was decreased (step 3). This behavior was derived from the mismatching of the mobile phase between the resistance tubing and the capillary column. In step 1, the increase in water content, i.e., increase in viscosity, enlarged the pressure at column inlet. However, replacement of the mobile phase in the column had not proceeded sufficiently. The elution time of t_0 marker ranged from 1.2 to 2.0 min in this experiment, so it took at least 1.2 min to flush out the mobile phase in the column. In addition, the delay volume in the “low delay volume connector” also inhibits the replacement of the mobile phase in the column. However, the replacement in the resistance tubing was achieved in a brief period of 0.03 s. Therefore, the increase in the pressure could accelerate the flow rate with less of an effect of increasing flow resistance in the column. In step 2, the replacement of the mobile phase in the column was progressing, and the flow resistance in the column was increased, thereby moderately increasing the flow rate. In step 3, the pressure at column inlet became constant, because the gradient program had finished. However, the mobile phase in the column had not yet been replaced completely. Since the replacement of the mobile phase increased the flow resistance in the column, the flow rate was decelerated. The mismatching of the mobile phase resulted in variation in the flow rate in the three steps. The overshoot in the flow rate shown in Fig. 2A was derived from step 3. When the flow velocity in the resistance tubing becomes the same as in the column, the matching of mobile phase would be achieved. The synchronization in viscosity of the mobile phase in the resistance tubing and the column might reduce the variation of the flow rate in the gradient elution. Therefore, the design

of the resistance tubing is quite important for the pressurized gradient capillary electrochromatography using a splitter.

The effect of the application of voltage on the pressure was not observed as shown in Fig. 2B. However, Fig. 2A shows that the flow rate with application of +5 kV is higher than that without application voltage. Since the TSKgel Amide-80 could not generate an electroosmotic flow in this experimental condition, the decrease in viscosity by Joule heating may accelerate the flow rate. Therefore, the temperature control for the mobile phase in both the column and the resistance tubing is required to achieve a stable flow rate in the column.

3.2. Typical enhancement in separation of peptides in isocratic elution

Separations of Gly–Thr (peak 1) and Gly–Glu (peak 2) with and without application of voltage in the isocratic elusion mode are shown in Fig. 3. Both peaks were identified using addition method. Fig. 3 shows typical enhancement in separation induced by the application of voltage. The two peptides could not be separated completely without application of voltage, i.e., liquid chromatography mode. Since isoelectric points of Gly–Thr and Gly–Glu are 6.0 and 3.3, respectively, both peptides have positive charges in this mobile phase. An application of positive voltage at the column outlet induced electrophoretic migration of the cation toward the column inlet, and delayed the elution time of the cation. The delay of Gly–Glu became larger than that of Gly–Thr, because of the higher electrophoretic mobility of Gly–Glu. Though the net charge of Gly–Thr might be larger than that of Gly–Glu in this experimental condition, other factors, such as molecular size and solvation, would result

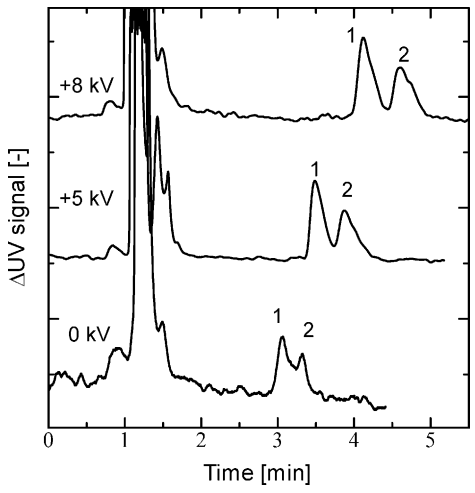

Fig. 3. Enhancement in separation by application of voltage in isocratic elution. Peaks of 1 and 2 are Gly–Thr and Gly–Glu, respectively. Column: i.d. 0.15 mm, packed length 120 mm whole length 205 mm, packed with TSK gel Amide-80; Eluent: 80% (v/v) acetonitrile containing 0.1% TFA; Flow: constant pressure mode of 4.9 MPa; Other conditions are the same as in Fig. 2.

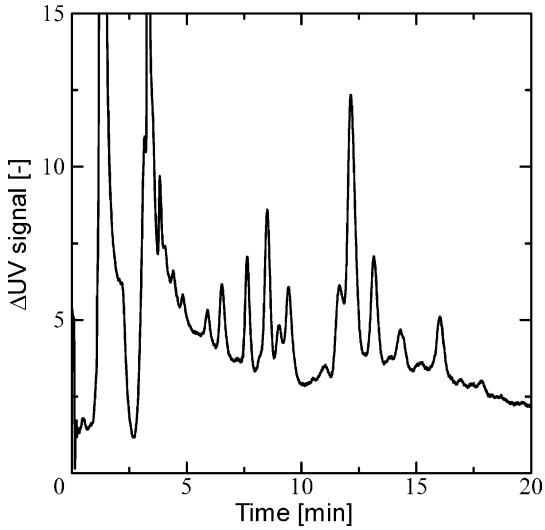

Fig. 4. Separation of tryptic digests of cytochrome *c* in pressurized gradient capillary electrochromatography. Gradient elution: (a) 100% acetonitrile containing 0.01% TFA, (b) 50% acetonitrile containing 0.01% TFA, 100% (a) in 0.5 min, 0–10% (b) in 0.5 min, 10–50% (b) in 19 min; Column: i.d. 0.15 mm, packed length 115 mm, whole length 206 mm, packed with TSK gel Amide-80; Applied voltage: +5 kV; Other conditions are the same as in Fig. 2.

higher electrophoretic mobility of Gly–Glu than Gly–Thr. The application of positive voltage can enhance the separation, as shown in Fig. 3.

3.3. Separation of peptides in gradient capillary electrochromatography

The separation of tryptic digests of cytochrome *c* (cyt C) using capillary electrochromatography in gradient elution is performed to check the delay period in our electrochromatograph, and the result is shown in Fig. 4. The gradient programming for Fig. 4 is as follows: 100% acetonitrile containing 0.01% TFA in 0.5 min, 100–95% acetonitrile containing 0.01% TFA in 0.5 min, 95–75% acetonitrile containing 0.01% TFA in 19 min. As shown in Fig. 4, the tryptic digests of cyt C were well separated within 20 min. The presented pressurized flow-driven gradient capillary electrochromatograph did not exhibit a serious delay of the sample elution.

The enhancement in separation induced by application of voltage is demonstrated in Fig. 5. In Fig. 5, the tryptic digests of bovine serum albumin (BSA) were separated without application of voltage, and about 30 peaks were observed. When +5 kV was applied to the column outlet, the number of peaks increased compared with 0 kV. About 40 peaks were observed in the electrochromatogram with application of +5 kV. Since the peptides had positive charges in this experimental condition, the application of positive high voltage delayed the elution time of the peptides. The difference in electrophoretic mobility of peptides produced electrophoretic separations of peptides under the electric field

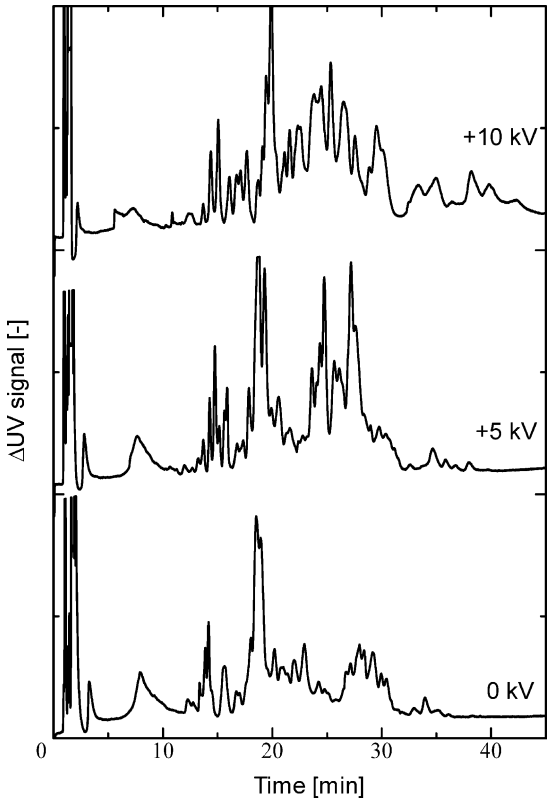

Fig. 5. Enhancement in separation of tryptic digests of bovine serum albumin by application of high voltage. Gradient elution: (a) acetonitrile containing 0.01% TFA, (b) water containing 0.01% TFA, (a) in 2 min, 0–30% (v/v) (b) in 30 min, (b) in 13 min; Column: i.d. 0.15 mm, packed length 140 mm, whole length 222 mm, packed with TSK gel Amide-80; Other conditions are the same as in Fig. 2.

(see Fig. 3). The combination of chromatographic and electrophoretic separations is effective in separating peptides. We believe that the voltage-induced enhancement in peak numbers was produced by electrophoretic separation, but a further study is necessary to clear this phenomenon.

The peak number with application of +10 kV is almost the same as that with +5 kV. The separation was not enhanced significantly by the application of +10 kV. The peaks with application of +10 kV are broader than those with +5 and 0 kV. This phenomenon is especially significant in the peaks eluted later. As shown in Fig. 2, the flow velocity in the column increased at a latter time in this gradient elution. This increase is an advantage over the decrease in peak boarding of the sample solute eluted later. The elution of peptides with high retention is accelerated by the two gradients of the mobile phase composition and the flow.

4. Conclusion

The flow rate in gradient elution was varied with three steps in our pressurized flow-driven electrochromatograph using a splitter. We guess that the similar phenomenon might be observed in gradient elution using other capillary liquid chromatograph with a splitter. The use of suitable resistance tubing may improve the variation of the flow rate. The electrophoretic migration in electrochromatography could enhance the separation of peptides. Electrochromatography might be a suitable method to separate a mixture of peptides, such as tryptic digests.

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