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Elution Behavior of Proteins in Capillary Chromatography Using an Untreated Fused-silica Capillary Tube and a Water–Hydrophilic–Hydrophobic Organic Mixture Carrier Solvent

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We examined the elution behavior of isoluminol isothiocyanate (ILITC)-labeled proteins (alcohol dehydrogenase (ADH) and bovine serum albumin (BSA)) in a capillary chromatography system using an untreated fused-silica capillary tube and a water–acetonitrile–ethyl acetate mixture carrier solution. The mixture of ILITC and ILITC-labeled protein (ADH or BSA) was analyzed using this system with a chemiluminescence detector; the ILITC and the labeled protein were separated in this order with an organic solvent-rich carrier solution, while they were eluted in the reverse order with a water-rich carrier solution. In addition, the mixture of ILITC, ILITC-labeled ADH, and ILITC-labeled BSA was added to this system, and the analytes of the mixture were separated and confirmed with the individual peaks on the chromatograms.

A capillary chromatography system using an untreated capillary tube made of fused-silica, polyethylene, or poly(tetrafluoroethylene) and a water–hydrophilic–hydrophobic organic mixture carrier solution has been reported. We call this a tube radial distribution chromatography (TRDC) system that works under laminar flow conditions.[1–3]

Briefly, separation in the TRDC system is explained as follows.[1–3] Aqueous and organic solvents in the carrier solution are dispersed nonuniformly in the capillary tube under laminar flow conditions, generating an organic solvent-rich and a water-rich phase in the capillary tube. An organic solvent-rich carrier solution generates an organic solvent-rich inner phase and a water-rich outer phase, while a water-rich carrier solution results in a water-rich inner phase and an organic solvent-rich outer phase. That is, a major inner phase is formed around the center of the tube away from the inner wall and a minor outer or capillary wall phase is generated near the inner wall, based on the tube radial distribution of the carrier solvents. The analytes that are delivered through the capillary tube are distributed between the inner and outer phases undergoing chromatographic separation. In the TRDC system, the order of analyte elution times can be changed easily by altering the solvent component ratios in the carrier solution.

To date, various mixtures of hydrophilic and hydrophobic analytes have been separated using the TRDC system. However, most of these have been organic compounds with low molecular weights: 1-naphthol and 2,6-naphthalenedisulfonic acid were typical model analytes.[1–3] As investigation of the TRDC system has just begun, it is important to examine the elution behavior of various analytes with the system to expand our knowledge regarding its separation performance. In this study, we examined the elution behavior of biopolymer analytes, isoluminol isothiocyanate (ILITC)-labeled proteins, in the TRDC system with chemiluminescence (CL) detector.

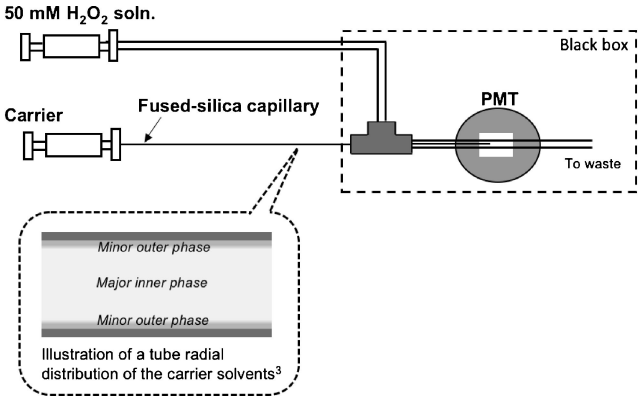

Figure 1. Schematic diagram of the present capillary chromatography with CL detection system.

Water was purified using an Elix UV 3 (Millipore Co.). All reagents used were commercially available and of analytical grade. Acetonitrile, ethyl acetate, hydrogen peroxide solution (30 wt %), and alcohol dehydrogenase from yeast (ADH) (MW 140000) were purchased from Wako Pure Chemical Industries, Ltd. ILITC and bovine serum albumin (BSA) (MW 66000) were purchased from Tokyo Chemical Industry Co., Ltd. and Sigma-Aldrich Chemical Co., respectively. A fused-silica capillary tube (75 μm i.d. and 150 μm o.d.) was purchased from GL Science.

A schematic representation of the present TRDC system is shown in Figure 1. The system consists of a fused-silica capillary tube (70 cm in length), a microsyringe pump (MF-9090; Bioanalytical Systems, Inc.), and a CL detector (Model EN-21, Kimoto Electric Co., Ltd.) that takes advantage of the luminol reaction. A flow CL detection cell (0.5 mm i.d. poly(tetrafluoroethylene) tube)[4] was used in this system.

A water–acetonitrile–ethyl acetate mixture was prepared in a volume ratio of 3:8:4 as an organic solvent-rich carrier solution or in a volume ratio of 15:3:2 as a water-rich carrier solution, where the water component was 10 mM carbonate buffer at pH 11.8 including 4 μM microperoxidase. Protein (ADH or BSA) was labeled with ILITC as follows. Protein (0.5 μmol) and ILITC (1.0 μmol) were dissolved in a solution of water–triethylamine (95:5 volume ratio) (100 μL). The mixture was stirred for 1 min and left in the dark for 20 min. After evaporation of the solvent, the residue was redissolved in the carrier solution (1 mL) to give the analyte solution including an excess (or free) ILITC and ILITC-labeled protein. The analyte solution was injected into the capillary tube inlet by gravity from a height of 25 cm for 10 s. The analytes were then delivered into the capillary tube with the carrier solution using a microsyringe pump at a flow rate of 0.5 μL min^{-1}. The oxidant reagent

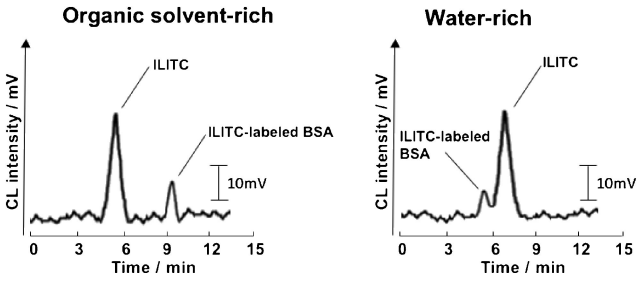

Figure 2. Chromatograms of a mixture of ILITC and ILITC-labeled BSA obtained by the present system. Conditions: Capillary tube, 70 cm length of 75 μm i.d. fused-silica; carrier, water–acetonitrile–ethyl acetate (3:8:4 v/v/v) mixture solution (organic solvent-rich) and water–acetonitrile–ethyl acetate (15:3:2 v/v/v) mixture solution (water-rich); sample injection, 25 cm height (gravity) × 10 s; flow rate of the carrier solution, 0.5 μL min^{-1}; and ILITC-labeled BSA concentration, 50 μM.

solution of 50 mM hydrogen peroxide (10 mM carbonate buffer, pH 10.8) was delivered at a flow rate of 10 μL min^{-1} to the capillary outlet where the analytes were mixed with the reagents to generate CL.

Figure 2 shows the chromatograms of the mixture of ILITC and ILITC-labeled BSA obtained with the TRDC with the organic solvent-rich and water-rich carrier solutions. ILITC and ILITC-labeled BSA were separated and detected in this order with the organic solvent-rich carrier solution, while they were detected in the reverse order with the water-rich carrier solution. Clearly, the elution times of the analytes were changed by altering the component ratios of the carrier solvents. In addition, the first peaks on the chromatograms roughly appeared with the average linear velocity and the second peaks were eluted with lower velocity under laminar flow conditions. The average linear velocity was confirmed by the experiment with a normal aqueous carrier solution not including any organic solvents. The mixture of ILITC and ILITC-labeled ADH was also analyzed with the TRDC system, and these components showed similar elution behaviors to those on the chromatograms shown in Figure 2. The elution orders obtained for the mixture of ILITC and ILITC-labeled BSA or ADH were reasonable considering the free ILITC to be more hydrophobic than the ILITC-labeled protein.

Furthermore, we examined the mixture of ILITC, ILITC-labeled ADH, and ILITC-labeled BSA with the present TRDC system. The obtained chromatograms are shown in Figure 3. ILITC, ILITC-labeled ADH, and ILITC-labeled BSA were detected in this order with the organic solvent-rich carrier solution, while they were eluted in the reverse order with the water-rich carrier solution. The orders of analyte elution on the chromatograms indicated that the hydrophilicity of the ILITC-labeled BSA must be larger than that of the ILITC-labeled ADH. The isoelectric points of BSA and ADH are 4.8 and 5.8, respectively.[5] It is quite difficult to describe the dissociation and protonation of carboxyl groups and amino groups on the labeled proteins in an aqueous–organic solvent mixture. However, for the moment, the difference in gaps between the isoelectric points (4.8 for BSA and 5.8 for ADH) and pH 11.8 of the water

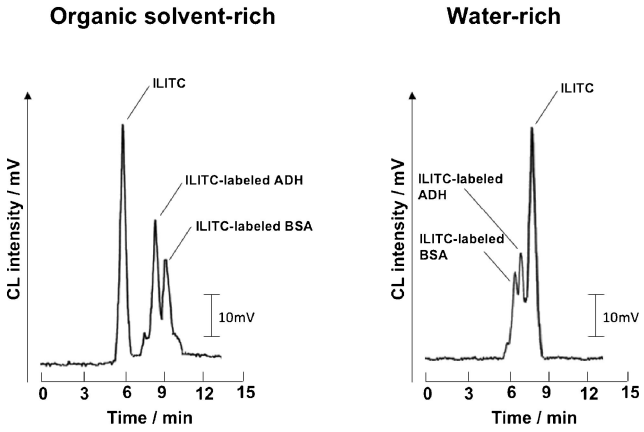

Figure 3. Chromatograms of a mixture of ILITC, ILITC-labeled ADH, and ILITC-labeled BSA obtained by the present system. Conditions: Capillary tube, 70 cm length of 75 μm i.d. fused-silica; carrier, water–acetonitrile–ethyl acetate (3:8:4 v/v/v) mixture solution (organic solvent-rich) and water–acetonitrile–ethyl acetate (15:3:2 v/v/v) mixture solution (water-rich); sample injection, 25 cm height (gravity) × 10 s; flow rate of the carrier solution, 0.5 μL min^{-1}; and ILITC-labeled protein concentration, 50 μM.

component in the carrier solution seemed consistent with the nature of hydrophilicity or hydrophobicity of the labeled proteins observed on the chromatograms.

In conclusion, the model biopolymers BSA and ADH were labeled with ILITC for analysis using the TRDC with CL detection system. ILITC, ILITC-labeled ADH, and ILITC-labeled BSA in the mixture solution were separated and confirmed with the individual peaks on the chromatograms. Separation was performed using an untreated open fused silica capillary tube and a water–acetonitrile–ethyl acetate mixture carrier solution without any additives, such as gels, or applying a high voltage. To date, the TRDC has mostly been applied to the analysis of organic compounds with low molecular weights. The results obtained for biopolymer analysis here provide insight to expand the TRDC system to future research.

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