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Band broadening caused by the multiple labeling of proteins in micellar electrokinetic chromatography with diode laser-induced fluorescence detection

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Abstract

When a labeling reagent is used, in the determination of proteins by capillary electrophoresis with laser-induced fluorescence detection, the multiple labeling of proteins frequently occurs, which can degrade the separation efficiency. In order to understand the influence of the multiple labeling of proteins on separation efficiency, the band broadening caused by a labeling reaction between bovine serum albumin (BSA) and a cyanine fluorescent dye (Cy5) was investigated using micellar electrokinetic chromatography in conjunction with diode laser-induced fluorometry. With the aid of an internal standard, methylene blue, the height equivalent to the theoretical plate (HETP) ratio of BSA to methylene blue was used as an indicator for band broadening under optimum separation conditions. Labeling conditions, including reaction buffer pH, reaction time, and initial concentration of Cy5 to bovine serum albumin, were found to influence the HETP ratio. The separation efficiency for the labeled protein was degraded by experimental conditions employed in the labeling, which indicates an increase in the heterogeneity of the final products.

Keywords: Band broadening; Column efficiency; Derivatization; Capillary electrophoresis; Proteins

1. Introduction

Capillary electrophoresis (CE) is a rapid, simple, and efficient technique for protein separation. Detection is often achieved by means of an ultraviolet (UV) absorbance detector [1–3]. However, the sensitivity is limited due to the short optical path length of the capillary. The use of laser-induced fluorescence detection (LIF), in conjunction with CE can improve detectability by several orders of magnitude [4]. A number of research groups have reported on the use of CE–LIF in the trace analysis of proteins in biological samples [5,6]. Two strategies are often used: One uses native fluorescence emitted by the aromatic amino acid residues, e.g. tryptophan residues on proteins [7–9]; the other is to label the proteins using a fluorophore or fluorogenic reagent to form the derivatives that can produce a fluorescence signal [10–12]. The later method is more practical for this, because of the weak native fluorescence emitted by the proteins and the expense of lasers emitting in the UV region.

In most cases, the attachment of fluorophores or fluorogenic reagents to proteins is achieved through

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the reaction between the NH_2 groups of the proteins and an amine-reactive reagent. Unfortunately, unfavorable derivatization, multiple labeling, occurs since a protein typically contains a number of free amino groups, e.g. lysine, resulting in conjugation with more than one amine reactive fluorescent or fluorogenic dye [13,14]. Such multiple labeling often results in the incomplete separation of the heterogeneous labeled products in CE. Thus, if all the multiple labeling products derived from a protein have slightly different electrophoretic mobilities, the extent of peak broadening should be representative of heterogeneity, which would be directly dependent on the labeling conditions. Therefore, it is important to have a more complete understanding of the influence of labeling conditions on band broadening.

Previously, CE was used for the complete separation of the multiple labeling products derived from relatively simple peptides or small proteins which contain only a few free amino residues [15]. However, Zhao et al. estimated that if a protein has *n* free amino groups, $2^n - 1$ possible products would be expected [16]. Therefore, the complete separation and identification of multiple labeling products of large proteins would appear to be difficult due to small differences in molecular mass and similar electric charges of the labeled products.

In fact, multiple labeling products of proteins usually have different electrophoretic mobilities in CE because of the change in the molecular mass and the isoelectric points (p*I* values) of the products [13,17]. Therefore, peak broadening would be expected to depend on the heterogeneity of the multiple labeling products. Craig and Dovichi reported on band broadening phenomenon due to the labeling in the separation of a labeled protein [18]. However the issue of how the factors that affect the labeling influence band broadening have not yet been experimentally demonstrated at this time.

In this study, bovine serum albumin (BSA), which contains 60 amino groups per molecule [19], was reacted with an amine-reactive fluorescent dye, Cy5. Separation and detection were achieved by micellar electrokinetic chromatography (MEKC) combined with diode laser-induced fluorescence detection. An internal standard, methylene blue, was added to the reaction mixtures to evaluate the effect of multiple labeling on the band broadening of the protein peak. The relative height equivalent to a theoretical plate (HETP) of labeled BSA to methylene blue was used as an indicator of band broadening caused by the multiple labeling of proteins. The experimental factors directly related to the labeling reaction such as reaction buffer pH, reaction time, and concentration ratio of BSA to Cy5, were investigated.

2. Experimental

2.1. Instrumentation

All measurements were carried out with a capillary electrophoresis/diode laser-induced fluorescence system similar to that used in our previous paper [20]. In summary, a red diode laser (LDA1035, Life Laser-Innovation, Switzerland), emitting at 635 nm with an output power of 1.5 mW, was used as the excitation source for the fluorescence detection system. The laser beam was focused with a 10× lens (Nikon 22405, Tokyo, Japan) and the resulting fluorescence was collected with a 40× lens (Olympus 101247, Tokyo, Japan) perpendicularly. To eliminate the scattering light, an interference filter (CWL: 672 nm), a long pass filter (cut-off: <640 nm) and a 200 μm pinhole were placed in the front of a Hamamatsu model R3896 photomultiplier tube (PMT).

2.2. Procedures

The separation was carried out using an uncoated fused-silica capillary with an effective length of 28.5 cm (Polymicro Technologies, Phoenix, AZ, USA, 38.5 cm total length×50 μm I.D.×375 μm O.D.). Hydrodynamic injections were performed at 10 cm for 5 s. Prior to each run, the capillary was washed with a 0.1 *M* NaOH solution, deionized water and the separation buffer, respectively, for 1 min each. A high-voltage power supply (HCZE-30P, Matsusada Precision Devices) was used to apply the separation voltage, 12.5 kV. The room temperature was maintained at 25 °C by an air conditioner.

A Shimadzu ultraviolet spectrometer (UV2400-PC) was used to measure the concentration of Cy5 stock solution as reported previously [21]. The

following extinction coefficient was used: Cy5, $\lambda_{max} = 650$ nm, $\epsilon = 250\,000$ mol^{-1} cm^{-1}.

2.3. Data processing

The output from the PMT was directed to an Apple computer (Apple, Cupertino, CA, USA) through a Varian Computer Interface Module (Varian, Walnut Creek, CA, USA) and read out into digital files using Rainin ver. 1.4.5 (Varian) software. All digital files were processed on a personal computer and the peak area, migration time and peak width were measured by means of KaleidaGraph 3.09a (Synergy Software) software.

Peak efficiencies were calculated by using the following equation,

$$H = \frac{L}{5.54} \cdot \left(\frac{W_{1/2}}{t_m} \right)^2$$

where H is height equivalent to a theoretical plate, L the effective length to the detector, $W_{1/2}$ the peak width at half maximum in time unit and t_m the migration time.

2.4. Chemicals

All reagents used in the study were of either analytical or spectroscopic grade unless specially stated. Cy5 was purchased from Amersham Pharmacia Biotech UK. Methylene blue was obtained from Kanto (Tokyo, Japan). Sodium hydroxide and anhydrous sodium dihydrogenphosphate were obtained from Kishida (Osaka, Japan). Bovine serum albumin and all other reagents were obtained from Wako (Osaka, Japan). Deionized water was prepared with an Elix Purified-Water System (Millipore, France).

2.5. Labeling reaction

A Cy5 stock solution was prepared by the addition of 500 μl of anhydrous dimethylformamide (DMF) to a plastic vial containing less than 100 μg of Cy5. A phosphate buffer (0.1 *M*, pH 7.0) and a carbonate buffer (0.1 *M*, pH 9.3) were used for the labeling reactions. A standard solution of methylene blue was prepared by adding appropriate amounts of methylene blue to 100 ml deionized water. BSA solutions, at concentrations of up to 1 mg/ml or 10 mg/ml were prepared using the phosphate or carbonate reaction buffers. Prior to initiating the labeling reaction, BSA and 0.5 μl of methylene blue solutions were added into a vial and mixed with an appropriate volume of the reaction buffers up to 90 μl. After mixing the contents of the vial for 1 min by swirling, 10 μl of the Cy5 solution was added to initiate the labeling reaction.

3. Results and discussion

To investigate band broadening caused by the labeling reaction conditions, it is necessary to suppress the influence on band broadening caused by operational and separation conditions. However, imprecision in run-to-run or day-to-day separation and operation conditions, is unavoidable since it is difficult to maintain the surface conditions of the capillary constant and to inject samples precisely by a manual hydrodynamic method on the laboratory-made electrophoresis system used. Such imprecision can induce large changes in the plate heights because of various band dispersion effects [22,23]. Therefore, a direct comparison of the absolute value of the plate height was deemed to be unworkable. The use of an internal standard would be helpful to minimize the effect of band broadening related to imprecision in the operational conditions. It would be expected that the effect of band broadening on the labeled protein would be similar to that on the internal standard, e.g. an increase in the length of the sample plug results in the increases in HETP for both the labeled protein and the internal standard. Therefore the use of the ratio of the HETP of the labeled protein to the internal standard would be an advantage, in terms of minimizing the effect of the changes in the experimental conditions on band broadening.

An electropherogram of reaction mixtures containing an internal standard, methylene blue under optimized separation conditions is shown in Fig. 1. In the optimized separation conditions, 3.5% methanol was used to separate the internal standard from the mixtures of labeled products. The peak corresponding to the labeled BSA was identified by comparison with a separation of a Cy5 sample

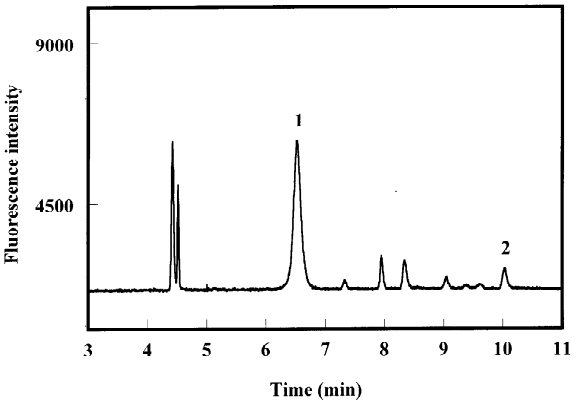

Fig. 1. Electropherogram of a reaction mixture containing methylene blue, an internal standard. Condition for the labeling: reaction time, 67 min; molar concentration ratio of Cy5 to BSA, 1:1. Separation condition: borate buffer 10 m*M*, pH 8.6; 6% (m/v) sodium dodecylsulfate; 3.5% (v/v) methanol. Peaks: 1 = labeled BSA; 2 = methylene blue.

without added BSA under identical separation conditions. As the figure indicates, the labeled BSA and internal standard were completely separated under those optimized conditions.

In order to evaluate reproducibility for the HETP ratio of labeled BSA to methylene blue, the same experiment was performed on different days: An initial concentration of 0.5 mg/ml BSA was reacted with 10 μl of the Cy5 solution in 0.1 *M* pH 9.3 carbonate buffer. Data on the relative area and the HETP ratio of labeled BSA to methylene blue for the two batches, sample A and sample B are shown in Table 1. The results were reproducible within an error of 5% for peak areas. However, poor reproducibility of the HETP ratio was found because the measurement of HETP is more sensitive to small changes in the experimental conditions, e.g. the condition of the capillary surface, than that for peak area. Such larger deviations in HETP ratio were also found in the run-to-run measurements. As Table 1 shows, the relative standard deviation (RSD) of HETP ratio for samples A and B is 15 and 19%, respectively. Therefore it must be assumed that the ratio of the plate heights contains an average RSD of 17%, in terms of evaluating the band broadening.

Table 1
Reproducibility of the relative area and HETP ratio of labeled BSA to an internal standard

	Sample A	Sample B
Injection times	5	8
Relative area	8.4±0.6	8.0±0.2
HETP ratio	14.8±2.2	13.0±2.5

Sample A and B: 0.5 mg/ml BSA + 10 μl of Cy5 solution. Reaction buffer: 0.1 *M*, pH 9.3 carbonate buffer.

The relationship between the molar concentration ratio of Cy5 to BSA in the reaction mixture and the HETP ratio of labeled BSA to methylene blue is shown in Fig. 2. The standard deviation, as indicated by the error bar, was calculated from the results of at least five runs. The results suggest that band dispersion was strongly dependent on the ratio of the concentrations of Cy5 to BSA. With an increase in the concentration ratio, the HETP ratio dramatically increases, indicating that the change in the heterogeneity of the labeled BSA molecules is the result of a change in the concentration ratio of Cy5 to BSA. At the ratios from 1.5 to 0.75, the relative HETP ratio is nearly constant, indicating that no change in the heterogeneity of the labeled BSA molecules occurred at these labeling conditions. Furthermore, the results suggest that a lower concentration ratio of

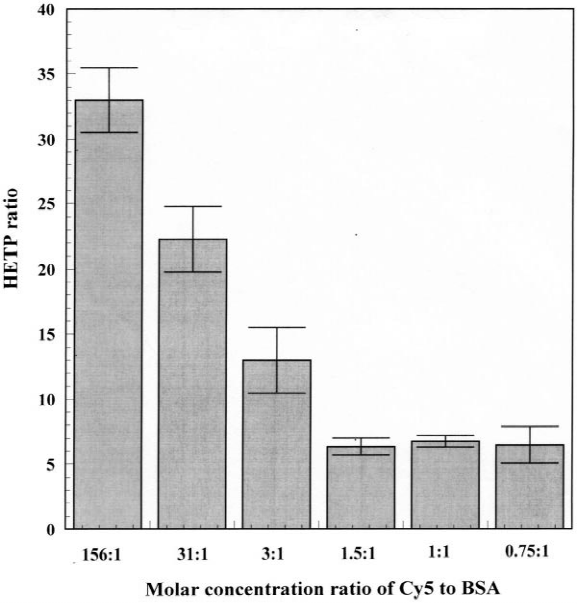

Fig. 2. Relationship of HETP ratio of labeled BSA to an internal standard and the molar concentration ratio of Cy5 to BSA (labeling in 0.1 *M* pH 9.3 carbonate buffer).

Cy5 to BSA would be expected to decrease the production of heterogeneous multiple labeling derivatives.

A measurement for the reaction time dependence of the HETP ratio of labeled BSA to methylene blue was carried out for a sample in which 0.5 mg/ml BSA was reacted with 10 μl Cy5 at 0.1 *M*, pH 7.0 phosphate buffer (Fig. 3). A change in the band broadening was clearly observed with increasing reaction time. Fig. 3 suggests that more heterogeneous labeled BSA molecules are produced as the reaction time is increased. On the other hand, the pH of the reaction buffer also has influence on the reaction, as shown in Fig. 3. In comparison with the results in Table 1, the HETP ratio at pH 9.3 was determined to be an average of 14; whereas this value was 8 at pH 7.0 when the reaction was nearly complete (note: no change was found for the relative peak area of BSA to methylene blue after a reaction time of 80 min). On the other hand, the relative peak area of BSA to the internal standard at pH 7.0 was measured to be 3.6 after 80 min of reaction time, which is much less than 8.2 at pH 9.3 (mean of the values shown in Table 1). The fluorescence intensity for the labeled BSA formed at the neutral pH is lower than that at alkaline pH, which should be mainly attributed to the difference in the average number of fluorophores on a labeled protein molecule [21], namely, degree of labeling (*D*/*P*), defined as the average number of fluorophores on a labeled protein molecule, is lower at pH 7.0 than that at pH 9.3. Thus, in term of sensitivity, an alkaline reaction buffer is preferable to a neutral one. However, a higher pH of the reaction buffer results in more extensive band broadening, suggesting that more heterogeneous protein derivatives would be produced in the alkaline reaction buffer.

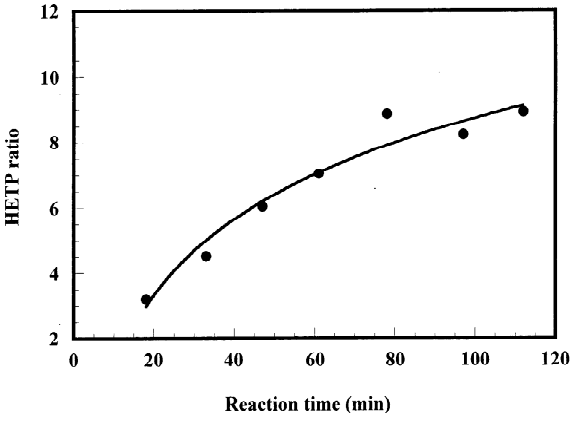

Fig. 3. The reaction time dependence of the HETP ratio of labeled BSA to internal standard (0.5 mg/ml BSA + 10 μl of Cy5 solution, reaction buffer: 0.1 *M*, pH 7.0 phosphate buffer).

It has been reported that the experimental factors, such as reaction buffer pH, reaction time and the initial concentration of dye to protein, have a direct influence on the degree of labeling (*D*/*P*), i.e. higher pH, longer reaction time, and larger initial concentration ratio of dye to protein resulted in a higher *D*/*P* [21,24]. The higher *D*/*P* ratio can be explained by taking into account the number of amino groups on the BSA molecule and their reactivity with Cy5. The labeling reaction of BSA with Cy5 is achieved by the reaction between deprotonated amino groups of BSA and succinimidyl ester of Cy5. Since BSA has several amino groups that have different pK_a values, some of the amino groups are protonated even at a neutral pH, e.g. ε-NH_2 group of lysine is usually protonated at pH 7. The protonated amino groups cannot react with Cy5, that is, the number of amino groups that can react with Cy5 is smaller at pH 7.0 than at pH 9.3. As a result, the number of Cy5 conjugated with BSA will increase with increasing the pH of the reaction buffer. Long reaction time and large initial concentration ratio of dye to protein also result in a high degree of labeling, since deprotonated amino groups could react with Cy5 as long as reactive Cy5 molecules are present in the solution.

The same influences were also found on band broadening (the heterogeneity) of the labeled protein in this research. The production of heterogeneous multiple labeling derivatives would be attributed to the difference in the reactivity of each amino group on the molecular structure of BSA. The labeling reaction of more than two Cy5 with BSA is expected not to be simultaneous, but to be gradual. Therefore, at the start of the labeling reaction, most of BSA would react with one Cy5 molecule at a site of the amino group with highest reactivity and high efficiency of conversion. As the reaction proceeds under a condition to form the labeled proteins with higher *D*/*P*, the other amino groups with lower reactivity would react with Cy5 further. However,

due to the lower reactivity of the amino groups, only some of them can react with Cy5, resulting in the formation of the BSA molecule labeled with two Cy5 molecules. Similarly, the reaction between the Cy5 molecule and the BSA molecules labeled with two Cy5 molecules yields the BSA labeled with three Cy5 molecules, but the reaction yield is expected to be smaller than those of BSA labeled with one or two Cy5 molecules. As a result, at the end of the reaction the labeled products should be a mixture of BSA derivatives attached with one, two, or more Cy5 molecules. In this study, all the labeled BSA molecules eluted as a single peak, but band broadening of the BSA was observed at the labeling condition where multiple labeling is expected to happen. This fact indicates that more heterogeneous labeled BSA molecules were involved in the single peak. Therefore, as the D/P value increases, the number of possible products will increase, resulting in band broadening (high heterogeneity) of labeled BSA peak. Obviously, higher heterogeneity is also induced by the labeling condition of higher pH, longer reaction time, and larger concentration ratio of Cy5 to BSA. Therefore it can be concluded that the labeling conditions that lead to larger degree of labeling (D/P) are also associated with the more serious band broadening problem.

It seems to be difficult to perform quantitative analysis for proteins directly using labeling reaction since the D/P value is affected by the labeling reaction conditions as shown in this study. In fact, our preliminary experiments indicated that no linear relationship between relative area of BSA and its concentration were found within a concentration range (0.01–2 mg/ml BSA) when the labeling reactions were carried out in the presence of a constant amount of Cy5, where Cy5 is an excess component. In spite of that, labeled proteins are practically used as a probe for immunoassay. We have also reported immunoassay of human serum albumin using CE [25], in which human serum albumin labeled with Cy5 was employed as a fluorescent probe. In immunoassay, a labeled protein must retain its biological activity. However, the labeled protein would lose its activity gradually as more and more fluorophores are attached on the protein, so that a lower D/P value would be preferable. Therefore, low concentration ratio of Cy5 to the protein and the neutral pH buffer should be recommended for the labeling reaction to prepare a labeled protein as a probe in immunoassay.

Heterogeneously labeled products induced by the multiple labeling of proteins resulted in a decreased separation efficiency for the products. Therefore, in practical applications of CE-LIF in the trace analysis of proteins, labeling conditions must be carefully chosen. To improve the detection sensitivity for proteins, the labeling conditions should be selected so that more fluorophores are attached to the proteins, that is, a large D/P is favorable for achieving higher sensitivity of detection. However, on the other hand, the higher degree of labeling, unfortunately, results in the production of a wide variety of heterogeneous protein derivatives, which decrease the separation efficiency and potentially degrades the resolution of CE. In this case, a compromise between separation efficiency and detection sensitivity for proteins of interest is required in the selection of labeling conditions.

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