Retention Characteristics of Various Proteins in Flow Field-Flow Fractionation : Effects of pH, Ionic Strength, and Denaturation

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The study investigated the effect of carrier composition (ionic strength and pH) on the retention of various proteins in flow field-flow fractionation (Flow FFF) as well as the conformational change of Bovine Serum Albumin (BSA) with urea concentration, storage time and temperature. The study found that the retention of protein in Flow FFF increased with the ionic strength of the carrier liquid. Most proteins were well solubilized at pH = 7-8. The hydrodynamic diameters obtained from Flow FFF retention data agree well with theoretical values. The retention increased and the peak shape became distorted at extreme pH conditions of the carrier solution. The selected carrier composition for comparison between the literature value of proteins was 0.05 M tris buffer solution with a pH of 8. Storing BSA at 4 ± 2 °C over a period of three months resulted in slow dimerization. Also, in case of the storage of BSA at 37 ± 5 °C for one week, the retention of both BSA monomer and dimer increased with the urea concentration. Finally, the structural composition of specific enzymes: malonyl-CoA decarboxylase (MCDC) and malonyl-CoA synthesis (MCS) was determined by using Flow FFF at specific carrier solutions. The molecular weight of the natural MCDC was determined to be 208 kDa, which means it is a homotetramer, while that of the MCS was determined to be 47 kDa, which means it is a monomer.

Introduction

Proteins control physiological functions and are involved in metabolism. The clarification of that mechanism is of interest to many researchers. Rapid separation is required for the characterization of proteins without disrupting the unique three-dimensional structure which affects the protein activity.[1] Many methods exist for the characterization of proteins. Existing methods for separation and analysis of proteins include capillary electrophoresis,[2,3] gel filtration chromatography,[4] hydrophobic interaction chromatography,[5] sodium dodecyl sulfate-polyamide gel electrophoresis,[6] ion exchange chromatography,[7] reverse phase liquid chromatography,[8] and affinity chromatography.[9] The influence of the mobile phase ionic strength and pH on the retention mechanism is well known.[7,10-13] Most of existing methods contain the stationary phase, and the presence of the stationary phase could cause some problems such as the sample adsorption of the sample due to undesired interaction between the sample and the stationary phase.[1] Also, the choice of the carrier liquid is limited, as the carrier must be compatible with the stationary phase.

Field flow fractionation (FFF) is a family of separation techniques that applies to the analysis of macromolecular, colloidal materials and proteins.[14,15] Utilization of the flow FFF methods for the separation of proteins and for the simultaneous measurement of their diffusion coefficients D was demonstrated.[16,17] FFF uses a thin ribbon-like channel that is open (does not contain the stationary phase), and thus minimizes the adsorption or shear degradation of the sample. Unlike chromatographic methods where the sample component is retained by the interaction between the sample and the stationary phase, the external field perpendicularly acting to the channel flow induces the retention of the sample in FFF. The application of various external fields gives rise to various subtechniques of FFF. The type of FFF fields are sedimentation (sedimentation FFF), cross flow (flow FFF), temperature gradient (thermal FFF), and electrical field (electrical FFF). The channel flow is a laminar having a parabolic profile, and the unequal velocities of the laminar flow cause the separation in FFF channel.

In flow FFF, the cross flow is employed as the external field that enters through the upper block of the channel and exits through the bottom block. The driving force in flow FFF is thus the viscous force exerted on sample components toward the bottom wall of the channel by the cross flow stream. The retention time in flow FFF is approximately proportional to the hydrodynamic diameter of sample materials. Flow FFF has unique advantages in protein analysis. First, there is very little (if any) mechanical stress on sample components as the channel is open. Second, flow FFF is so versatile that various carrier liquids can be used. Third, the diffusion coefficient of sample components can be directly calculated from the retention measurement.

The purpose of this study is to investigate the effect of the carrier liquid on the retention of protein in flow FFF. As compared with published experiments, it was commonly performed at the neutral pH and ionic strength of 0.05-0.1 M. In this experiment, the retention behavior of various proteins, that was chosen to have different values of molecular weight and isoelectric point, is examined by varying the ionic strength and pH of the carrier liquid. The conformational change (and thus the denaturation) of the Bovine Serum Albumin (BSA) is studied by varying the storage

times and temperature. Finally, based on earlier experiments, real samples were analyzed at optimum condition. The study determined the structural composition of enzymes: malonyl-CoA decarboxylase (MCDC), which plays an important role in the symbiosis of bacteroid; and malonyl-CoA synthesis (MCS), which catalyzes the formation of malonylCoA directly from malonate and CoA with hydrolysis of ATP to AMP and PP.[18] The structural information of these enzymes is important for the investigation of metabolism.

Theory

In FFF channel, the sample concentration c is defined by

$$c = c_0 \exp(-x/l) \tag{1}$$

where c_0 is the concentration of sample materials at the accumulation wall (bottom wall), x the distance from the accumulation wall, l the mean layer thickness of the sample materials.[19] The retention of the sample is measured by the retention ratio R.

$$R = \frac{t^0}{t_r} = \frac{V^0}{V_r} = 6\lambda\left[\coth\left(\frac{1}{2\lambda}\right) - 2\lambda\right] \tag{2}$$

where t^0 is the void time, t_r retention time of the sample component, V^0 the channel void volume, V_r the retention volume, and w the channel thickness. The retention parameter l is defined by

$$\lambda = \frac{l}{w} = \frac{kT}{Fw} \tag{3}$$

where k is the Boltzmanns constant, T the absolute temperature, and F the force exerted on the sample by the external field. In FFF, separation is achieved by the difference in l (see Eq. 2), and eventually by the difference in F (see Eq. 3). The force F in flow FFF is given by

$$F = fU = 3\pi\eta dU \tag{4}$$

where d is the hydrodynamic diameter of the sample component, η the viscosity of the carrier liquid, and U the linear velocity of cross flow. It is noted that the force in flow FFF depends only on the hydrodynamic diameter of sample components. The field-induced velocity U can be obtained by

$$U = \frac{V_c}{bL} \tag{5}$$

where V_c is the volumetric flow rate of cross flow, b the breadth of the channel, and L the length of the channel. By combining Eqs. 3, 4, and 5, the dimensionless parameter λ is obtained as

$$\lambda = \frac{bLkT}{3\pi\eta w dV_c} = \frac{V^0 kT}{3\pi\eta w^2 dV_c} \tag{6}$$

Using Eq. 6, the hydrodynamic diameter of proteins or particle samples is determined from an experimental measurement of λ (see Eq. 2). The relationship between the diffusion coefficient D and t_r is

$$D = \frac{w^2 V_c}{6 t_r V} \tag{7}$$

Using Eq. 7, the diffusion coefficient D can be also determined by measuring the retention time t_r of the sample in flow FFF. To get the more reasonable values of the applied samples, the calibration curve was also used. The molecular weight and structural information of a real sample can be determined using the calibration curve of some protein standards (ribonuclease, BSA, γ-globulin, ferritin) along with those of a real sample which was indicated by log λ *versus* log MW.

Experimental Section

The Flow FFF system used in this work is a Model F-1000 Universal fractionator purchased from the FFF ractionation, LLC (Salt Lake City, UT, USA). The flow FFF channel has a Mylar spacer sandwiched between two porous frits. The channel has the length of 29.9 cm, breadth of 2 cm, and the thickness of 0.0254 cm. The YM-10 regenerated cellulose membrane with a molecular cut-off value of 10,000 (Amicon, Inc., USA) was used as the accumulation wall. Two Eldex CC-100-S metering pumps (Eldex Laboratories Inc., Nape, CA, USA) were used to deliver the cross and channel flows. Flowrate was measured by using a buret. Eluted samples were monitored using an M-720 UV detector purchased from the Young-In Scientific Inc. (Seoul, Korea). The detector signal was collected and processed using the FFF data analysis software version 2.0 provided by the FFFractionation, LLC. To measure the actual channel thickness, polystyrene latex 50, 105, 155nm standards obtained from the

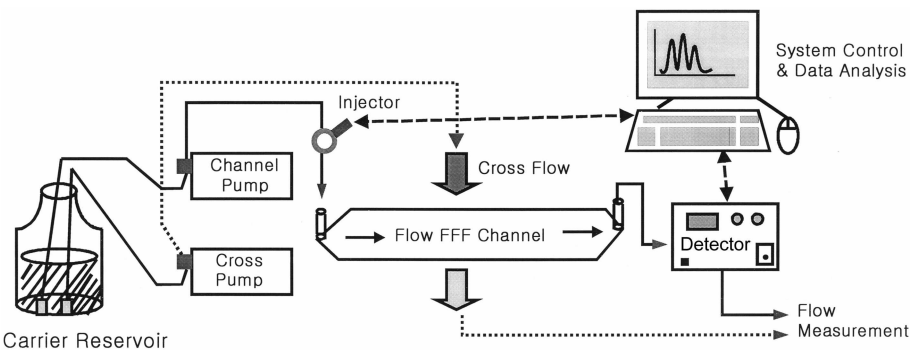

Figure 1. Schematic diagram of flow FFF system.

Table 1. Molecular Weight and Isoelectric Point (pI) of Proteins Used in This Work

Proteins	MW*	pI*	Source
Ribonuclease	13.700	9.0	Bovine Pancreas
β-lactoglobulin	35.000	5.2	Bovine Milk
Hemoglobin	65.000	6.9	Horse
Albumin(BSA)	66.000	4.9	Bovine Serum
γ-globulin	150.000	7.0	Bovine
Ferritin	443.000	5.0	Horse Spleen

*see ref. 13.

Duke Scientific (Palo Alto, CA, USA) were used with a carrier solution of 0.1% FL-70 (Fisher Scientific, Fair Lawn, NJ, USA) and 0.02% NaN_3 as a bactericide.

A schematic diagram of the flow FFF system is shown in Figure 1. Protein samples that were used in this study are listed in Table 1. For all flow FFF experiments, the protein samples is dissolved to 2 mg/mL at pH 8, 0.01 M tris buffer solution and 20 mL of sample solutions were injected, using a Rheodyne 7725 loop injector (Rheodyne, L. P., CA, USA). The carrier solution for proteins analysis was 0.005-0.1 M tris buffer solution. To adjust the pH and ionic strength of the carrier liquid, tris (hydroxymethyl) aminomethane hydrochloride (Trizma HCl, Sigma Chemical Co, MO) and Trizma Base were mixed. 0.1 M H_3PO_4 was added to adjust the pH 3, 5 of the carrier liquid, and 0.1 M NaOH was added to adjust the pH 11, 13 of the carrier liquid, respectively. The denaturation of BSA was studied by varying the concentration of urea and by storing them at different temperature. A Hitachi F-4500 Fluorescence spectrometer (Hitachi, Japan) was used for fluorescence spectrometry. The excitation wavelength was 280 nm and the emission wavelength, 270-500 nm. For the analysis of the MCDC and MCS, each specific carrier solutions was used to provide the same condition as the stock solutions. MCDC was dissolved at pH 7.3, 0.05 M potassium phosphate buffer, and MCS was dissolved at pH 7.1, 0.005 M potassium phosphate buffer containing 15% glycerol and 10mM $MgCl_2$.

Results and Discussion

Effect of ionic strength on carrier solution. The effect of the ionic strength of the carrier on the retention of proteins in flow FFF is investigated. As the ionic strength of the medium increases, molecules can move closer to each other due to a reduction in the electrical double layer thickness.[20] Thus, at higher ionic strength, molecules can move closer to the accumulation wall of the FFF channel, resulting in increase in retention time and also in measured diameter. At lower ionic strength of the carrier solution, retention time (and the measured diameter) decreased due to increased electrostatic repulsion between charged molecules. Figure 2 shows the hydrodynamic diameter measured at various ionic strengths. As expected, the retention of proteins increased with the ionic strength of the carrier, and thus the measured hydrodynamic diameter increased. The measured diameter

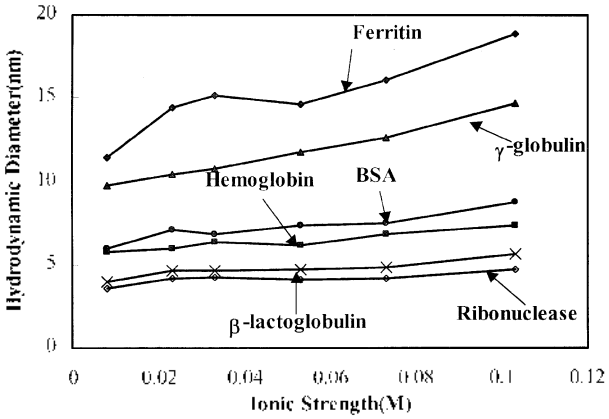

Figure 2. Hydrodynamic diameter of six proteins at various ionic strength. Flow conditions are channel flow of V_{out} = 0.50 mL/min and cross flow of V_c 2.65 mL/min (±0.05 mL). Carrier is tris buffer solution of pH 8.00.

increases more rapidly at higher ionic strengths than at lower ones. The difference between the diffusion coefficient obtained at 0.005 M and 0.1 M was 20-39%. The change in diffusion coefficient was not significant for small spherical proteins such as ribonuclease. The change became more significant as the molecular size increases. This is due to the larger proteins are affected a lot for longer period of time by the field. Moreover, the decrease of electrical double layer under the strong ionic condition enables the sample materials to approach the channel surface closely and it resulted in the larger retention of proteins.

Effect of pH on carrier solution. It is generally known that the solubility and conformation (thus the denaturation) of proteins are affected by pH, temperature, salt concentration, and other factors. To study the effect of carrier pH on the retention of proteins in flow FFF (and thus to find an optimum pH range for protein analysis), fractograms were obtained for each protein at various pH conditions. At pH ~ 7, protein molecules have appropriate charge density and maintain their unique three-dimensional structure. It can minimize the nonideal retention due to the aggregation or dissociation of protein molecules. As shown in Figure 3, the retention time and the shape of fractograms changed with pH. The most appropriate pH condition for protein analysis seemed to be between 7 and 8. The hydrodynamic diameters measured at pH 3 were different from those at the pH ~ 7, which is probably due to the dissociation with the subunits and adsorption between molecules and membrane. The tendency of retention behavior of each protein did not proportional to their molecular weight, because they have different isoelectric point and structural character. Also, fractograms obtained at their isoelectric points were broader and retained more. At that condition, because the net charge of the proteins became zero, the reduction in the electrostatic repulsion facilitated the aggregation of molecules and resulted in an increase in measured diameter. For example, β-lactoglobulin exists in various oligomeric forms depending upon the composition of the medium.[21,22] At pH ~ 13, the measured hydrodynamic diameter was much larger than that measured

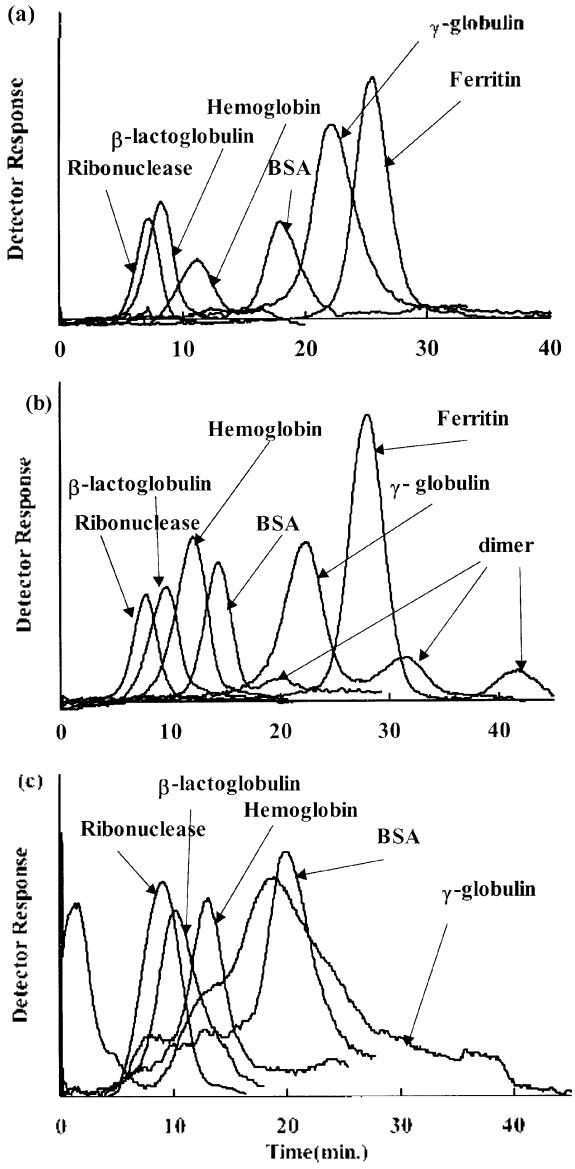

Figure 3. Fractograms of proteins (a) at pH 3. (b) at pH 7 and (c) at pH =13. Flow conditions are V_{dot} = 1.01 mL/min and V_c = 3.41 mL/min (±0.05 mL). Carrier is tris buffer solution of I = 0.053 M.

at pH – 7. The structural variants contributed differently at different pH range of the carrier. The carrier condition affected the alteration of the hydrogen bond and the disulfide bond or the ionization of amino acid residue, which was correlated with the unfolding or the separation of subunits. Figure 4 shows the variation of the measured hydrodynamic diameter with the pH. Certainly a simple comparison of the measured diameter alone can not accurately explain the conformational change of the protein molecules, thus more investigation is needed. For an accurate size analysis of proteins using flow FFF, the aggregation and the denaturation of protein molecules must be prevented, and optimum conditions of the pH and the ionic strength of the carrier liquid must be found.

Denaturation of BSA by urea. BSA has a free cysteinyl residue, a molecular weight of 66,000 and an isoelectric

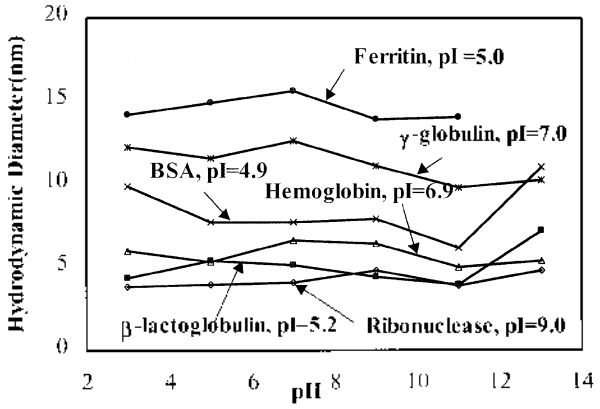

Figure 4. Hydrodynamic diameter of six proteins at various pH. Flow conditions are V_{dot} = 1.01 mL/min and V_c = 3.41 mL/min (±0.05 mL). Carrier is tris buffer solution of I = 0.053 M.

point of 4.9. BSA has a tertiary structure consists of three spatially distinct domains and several subdomains. Also, its several isomers exist at various pH ranges. Proteins are easily denatured as a result of conformational alteration. Protein molecules end up with a random conformation by a change in the non-covalent bond without breaking the covalent bond.

Figure 5-7 shows the fractograms of BSA stored at different urea concentrations. Because the original sample contains had a small amount of dimers, there is a shoulder beside the main monomer peak. The dimer shoulder grew with the urea concentration. Most of the protein molecules were denatured at 8 M-urea solution. When urea was removed from the sample solution, the conformational change was reversible due to the reconstruction of noncovalent bond between the amino acid residues each other. When BSA was stored at 4 + 2 °C in different urea concentration for three months, what resulted was additional changes that were conformational and chemically modified in nature that is, not easily reversed upon urea dilution. After storing BSA at 4 ± 2 °C, that sample solutions were stored at 37 °C ± 5 for

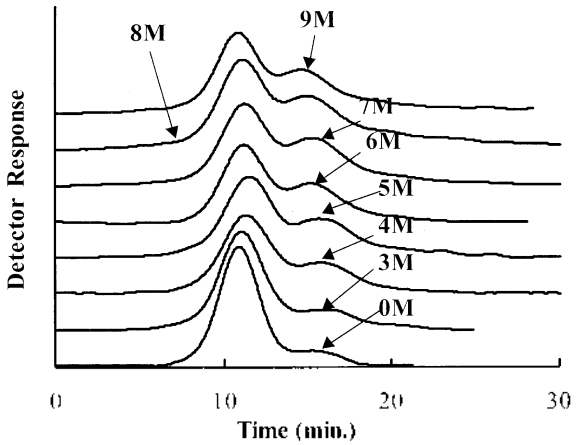

Figure 5. Fractograms of BSA denatured by various urea concentrations. All samples are stored at 4 °C±2 during 3 months. Flow conditions are V_{dot} = 0.50 mL/min and V_c = 2.65 mL/min (±0.05 mL). Carrier is tris buffer solution of pH 8.0, I = 0.053 M.

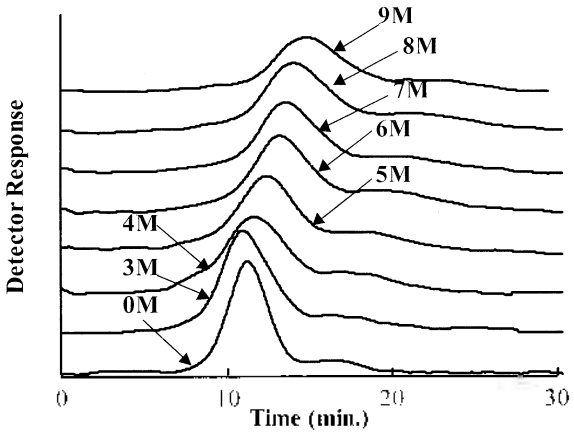

Figure 6. Fractograms of BSA denatured by various urea concent-rations. All samples are stored at 37 °C ⊥ 5 during 1 week. Flow conditions are V_{dot} = 0.50 mL/min and V_c = 2.65 mL/min (+0.05 mL). Carrier is tris buffer solution of pH 8.0. I = 0.053 M.

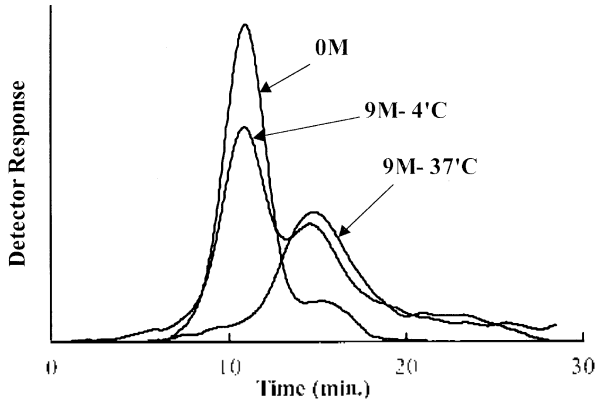

Figure 7. Fractograms of BSA denatured by various urea concentrations. Flow conditions are V_{dot} = 0.50 mL/min and V_c = 2.65 mL/min (±0.05 mL). Carrier is tris buffer solution of pH 8.0. I = 0.053 M.

one week additionally and also analyzed by Flow FFF. This resulted in increased retention time for both monomer and dimer with urea concentration. The retention time of the monomer peak of the BSA denatured by 8-9 M urea solution was similar to that of the dimer peak of the native species in 0 M urea solution. Figure 7 shows the fractograms of BSA stored at 37 °C ⊥ 5 for one week as well as the most distinguishable results of 9 M-urea BSA solution stored between 4 °C ⊥ 2 and 37 °C ⊥ 5.

Figure 8 shows the fluorescence spectrum of BSA samples that are the same as those in Figure 6. There are three amino acids: phenylalanine, tryptophane, and tyrosine; that have the delocalized π-electrons. The hydrophobicity of tryptophan was larger than that of other residues and is sensitive to environmental conditions. The intensity of fluorescence emission was mostly due to this amino acid residue. Conformational information was extracted by investigating the tendency of this residue. Tryptophan had maximum fluorescence emission at 348 nm, which was moved to a higher wavelength due to the polarity of the aqueous solution. On the other hand, the peak maximum shifted to a shorter wavelength at non-polar condition. Peak intensity was proportional to the dynamic motion of the surrounding residues and water molecules. In the process of denaturation of BSA, intensity is quenching induced by exposure of tryptophan that had been folded inside of protein. Maximum intensity of fluorescence emission of BSA is at 345 nm. There was no shift in the wavelength from 0 M to 5 M of the urea solution, but from 6 M to 9 M of the urea solution, peak maximum shifted to a longer wavelength and intensity decreased. These results indicate that BSA was in an unfolding process and further supported the result of the flow FFF data.

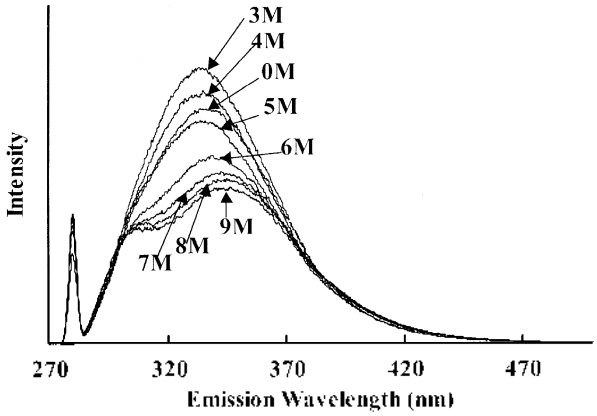

Figure 8. Fluorescence spectra of BSA. Excitation wavelength is 280 nm and emission wavelength is 270-500 nm. All samples are stored at 37 °C ± 5 during 1 week.

Application to real samples (MCDC, MCS). The molecular weight and structural information of a real sample can be determined by using Flow FFF. In some chromatographic methods, to avoid the interaction between the stationary phase and samples, the solvents ionic strength is increased and the pH range of the carrier solution is limited. But in Flow FFF, the carrier condition is gentler and can be adjusted to a wide range of pH and ionic strength. In this experiment, a specific carrier solution was used to maintain the stable condition of these enzymes. Malonyl-CoA decarboxylase (MCDC) has a calculated molecular weight of 51 kDa and consists of 462 amino acids, while Malonyl-CoA

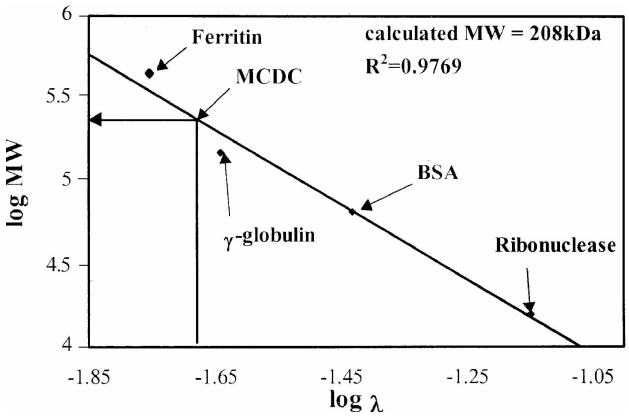

Figure 9. Calibration curve of MCDC with some protein standards. Carrier is phosphate buffer saline pH 7.3. Flow conditions are V_{dot} = 0.50 mL/min and V_c = 2.55 mL/min (± 0.05 mL).

synthetase (MCS) has a calculated molecular weight of 55 kDa and consists of 504 amino acids. These two proteins have been known to malonate-specific enzyme related with the metabolism of malonate. Structural information on these proteins is essential for clarifying the metabolism of malonate. Figure 9 shows the calibration curve of some protein standards (ribonuclease, BSA, γ-globulin, ferritin) along with those of MCDC. The molecular weight of the native MCDC was determined to be 208 kDa by Flow FFF, and the linearity of calibration curve is about $R^2 = 0.97$. The subunit molecular weight was determined to be 53 kDa by SDS-PAGE.[18] From these results, we can conclude that MCDC has a homotetrameric structure.[23] By the same manner, the molecular weight of MCS was determined to be 47 kDa which has suitable linearity of $R^2 = 0.97$. It means that MCS has a monomeric structure.

Conclusion

The study has found that the ionic strength and the pH of the carrier significantly affect the retention of proteins in flow FFF. Proteins were analyzed by varying the ionic strength and the pH of the carrier. At the pH of 8.0 and the salt concentration of 0.05 M, the measured hydrodynamic diameters agree well with literature values. To study the denaturation of BSA, urea was added in various concentrations and different storing temperatures. A change in condition caused changes in molecular conformation and the degree of aggregation. More information on structural change can be acquired by fluorescence spectrometry and the structural composition of enzymes can be determined through Flow FFF.

The Flow FFF method is intrinsically gentle and non-denaturing, thus particularly useful in the analysis of biomolecules such as proteins. After fractionation, each slice of the sample can be subjected to additional (or so-called secondary) analysis. Combined with other methods, Flow FFF can provide useful information on structural changes in proteins. Flow FFF has shown a potential for determining the molecular weight and characterization of oligomers of biological materials.

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