A Study of the Retention Behavior of Proteins in High-Performance Liquid Chromatography(II): The Effect of salt and Temperature on Retention Behavior of Proteins in Hydrophobic Interaction Chromatography

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The retention behavior of proteins was investigated by using hydrophobic interaction chromatography (HIC), comparing to the results obtained in reversed-phase chromatography (RPC) described in the previous paper. A SynChropak propyl column was employed with 0.05 M phosphate buffer (pH 7.0) containing sodium sulfate. Conformational changes were recognized by examining Z values as a function of sodium sulfate concentration over a range of temperature between 5 and 65℃. Z values did not change significantly at the range of the temperature showing the consistent ΔH° and ΔS° values. The sign and the magnitude of ΔH° and ΔS° of proteins in HIC were compared with those obtained in RPC. The signs of ΔH° and ΔS° of proteins in HIC were all positive, while those of proteins in RPC were all negative. These results suggested that the retention of proteins in HIC and in RPC were entropy-driven and enthalpy-driven process, respectively. From the two different investigations, it was concluded that the retention mechanism of RPC and HIC was based on the same fundamental principle in which separation is dependent on hydrophobicity, but the retention behavior of the proteins in HIC is clearly different from that observed in RPC.

Introduction

Since Hjerten described the general aspects in HIC in 1973,[1] HIC has been an important separation mode for purification and characterization of proteins.[2-7] The hydrophobic nature of proteins facilitated the separation through the hydrophobic interaction with the ligand surface of the stationary phase. There are two different modes of separation depending on hydrophobicity, reversed-phase chromatography and hydrophobic interaction chromatography.[8,9] Since RPC has already been described at the previous paper, retention behavior of protein in HIC will be discussed in this report. The surface of the stationary phases used in HIC is coated with a highly polar hydrophilic organic layer and weak hydrophobic ligates such as small alkyl or aryl functional groups.[10-14] The use of weakly hydrophobic phases in HIC provides a milder adsorptive surface, leading to the elution of proteins in an active state.[15]

Horvath *et al.* suggested that retention behavior in HIC was explained by the framework of the solvophobic theory.[16-18] The retention of proteins in HIC was able to be modulated by the use of neutral salts in the mobile phase. The principal parameters which determine the effect of the salt on the retention were salt molality and the molal surface tension increment of the salt.[19] According to the solvophobic theory, retention of proteins can be increased by increasing the salt molaity in the mobile phase or by changing salt having a greater molar surface tension increment. There are two groups of salts used in HIC, chaotropes (water structure breaker) and kosmotropes (polor water structure maker).[20] These salts can be used in the eluents simultaneously. At a constant eluent surface tension, gradient elution with two or three aqueous salt solutions was found to be superior to the gradient operation with single salt gradients in terms of modulating the retention and selectivity in HIC.[21] The initial salt concentration as well as the salt type influences the separation of proteins.[22] According to Wetlaufer *et al.*, non-ionic surfactants can be used in HIC to improve the solubility of the proteins and the selectivity in separation.[23,24]

Temperature is a very important parameter in the modulation of column selectivity.[25] Depending on the temperature and the type of proteins, conformational changes can occur during the chromatographic operation. The change of retention and peak width variations with temperature, Z value (the slope of the plot of the $\log k'$ *vs.* log [displacer salt])[26,27] and thermodynamic parameters (ΔH°, ΔS°, and ΔG°)[28,29] were used in methods for recognizing conformational changes. Recently, Chang *et al.* proposed a stoichiometric displacement retention model for protein based on water as a displacing agent in HIC instead of salt.[9]

Experimental

Equipment. The chromatographic system employed in this study consists of a Waters M-6000A and M-45 Solvent Delivery System with a Model 660 Solvent Programmer, a M-U6K Universial injector, a M-740 Data Module, a series 440 Absorbance Detector (254, 280, and 405 nm), and a Waters 991 Photodiode Array Detector (Water Associates Inc., Milford, Mass, U.S.A.). Doubly-distilled water was purified by passing it through a Milli-Q Water purification System (Millipore, Bedford, MA, U.S.A.). HIC was performed on a SynChropak propyl column, 25×0.46 cm I.D., from SynChrom (Lafayette, IN, U.S.A.).

Reagents. Sodium phosphate monobasic, sodium phosphate dibasic sodium sulfate, and all protein standards were purchased from Sigma Chemical Company (St. Louis, Mo, U.S.A.). Proteins used in this study were lysozyme, hemoglobin, cytochrome c, myoglobin, protease, transferrin, carbonic anhydrase, ovalbumin, α-lactoalbumin, and albumin.

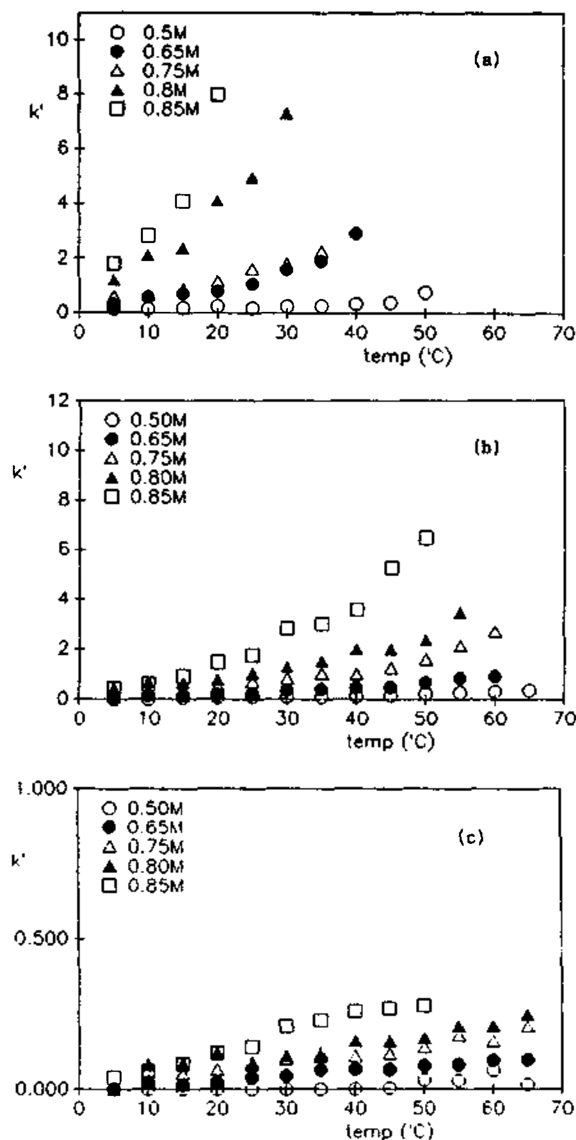


Figure 1. Plots of k' *vs.* temperature for α-lactoalbumin (a), carbonic anhydrase (b), and myoglobin (c): column, SynChropak propyl column; mobile phase, 0.05 M phosphate buffer (pH 7.0) containing 0.50 (○), 0.65 (●), 0.75 (△), and 0.80 (▲), and 0.85 (□) M sodium sulfate; flow-rate, 1.0 m*l*/min.

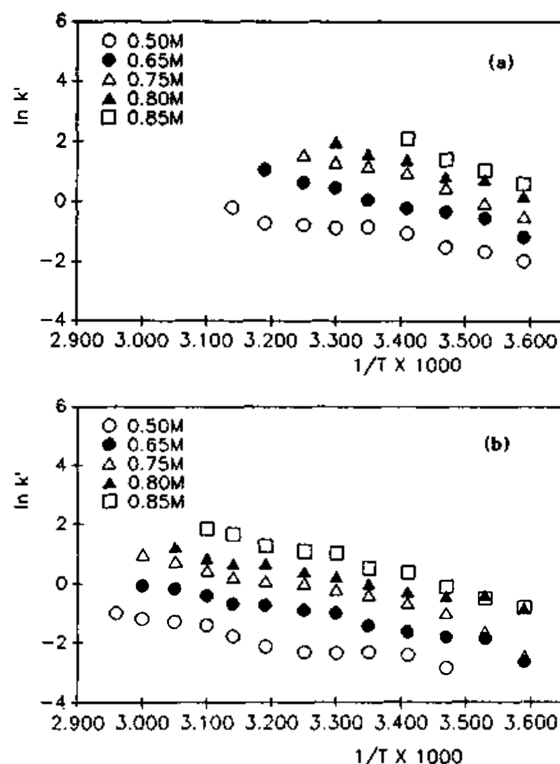


Figure 2. Plots of ln k' *vs.* 1/T for α-lactoalbumin (a) and carbonic anhydrase (b). Other conditions are same Figure 1.

Chromatographic Procedure. The mobile phase A was 0.05 M phosphate buffer, adjusted at pH 7.0, and the mobile phase B contains 1.5 M sodium sulfate added to the mobile phase A. The mobile phase A and B was filtered and degassed by Waters filter kits. By combining A and B, different concentrations of sodium sulfate as a practical solvent were prepared as 0.50, 0.65, 0.75, 0.80, and 0.85 M. The retention time data was obtained over a range of temperature between 5 and 65℃. Protein solutions were prepared by dissolving in 0.05 M phosphate buffer (pH 7.0) at a concentration of 3 mg/m*l*. Injection size was 5 μ*l* and the flow-rate was 1 m*l*/min.

Results and Discussion

Retention Behavior of Proteins by Changing Salt Concentration and Temperature. The retention behavior of a set of proteins on a propyl ligand column was investigated as a function of salt concentration and temperature. As shown in Figure 1, k' increases with the increase of temperature in opposition to the observations from RPC. This trend implies that retention of proteins in HIC is entropy-driven process. Generally, the linearly increasing part of the elution volume with increase in temperature is absent or very small due to a conformational change.[31] The temperature region showing a non-linear increase in retention time in Figure 1 is in part due to the conformational change of the protein at high temperature which results in an increase in the conformational entropy. Temperature dependence of k' increases when the concentration of sodium sulfate increases from 0.50 M to 0.85 M. A higher concentration of sodium sulfate (a high ionic strength) raises the surface tension of the mobile phase, thus it promotes the hydrophobic interaction between proteins and the stationary phase. It is well known that the long contact period of the protein to the adsorbed surface can lead to the denaturation of proteins and harsh conditions are required for strongly adsorbed protein to be eluted.[32] It was shown in Figure 1 that the k' value of myoglobin was nearly zero throughout the all experimental conditions. This means that they interact very weakly with column surface and their retentions were little or less influenced by temperature effect or salt concentration than other proteins. While lysozyme and carbonic anhydrase were relatively stable for a wide range of temperature, α-lactoalbumin was denatured very sensitively by the temperature effect. From the experimental chromatogram, the elution of α-lactoalbumin appeared as a broad peak when tempera-

Table 1. Changes in Enthalpy and Entropy for the Carbonic Anhydrase and α-Lactoalbumin

Na_2SO_4	Carbonic anhydrase		α-Lactoalbumin	
M	$\Delta H°$ (kcal/mol)[a]	$\Delta S°$ (cal/mol·K)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/mol·K)
0.50	16.02^{40-50}	49.87	9.64^{5-20}	33.66
	5.44^{50-65}	17.09		
0.65	4.91^{10-25}	16.59	8.62^{10-35}	32.23
	6.42^{30-60}	22.10		
0.75	8.88^{15-35}	32.07	16.09^{5-20}	59.79
	10.51^{40-60}	36.54	7.11^{20-35}	29.17
0.80	7.97^{15-40}	29.77	11.65^{5-30}	45.42
0.85	12.30^{5-30}	45.52	15.90^{5-20}	61.18

[a] Superscripts denote the temperature region (℃) of the van't Hoff plot used to derive the corresponding parameters.

Table 2. Comparison of $\Delta H°$ and $\Delta S°$ for Hemoglobin and Myoglobin

Na_2SO_4	$\Delta H°$ (kcal/mol)		$\Delta S°$ (cal/mol·K)	
M	Hemoglobin	Myoglobin	Hemoglobin	Myoglobin
0.50	10.85^{35-65}		35.49	
0.65	11.88^{20-45}	3.28^{35-65}	41.85	8.09
0.75	8.68^{15-45}	3.91^{25-65}	33.26	11.29
0.80	12.58^{5-15}	3.93^{40-65}	47.43	11.80
0.85	17.53^{5-25}	2.90^{30-50}	65.07	9.54

ture was raised. When the experiment was carried out at 0.85 M sodium sulfate, elution peak disppeared to the baseline even though the temperature was near 20℃. The k' values of myoglobin and hemoglobin were similar in RPC but they were greatly different from each other in HIC.

The Thermodynamic Considerations of Protein Retention in HIC. Figure 2 shows the plots of $\ln k'$ *vs.* reciprocal temperature obtained at different salt concentrations for α-lactoalbumin and carbonic anhydrase. $\Delta H°$ and $\Delta S°$ of each data point were calculated for the linear range of temperature, and these are listed in Table 1. Compared to the negatively charged values obtained by RPC, $\Delta H°$ and $\Delta S°$ values by HIC are positive (all other proteins used in this paper showed the same trend). This suggests that the association of the proteins with the ligand surface of HIC is an entropy-driven process, thus the retention process of proteins is more favorable with increase of temperature. The fact that HIC can be performed at a low temperature without an excessive interaction with the ligand surface of the stationary phase suggests that HIC is a powerful technique for protein separation without incurring the denaturation of proteins at ambient temperature. The calculated values of $\Delta H°$ and $\Delta S°$ for hemoglobin and myoglobin were compared in Table 2. In RPC, $\Delta H°$ and $\Delta S°$ for hemoglobin and myoglobin were very similar, but in HIC these values for hemoglobin are larger than those for myoglobin. It means that the magnitude of the hydrophobic interaction of hemoglobin

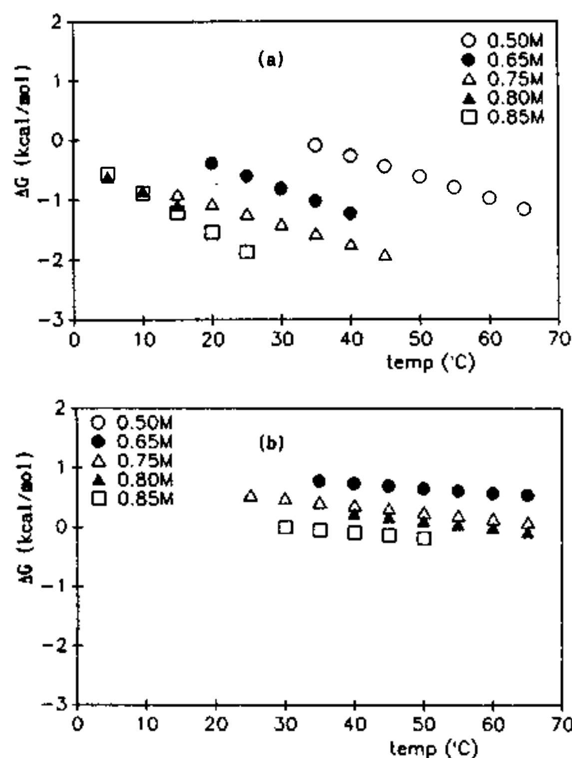


Figure 3. Plots of $\Delta G°$ *vs.* temperature for hemoglobin (a) and myoglobin (b). $\Delta G°$ was calculated from Table 2.

with the ligand surface is greater than that of myoglobin. For the better understanding of thermodynamic properties of these samples, the change in free energy ($\Delta G°$) calculated from the data points (Table 2) were plotted against temperature in Figure 3. The $\Delta G°$ values of myoglobin are slightly above zero, except in the case of 0.85 M sodium sulfate. It means that translation of myoglobin in mobile phase to stationary phase is an unfavorable process. However, $\Delta G°$ for hemoglobin are negative value and the affinity of hemoglobin to the ligand surface is considerably large even at the low temperature. The value of $\Delta G°$ of hemoglobin appears to change more sensitively than that of myoglobin upon the change of temperature or the concentration of sodium sulfate. All these facts show that the retention behavior of hemoglobin and myoglobin has a considerable selectivity in HIC.

Considerations of Conformational Changes by *Z* Value. Figure 4 shows that the plots of Z (the slope of plot of $\log k'$ *vs.* $\log [Na_2SO_4]$) *vs.* temperature were nearly unchanged with the increase of temperature. It is suggested that at the temperature range giving consistent $\Delta H°$ and $\Delta S°$, change of surface contact area of proteins with stationary phase may not happen. The values of $\log I$ little or slightly increase with the increase of temperature in Figure 4. It is likely that the affinity of proteins for ligand surface slightly increases with the increase of temperature. For the comparison with the retention behavior observed in RPC, the plots of Z *vs.* temperature and the plots $\log I$ *vs.* temperature for hemoglobin and myoglobin in HIC are illustrated in Figure 5. The Z values of hemoglobin and myoglobin are similar to each other throughout the overall temperature ra-

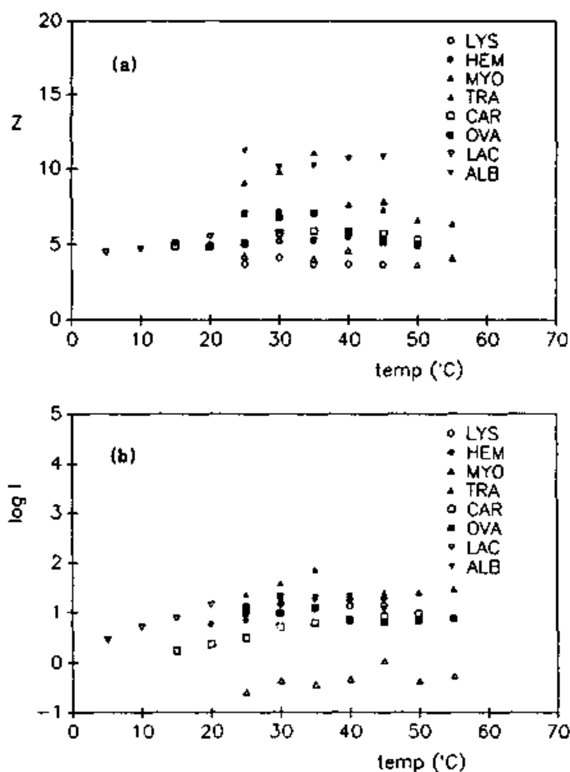


Figure 4. Plots of Z *vs.* temperature (a) and $\log I$ *vs.* temperature (b) at the temperature range with constante ΔH° and ΔS° for each proteins.

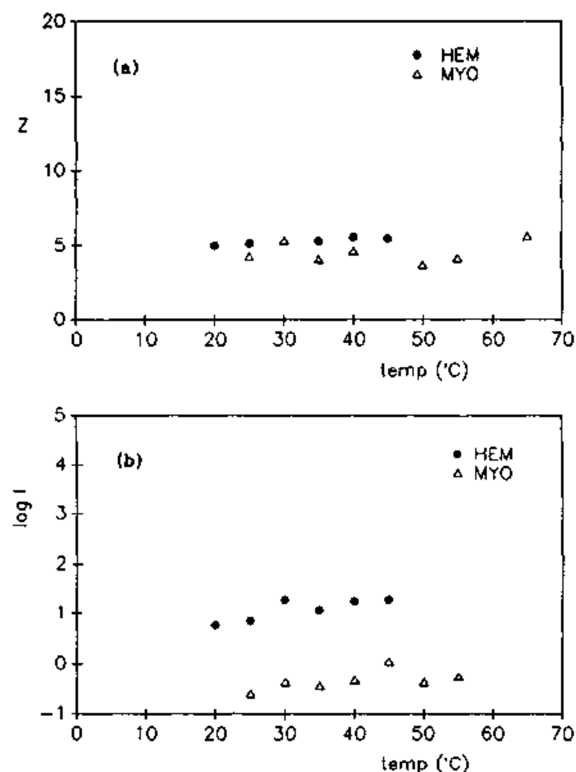
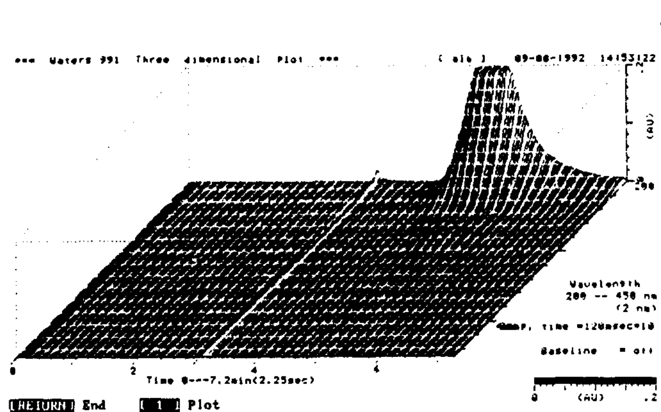
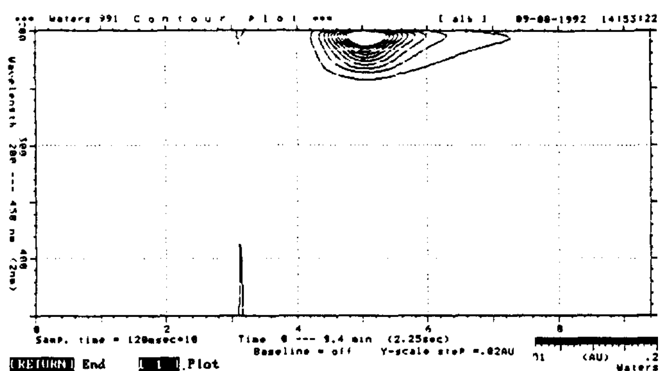


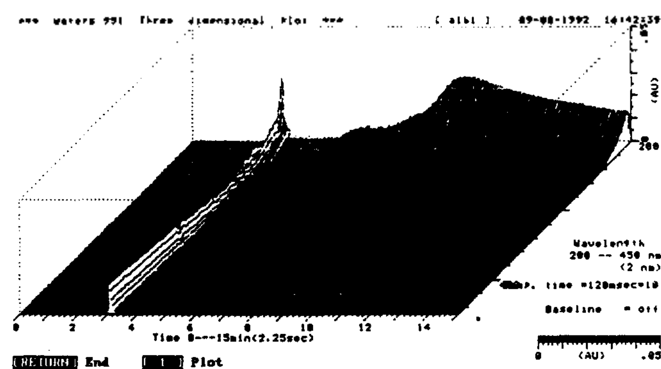
Figure 5. Plots of Z *vs.* temperature and $\log I$ *vs.* temperature for hemoglobin and myoglobin in HIC.

(a)





(b)



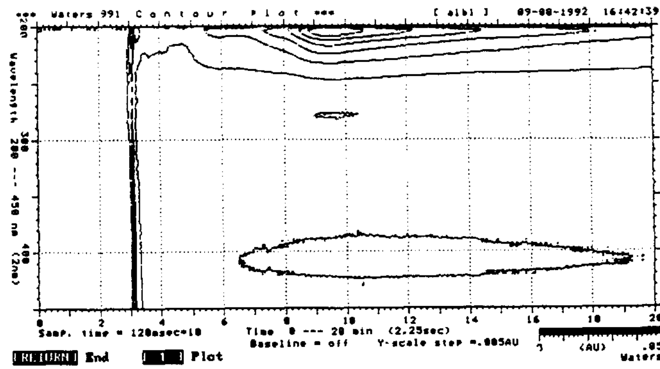


Figure 6. UV spectra with the photodiode array detector for albumin eluted in 0.05 M phosphate buffer (pH 7.0) containing 0.85 M sodium sulfate at 30℃ (upper) and 50℃ (lower).

nge, however the $\log I$ values of hemoglobin are greater than those of myoglobin. These facts indicate that the hydrophobic contact area of the two proteins for the hydrophobic ligand surface of stationary phase is similar, but the interaction forces for stationary phase or column affinity of hemoglobin are larger than those of myoglobin. The difference in the interaction forces results in a distinct change in the retention behavior of hemoglobin and myoglobin. The plots of Z *vs.* $\log I$ for a set of proteins-lysozyme, hemoglobin, transferrin, carbonic anhydrase, ovalbumin, α-lactoalbumin, albumin-do not show any correlation in HIC used in this paper (data not shown).

Futher Study for Detection of Conformation Changes of Proteins. Spectroscopy is a well developed method for the chanacterization of protein conformational changes. However, some spectral changes are reversible, hence important information may be lost by off-line spectroscopy. Since the on-line photodiode array detector can provide the whole spectrum immediately, it is useful to explore protein conformational changes as a function of column temperature. As shown in Figure 6, albumin peak obtained at 50℃ was smaller and broader than it was obtained at 30℃. This fact suggests that the conformational change of albumin may occur at the higher temperature. Futher study for the detection of conformational changes will include the method of size-exclusion chromatography (SEC). SEC methods can be used to monitor relatively minor changes in protein hydrodynamic volumes, apparent equilibrium constants, and Gibbs free energy of stabilization associated with the unfolding or refolding of a protein under different chromatographic conditions.

Conclusion

The retention behavior of proteins in HIC and RPC was investigated by changing temperature and mobile phase in this paper and the previous one, respectively. As shown in the preceding report, the retention behavior of proteins in RPC was predicted by applying the retention mechanism of small molecules. However, the retention behavior of the proteins is more complicated than that of small molecules since three-demensional structure of proteins is to be considered for the analysis of the proteins. Even though the retention mechanisms of RPC and HIC are based on the same fundamental principle which relies on the hydrophobic properties of the proteins and stationary phase, the retention behavior of the proteins in RPC is significantly different from that observed in HIC. This is due to the use of organic solvent in RPC. While hemoglobin and myoglobin showed a similar retention behavior in RPC when solvent and temperature were varied, they appeared to be selective in HIC as a function of salt and temperature. From these studies, we observed a sharp change in retention time or k' and broad or split peaks during the chromatographic elution. These might be from the denaturation of the proteins.

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