vacuo. The photoproduct **11** was separated by column chromatography followed by HPLC using *n*-hexane/ethyl acetate/methylene chloride: 8/1/1 (v/v/v) as an eluent to give **11** in 80 % yield.

Irradiation of 10 with AN. 10 (4 mM) solution in tetrahydrofuran (50 m*l*) with a mixture of AN (100 mM) was deaerated by nitrogen purging. Deaerated solution was irradiated with 300 nm UV light in a Rayonet Photochemical Reactor equipped with RUL 3000 Å lamps. After the irradiation for 24 hours, the reaction mixture was evaporated *in vacuo*. The photoproduct **11** was separated by column chromatography followed by HPLC using *n*-hexane/ethyl acetate/methylene chloride: 8/1/1 (v/v/v) as an eluent to give **11** in 20% yield.

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A Study of the Retention Behavior of Proteins in High-Performance Liquid Chromatography(I): The Effect of Solvent and Temperature on Retention Behavior of Proteins in Reversed-Phase Chromatography

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The retention behavior of proteins was investigated by using reversed-phase chromatography (RPC), comparing to the retention behavior of small molecules in RPC. The evaluation was carried out on a SynChropak RP-P(C_{18}) column with 0.1% aq. TFA-organic solvent modifier such as acetonitrile, isopropanol, and ethanol. The Z value (the number of solvent molecules required to displace the solute from the surface) was a general index for the characterization of protein retention as a function of organic concentration over a range of temperature between 5 and 70℃. Van't Hoff plots provided the basis for evaluating the enthalpic and entropic changes associated with the interaction between protein and the stationary phase. Z values did not change significantly at the range of temperature showing the consistent $\Delta H°$ and $\Delta S°$ values. From these investigation, it was concluded that the retention behavior of proteins in RPC was able to be predicted by the retention parameters applied to small molecules. Furthermore, myoglobin and hemoglobin in RPC as stated above showed a similar retention behavior regardless of their molecular weights.

Introduction

In the 1970s, high-performance liquid chromatography (HPLC) has become a powerful technique widely used for the separation of large biological substances and for their purification. In particular, RPC has been the prime method of peptide analysis and purification, and has also become a widly used chromatographic tool for protein analysis.[1-3] According to Snyder *et al.* linear solvent strength (LSS) gradient elution theory developed for small molecules can also be used to characterized the retention behavior of proteins even though there exist some differences between the RPC behavior of proteins and low-molecular-weight molecules.[4-7] It was suggested by Regnier *et al.* that the analysis of protein mixtures by RPC is complicated due to the nature of the solutions usually employed to affect high-resolution separation.[8,9] These conditions include low pH and the use of high concentrations of organic solvents as eluents, and both conditions are known to alter protein structure substantially. Karger *et al.* studied the changes in peak shape as a function

of temperature and the time taken for the protein to reside on the column prior to elution.[10-13] Since retention in RPC is a function of the number (Z) of solvent molecules requried to displace the solute from the surface, a simple mathmatical expression is derived from the relationship between the capacity factor of a protein to the concentration of the displacing agent and the stoichiometry of solvent-solute displacement. Z value is in part related to the size of the protein molecules and the contact area between proteins and the support.[14-16] Hearn *et al.* showed that van't Hoff plots confirmed significant changes in the free energy associated with retention. These observation provide the basis for the evaluation of the enthalpic and entropic changes associated with interaction between peptide and *n*-alkyl silicas.[17-19] In addition, they investigated the relevance of quantitative structure-retention relationships to the mechanistic basis of peptide and protein interactions with chromatographic surfaces.[20]

Our intention in this paper is to apply the theory derived from the small molecules in RPC to description of the retention behavior of proteins in RPC. For obtaining the detailed information of hydrophobic interaction of proteins in RPC, we calculated the Z values and the thermodynamic values from the retention data and attempted to explain the changes of interaction force and structure. The obtained results will be compared with those of HIC (hydrophobic interaction chromatography) of proteins in the following paper to understand the nature of hydrophobic interaction between proteins and hydrophobic ligand of stationary phase.

Experimental

Equipment. This study was carried out with a system consisting of a Waters M-6000A (Waters Associates Inc., Milford, Mass, U.S.A.) and a M-45 Solvent Delivery System with a Model 660 Solvent Programmer, a M-U6K Universal Injector, a Series 440 Absorbance Detector (254, 280, and 405 nm), and a M740 Data Module. The water used in this study was doubly-distilled and purified through a Milli-Q Water Purification System (Millipore, Bedford, MA. U.S.A.).

Reagents. HPLC-grade acetonitrile and 2-propanol from Merck (Hawthorne, NY, U.S.A.), HPLC-grade ethanol from Burdick & Jackson (Muskegon, MI, U.S.A.), trifluoroacetic acid (TFA) and proteins from Sigma Chemical Company (St. Louis, MO, U.S.A.) were used. Proteins used in this experiment were lysozyme, hemoglobin, cytochrome c, myogloblin protease, transferrin, carbonic anhydrase, ovalbumin, α-lactoalbumin, and albumin.

Chromatographic Procedure. RPC was performed on a SynChropak RP-P column, 25×0.46 cm I.D., from SynChrom (Lafayette, IN, U.S.A.). Bulk solvent was filtered and degassed by Waters filter kits. Mobile phase was 0.1% TFA in water containing organic solvent, whose compositions were varied from 30 to 40%. The retention times of various proteins were measured over a range of temperature between 5 and 70℃. Protein solutions were prepared by dissolving the solutes in 0.05 M phosphate buffer (pH 7.0) at a concentration of 3 mg/ml. Injection size was 5 μl and the flow-rate was 1 ml/min.

Results and Discussion

Table 1. Calculated and Experimental t_g Values of Protein in Gradient Elution

Proteins	$t_{g(cal)}$ (min)	$t_{g(exp)}$ (min)
Lysozyme	27.18	27.75
Hemoglobin	29.54	29.43
Cytochrome c	22.61	24.41
Myoglobin	29.47	29.45
Protease	32.58	33.27
Transferrin	29.66	29.45
α-lactoalbumin	32.16	32.46

Retention of Proteins by Gradient Elution in RPC. For isocratic systems of RPC, retention of small molecules is given by[4]:

$$\log k' = \log k_o - S\phi \quad (1)$$

where S is the slope of the plots of $\log k'$ *vs.* ϕ, volume fraction of organic solvent, and k_o is the extrapolated intercept. For large solute molecules, S values are far greater than those of small molecules. The gradient steepness parameter b is related to both gradient and solute parameters as:

$$b = (t_o \Delta\phi S) t_G \quad (2)$$

where $\Delta\phi$ is the change in ϕ during the gradient and t_G is the gradient time. The gradient retention time(t_g) was derived by Snyder *et al.*[21] as:

$$t_g = (t_o/b) \log (2.3bk_o' + 1) + t_o + t_D \quad (3)$$

in which t_D is the gradient delay time. Experimental t_g values are compared with the calculated values using Eqn. (3) (Table 1). Gradients were made wiht 20-50% acetonitrile in 0.1% aq. TFA for 30 min with a flow-rate of 1 ml/min. Experimental t_o and t_D values are 3.24 and 4.41 min, respectively. It is shown in Table 1 that experimental t_g values are in a good agreement with the calculated ones except the slight variation. It is concluded that gradient retention time for proteins can be predicted by the equations derived from the retention behavior of small molecules.

Retention Behavior of Protein Under Different Compositions of Organic Solvent Modifier. The retention behavior of a set of proteins on SynChropak RP-P column was investigated as the volumn fraction, ϕ, of the organic solvent modifiers such as acetonitrile, 2-propanol, and ehtanol, was systematically varied over a range $0.2<\phi<0.8$. Figure 1 shows that the capacity factor, k', changes rapidly especially for acetonitrile when the volume fraction of organic solvent modifiers is smaller than 45% and larger than 70%. Since solvophobic theory alone cannot explain the skewed U-shaped dependence between solute retention time and concentration of organic solvent, it has been suggested that silanophilic interactions may be responsible for this irregular behavior.[22,23] Proteins bound to the matrix under conditions of high organic solvent concentration can be recovered by introducing an inverse gradient of decreasing organic modifier concentration.[24] The capacity factor, k', for the proteins used in this paper decreases in the order of solvent modi-

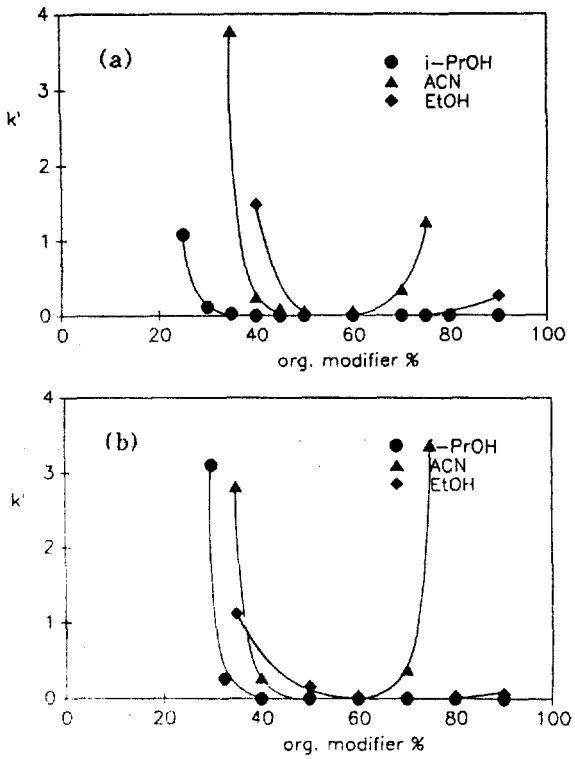

Figure 1. Plots of the capacity factors, k', for proteins against the volume fraction of the organic solvent in 0.1% aq. TFA-organic solvent mobile phases. Conditions: column, SynChropak RP-P; flow-rate, 1.0 ml/min; temperature, ambient; proteins, (a) lysozyme and (b) protease.

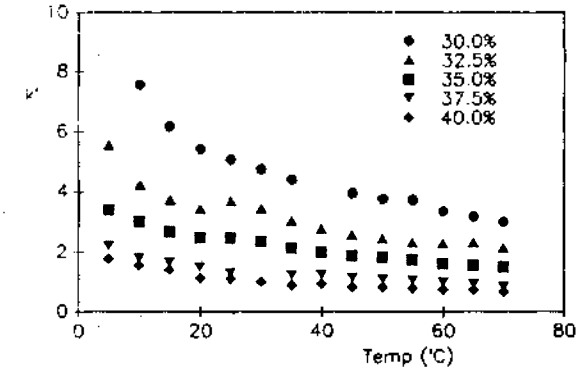

Figure 2. Plots of k' *vs.* temperature for myoglobin under five different proportions of acetonitrile, 30.0, 32.5, 35.0, 37.5, and 40.0%. Other experimental conditions are the same as used Figure 1.

fiers ethanol>acetonitrile>2-propanol at the same volume percentage.

Dependence of k' on Temperature. In general, for RPC of small molecules it has been observed that the retention time decreases with the increase of temperature. In Figure 2, temperature dependence of myoglobin on RPC shows a similar tendency as it can be expected from small molecules. A higher temperature condition induces more rapid mass transfer of the proteins, more rapid unfolding between native and disorganized species, and faster dynamics

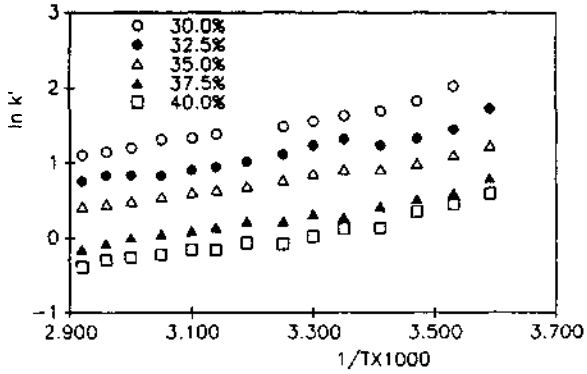

Figure 3. Plots of $\ln k'$ *vs.* $1/T$ for myoglobin. See legend to Figure 2 for experimental conditions.

Table 2. Changes in Enthalpy and Entropy for the Proteins Investigated

Organic solvent		$\Delta H°$ (kcal/mol)[a]		$\Delta S°$ (cal/mol·K)	
Type	%	Myoglobin	Hemoglobin	Myoglobin	Hemoglobin
Acetonitrile	30.0	-2.17^{20-70}	−2.42	−1.05	−1.87
	32.5	-2.48^{25-70}	−2.56	−2.80	−3.06
	35.0	-2.21^{10-70}	-2.32^{25-70}	−2.64	−2.98
	37.5	-2.17^{10-70}	-2.21^{10-65}	−3.52	−3.64
	40.0	-2.01^{20-70}	−1.97	−3.54	−3.40
2-propanol	30.0	-3.97^{30-70}	−4.25	−9.12	−9.99
	32.5	-2.96^{20-70}	−2.88	−6.66	−6.40
	35.0	-3.68^{25-70}	-3.22^{25-65}	−9.58	−8.15
	37.5	-3.68^{15-70}	-3.78^{15-65}	−10.13	−10.55
	40.0	-2.23^{20-70}	-2.30^{20-65}	−5.94	−6.26

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

associated with ion- and solvent-interaction phenomena. The dependence of k' on temperature, T, can be expressed as[17]:

$$\ln k' = -\Delta H°/RT + \Delta S°/R + \log \Phi \quad (4)$$

where Φ is a phase ratio and $\Delta H°$ and $\Delta S°$ are the standard enthalpy and entropy change for the transfer of the solute from the mobile phase to the stationary phase, respectively. From the linear regression of the plots of $\ln k'$ *vs.* reciprocal temperature (van't Hoff plots), it is possible to obtain $\Delta H°$ and $\Delta S°$ values associated with the protein unfolding at the stationary phase surface (Figure 3). The experimental data for the changes in enthalpy and entropy for myoglobin and hemoglobin are listed in Table 2. Even though hemoglobin is larger than myoglobin, $\Delta H°$ and $\Delta S°$ values for both proteins are not significantly different. This means that the retention behavior of hemoglobin is similar to that of myoglobin. The overall standard unitary free-energy changes ($\Delta G°$) associated with the transfer of the solute from the mobile phase to stationary phase can be calculated at the individual temperatures by:

$$\Delta G° = \Delta H° - T\Delta S° \quad (5)$$

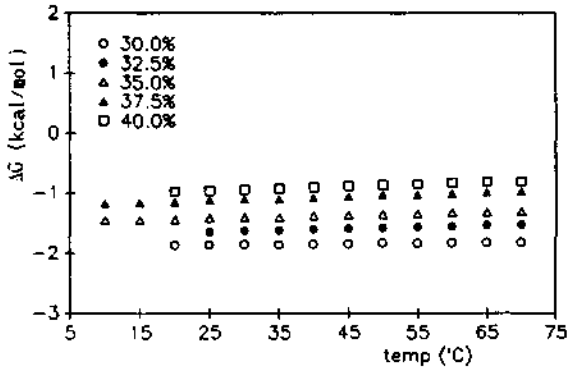

Figure 4. Plots of $\Delta G°$ *vs.* temperature (℃). See the text for other details.

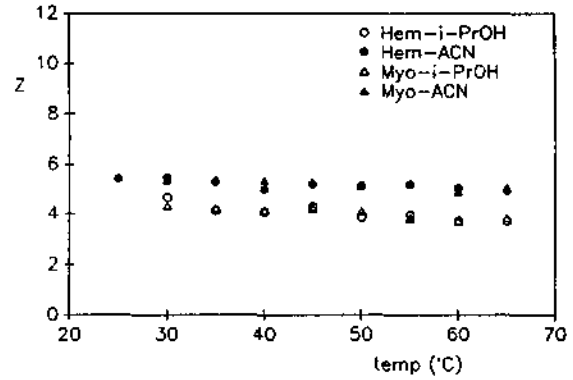

Figure 5. Plots of Z *vs.* temperature (℃). Identification symbols: hemoglobin-isopropanol (○); hemoglobin-acetonitrile (●); myoglobin-isopropanol (△); myoglobin-acetonitrile (▲).

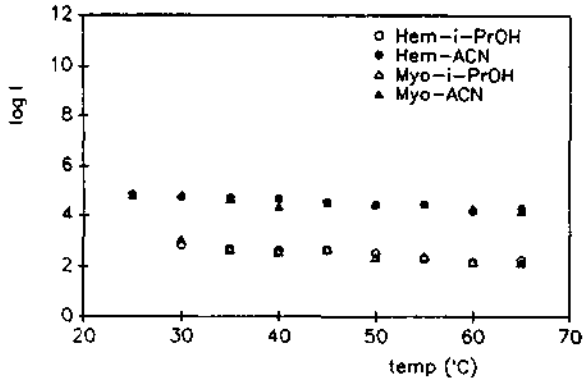

Figure 6. Plots of $\log I$ *vs.* temperature (℃). See legend to Figure 5 for symbol identification.

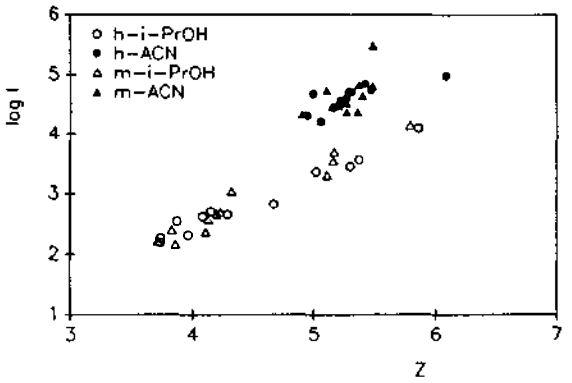

Figure 7. Plots of Z *vs.* $\log I$. See legend to Figure 5 for symbol identification.

In Figure 4, $\Delta G°$ values calculated from the experimental data shown in Table 2 are plotted against temperature. Since adsorption is a favourable process, the derived $\Delta G°$ values are all negative. Futhermore adsorption becomes less favourable when the acetonitrile concentration increases. As shown in Table 2, $\Delta H°$ and $\Delta S°$ values of proteins are all negative. It can be concluded that the association of the proteins with the stationary phase of RPC is an enthalpy-driven process, so the retention process of proteins is more favourable with decreasing temperature.

Stoichiometric Displacement of Proteins by Solvent in RPC. Based on the displacement model, retention can be written as[11]

$$\log k' = Z \log(1/[D_o]) + \log I \tag{6}$$

where $[D_o]$ is the molar concentration of organic modifier. The slope of a plot, Z, can be used as a measure of the contact area of the adsorbed protein and the value of k' at $[D_o]=1$ M, I, represents a measure of the relative binding strength of individual proteins under a fixed mobile phase composition. A relationship between solvent displacement stoichiometry (Z) and temperature is shown in Figure 5. As shown in Table 2, the common temperature ranges for each organic solvent are 25-65℃ for acetonitrile and 30-65℃ for 2-propanol. For these temperature ranges, Z values of myoglobin and hemoglobin appear to be consistent with a slight fluctuation. It is strongly suggested that the contact area of the adsorbed protein does not change during chromatographic procedure. Similarily, the plots of $\log I$ *vs.* temperature are given in Figure 6. The $\log I$ values did not vary significantly for the same temperature ranges as stated above. This means that the interaction force of two preteins stationary phase ligand is independent of tmeperature changes during elution. Since both Z and I values have been related to nonpolar contact surface area, it can be expected that plots of $\log I$ *vs.* Z is linear with a slope of J[24].

$$d(\log I)/dZ = J \tag{7}$$

This supposition is valid for small non-polor solute in RPC, but this may not always apply to proteins in RPC because the non-selective force controls the retention of proteins. When 2-propanol is used as an organic solvent modifier, J value for myoglobin and hemoglobin is calculated as 0.88 ($r=0.98$). However, we can hardly conclude a clear relationships between Z and I when acetonitrile is used (data points are shown as filled symbols in Figure 7), because data points are scattered ($r=0.65$). Further studies are required for the explanation of the selectivity at the two organic modifiers, 2-propanol and acetonitrile.

Conclusion

The results of these studies provide an evidence that the retention behavior of proteins in RPC can be understood

by the same principle underlying chromatographic separation of small molecules. However, the relationship between the retention behavior and chromatographic parameter is more complicated for proteins than for small molecules. It is suggested that the three-dimensional structure of protein can be a major determinant of retention in surface-mediated separation. Since Z value can be used as a measure of the contact area of the adsorbed proteins, it is recognized that the structure of the proteins remains unchanged at chromatographic conditions giving a consistant Z value. It is reinforced by the fact that the standard enthalpy and entropy changes for the transfer of the solute from the mobile phase to the stationary phase did not happen under the same chromatographic condition as stated above. However, there were some limitations to verify the unfolding of proteins during separation and to characterize intermediate species. For the detailed information of conformational changes of proteins, supplementary experiments are required. These works are required to elucidate the nature of the hydrophobic interaction of proteins with stationary phase.

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