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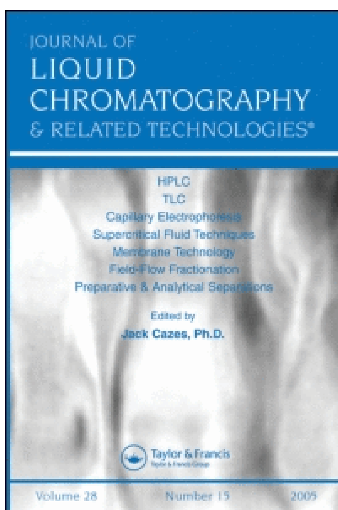

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Investigation of the Retention Behavior and Structural Change of Proteins in Reversed Phase and Hydrophobic Interaction Chromatography

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INVESTIGATION OF THE RETENTION BEHAVIOR AND STRUCTURAL CHANGE OF PROTEINS IN REVERSED PHASE AND HYDROPHOBIC INTERACTION CHROMATOGRAPHY

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ABSTRACT

The retention behavior of proteins was investigated in reversed phase chromatography(RPC) and hydrophobic interaction chromatography(HIC). The observation was performed in three viewpoints : (1) What are the differences in the retention behavior between small molecules and proteins? (2) What are the differences between RPC and HIC of proteins and the reason of the differences? (3) How can be detected the structural changes of proteins eluted from the column?

The retention behavior of proteins was able to be understood by the parameters used in describing the retention behavior of small molecules. However, applicable range of the parameters for proteins was limited and, sometimes, peculier behavior appeard in RPC and HIC for proteins ; that is, the retention time of proteins showed extreme difference for the small change of organic fraction and showed the skewed U-shaped dependence on fraction of organic solvent. Even though the retention mechanism of proteins in RPC and HIC was based on the same fundamental principle relied on the hydrophobic properties of the proteins and stationary phase, the retention behavior of the proteins in RPC differed significantly from that in HIC. The difference between RPC and HIC resulted from adding organic solvent in mobile phase of RPC to elute the protein strongly bound to the ligand of the stationary phase. From the

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view point of thermodynamics, the driving force of transferring proteins from the mobile phase to the stationary phase was ΔH° for RPC and ΔS° for HIC.

The investigation of structural change of the proteins eluted from the column was performed with UV and photodiode array detector as a function of the change of peak shape and the retention time. The number(Z) of solvent molecules required to displace the solute from the surface was useful for measuring the structural change of the proteins by combining the thermodynamic observation. For the identification of splitted peaks of hemeproteins eluted from RPC, apoproteins were prepared and compared with the retention time of heme proteins. K_D values were measured by using size-exclusion chromatography(SEC) with the same mobile phase used in RPC and HIC. From these values the phenomena such as aggregation or division into subunits of proteins were suggested.

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INTRODUCTION

In the 1970s, HPLC has become a powerful technique widely used for the separation of large biological substance and for their purification. In particular, RPC has been the prime method of peptide analysis, while HIC has been an important separation mode for the purification and characterization of proteins [1]. The retention behavior of proteins has been investigated by the same method used for small molecules ; Snyder et al. applied the linear solvent strength (LSS) gradient elution theory developed for small molecules to characterizing the retention behavior of proteins [2], and Hearn et al. showed that van't Hoff plots confirmed significant changes in the free energy associated with retention of peptides and proteins [3]. However, there exist some differences between the elution behavior of proteins and low-molecular-weight moleculs. Regnier et al. explained the fact as the structural changes of proteins during the chromatographic elution, particularly in RPC [4]. Guiochon et al. studied the changes in peak shape as a function of temperature and the type of the stationary phase [5]. Z value is in part related to the size of the protein moleculs and the contact area between proteins and the support. Z value can be used as a measure of the structural change of proteins[6].

Even though the retention mechanism of RPC and HIC is based on the same fundamental principle relied on the hydrophobic interaction between the proteins and stationary phase, they show a quite different character. This has already been reported in our previous reports [7-8]. From those reports, the following facts have been found. The association of the proteins with the stationary phase in RPC was an enthalpy-driven process, and the retention of proteins was more favourable with decreasing temperature, while in HIC transfer of proteins from mobile phase to stationary phase was an entropy-driven process and the retention became longer with increasing temperature. Furthermore, the retention behaviors of hemoglobin and myoglobin were very similar in RPC, while the retention behaviors of two proteins were significantly different in HIC. This may be explained only by investigating the structures of those two proteins eluted through the column.

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In the previous reports, structural change of proteins was observed along with the sudden change of retention time and the splitting and broadening of peaks by using UV and photodiode array detectors. The change of Z value with thermodynamic consideration was also used for the investigation of the structual change of proteins. However, precise structure of proteins eluted from the column and identification of splitted peaks were not clarified. For obtaining the information on the structural change of proteins, spectroscopic methods such as UV, fluorescence, and circular dichroism spectroscopy have been used[9-10]. When the structure of proteins changes, the UV and fluorescence spectra show changes in the aromatic acid on exposure to the solvent, while CD spectra can denote changes in secondary structures of the unfolded proteins. On-line conformational monitoring of proteins in HPLC is a proper method for the observation of the structural change of proteins during chromatographic elution[11]. SEC has been widely used for consideration of the change of molecular size and shape[12-14]. LC/mass spectroscopy[15] and LC/LALLS[16] may be applied to investigation of the change in the size of proteins by the occurrence of aggregation or division into subunits.

In this work, UV and photodiode array detectors were used to observe the structural change of proteins along with the changes in retention time and peak shape. For the identification of the splitted peaks, particularly in hemeproteins, apoproteins of hemoglobin and myoglobin were prepared and elution behavior of apoproteins was compared to that of native proteins. SEC was performed with the same solution conditions as employed in RPC and HIC to investigate the change in the size of proteins.

MATERIALS AND METHODS

Equipment

The chromatographic system employed in this work consists 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 Universial Injector, a M-740 Data Module, a series 440 Absorbance Detector (254, 280, and 405nm), and a Waters 991 Photodiode Array Detector. A SynChropak RP-P column, 25 X 0.46 cm I. D., from SynChrom (Lafayette, IN, U.S.A.) for RPC, a SynChropak propyl column, 25 X 0.46 cm I. D., from SynChrom for HIC, and two RPOTEIN-PAK 125 and a PROTEIN-PAK 300SW column, 30 X 0.78 cm I. D., from Waters for SEC, were employed.

Reagent

HPLC-grade acetonitrile and i-propanol from Merck (Hawthorne, NY, U.S.A.), HPLC-grade ethanol from Burdick & Jackson (Muskegon, MI, U.S.A.), trifluoroacetic acid (TFA), sodium phosphate monobasic, sodium phosphate dibasic, sodium sulfate, and proteins were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Proteins used in this work were lysozyme, hemoglobin, cytochrome

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c, myoglobin, protease, transferrin, carbonic anhydrase, ovalbumin, α-lactoalbumin, and albumin. Water was purified using a Millipore Milli-Q water purification system (Bedford, MA).

Chromatographic Procedure

RPC was performed with 0.1% aq. TFA containing organic solvent such as i-propanol, acetonitrile, and ethanol whose compositions were varied from 30 to 40%. For HIC, concentrations of sodium sulfate were 0.50 to 0.85M in 0.05M phosphate buffer (pH 7.0). SEC was performed under the same solution conditions as used in RPC and HIC. The retention times of various proteins were measured over a range of temperature between 5 and 70°C. Proteins were prepared by dissolving in 0.05M phosphate buffer (pH 7.0) at a concentration of 3mg/ml. Injection volume was 5μl and the flow-rate was 1ml/min. Apoproteins of hemoglobin and myoglobin were prepared according to the reported method [17]. Distribution coefficient, K_D, was calculated using the following equation

$$K_D = (Ve-Vo)/(Vt-Vo) \quad (1)$$

where Vo is the interstital volumn of the column, obtained from the retention volumn of blue dextrin (M.W.= 2,000,000), Ve is the retention volumn of protein, and Vt is the total liquid volumn of the column, obtained from the retention volumn of sodium azide (M.W.= 65).

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RESULTS AND DISCUSSION

Application of UV Detector in Conformational Monitoring

UV spectrophotometry is one of the most important tools for the investigation of the changes in the molecular conformation and

refolding of proteins. In our previous report[7], it was not mentioned that the chromatograms for hemeproteins showed the peaks splitted into two at a certain condition of RPC. As shown in Figure 1 and Figure 2, retention time of one peak (smaller peak in Figure 1 and unfilled symbol in Figure 2) change more sensitively than that of the other peak with increasimg temperature (Figure 1) and organic modifier fraction (Figure 2). The more sensitive peak for the change of chromatographic condition showed greater absorbance at 280nm than at 254nm; it means that the peak is unfolded form. Futhermore the peak didn't appear at 405nm as in Figure 3. The maximum absoption wavelengh of heme group is 405nm and proteins do not show absorption at this wavelengh. Those results indicate that the more sensitive peak may be an apoprotein and the stucture seems very unstable. Table 1 shows a comparison of the values for change of enthalpy and entropy for myoglobin and apomyoglobin.

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The fact that apomyoglobin has the greater negative value of ΔH° than myoglobin represents that apomyoglobin has the greater affinity for the stationary phase. The greater negative value of ΔS° for apomyoglobin suggests that apomyoglobin is more flexible in the mobile phase and more sensitive for the change of temperature ($\Delta G^{\circ} = \Delta H^{\circ} - T\Delta S^{\circ}$) than myoglobin. Two opposing processes may contribute to a change in entropy on unfolding process of protein [19]. First, the destruction of secondary structure requires increased solvation of the newly exposed hydrophobic amino acid residues which originally formed the internal core. This solvation would cause a corresponding decrease in entropy, owing to the ordering of water molecules around hydrophobic residues on the surface of the protein. However, this process will be compensated for by an increased disordering of the unbound structure due to the increase in the conformational freedom of the more flexible protein. The overall process would then be expected to yield negative ΔS° and this effect would be greater in apoprotein having the severe conformational disorder. Table 2 shows ΔG° values for hemoglobin and apohemoglobin at the various temperature.

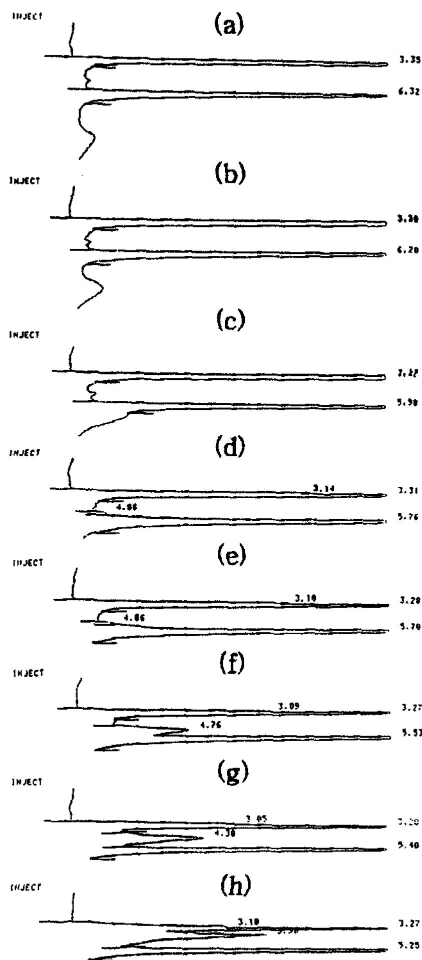

FIGURE 1. Chromatograms of myoglobin. Conditions: column, SynChropak RP-P; wavelength, 254nm; temperature, (a) 30, (b) 35, (c) 40, (d) 45, (e) 50, (f) 55, (g) 60, and (h) 65°C.

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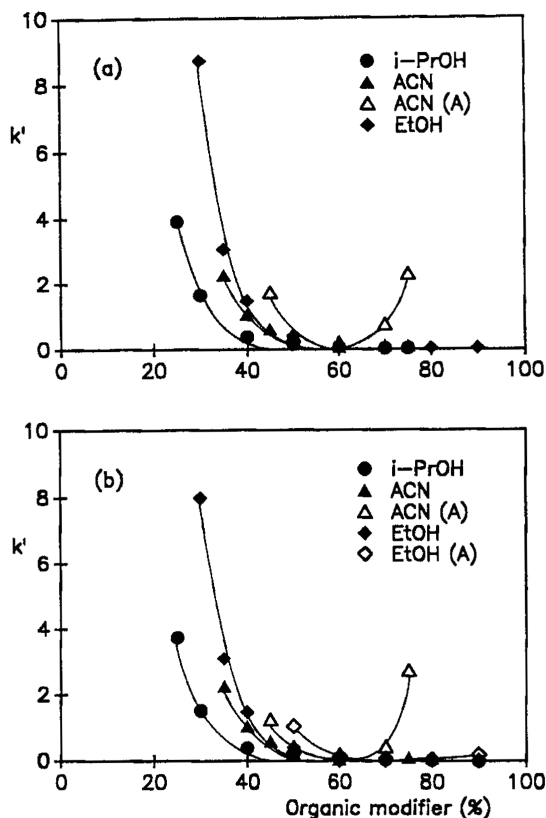

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FIGURE 2. Plots of k' vs. volumn fraction of organic solvent in 0.1% aq. TFA. Condition: column, SynChropak RP-P; temperature, ambient; proteins, (a) hemoglobin and (b) myoglobin.

TABLE 1. The Values of Change of Enthalpy and Entropy[18] for Myoglobin and Apomyoglobin

Proteins	i-PrOH (%)	Temperature (°C)	ΔH° (kcal/mol)	ΔS° (cal/mol·K)
Myoglobin	30.0	30-70	-3.97	-9.12
	32.5	20-70	-2.96	-6.66
	35.0	25-70	-3.68	-9.58
	37.5	15-70	-3.68	-10.13
	40.0	20-70	-2.23	-5.94
Apo-myoglobin	30.0	55-70	-24.34	-68.99
	32.5	45-70	-23.82	-70.48
	35.0	30-50	-28.93	-93.81
	37.5	15-30	-19.27	-65.15

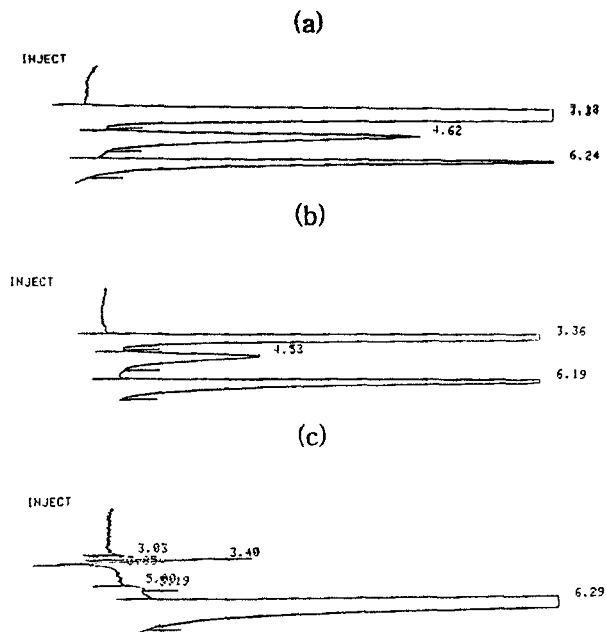

FIGURE 3. Chromatograms of myoglobin. Condition: column, SynChropak RP-P; temperature, 30°C; wavelength, (a) 280nm, (b) 254nm, and (c) 405nm.

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TABLE 2. ΔG° Values for Hemoglobin and Apohemoglobin in 0.1% TFA containing 35% i-propanol

	ΔG° (kcal/mol)	
	Hemoglobin	Apohemoglobin
Temterature	ΔH°=-3.22, ΔS°=-8.15	ΔH°=-17.37, ΔS°=-53.41
(°C)	(kcal/mol) (cal/mol·K)	(kcal/mol) (cal/mol·K)
25	-0.79	-1.45
30	-0.75	-1.19
35	-0.71	-0.92
40	-0.67	-0.65
45	-0.63	-0.39
50	-0.59	-0.12
55	-0.55	0.15
60	-0.51	0.42
65	-0.47	

In Figure 4, ΔG^o values calculated from Table 1 are plotted against temperature. The ΔG^o values of apoproteins change more rapidly with the increase in temperature than those of proteins containing heme group. The range of chromatographic condition shown in the apoproteins was quite limited and Z values for apoproteins was not able to be calculated.

Application of Photodiode Array Detector for Conformational Monitoring

UV spectra with photodiode array detector would offer more information on conformational changes than conventional UV detector owing to the ability of monitoring the overall wavelengh showing the absorpion for proteins only by one injection. In Figure 5, chromatogram obtained by photodiode array detector shows a elution profile for cytochrome c eluted with 0.1% aq. TFA containing 30% acetonitrile at 50°C. A portion eluted at 4.5 minutes has two peaks and they appear around 200nm and 400nm, respectively. The apex showed around 400nm is a peculier peak for heme group. A portion eluted around 10.2 minutes has only one peak showed the maximum around 200nm, which is caused by absorpion of amino acid constituting cytochrome c. The peak may be an apoprotein losing the heme group during chromatographic elution. Figure 6 also shows UV spectra for hemoglobin eluted with 0.1% aq. TFA containing 35% i-propanol at 30°C and 50°C. In the chromatogram eluted at 30°C, a portion containing heme group was eluted earlier than apohemoglobin. On the other hand, apohemoglobin was eluted earlier around void volumn at 50°C. The retention time of apohemoglobin changes more sensitively with changing of temperature.

Identification of Splitted peaks

Apoproteins of hemoglobin and myoglobin were prepared by according to the reported method. Figure 7 shows the chromatograms

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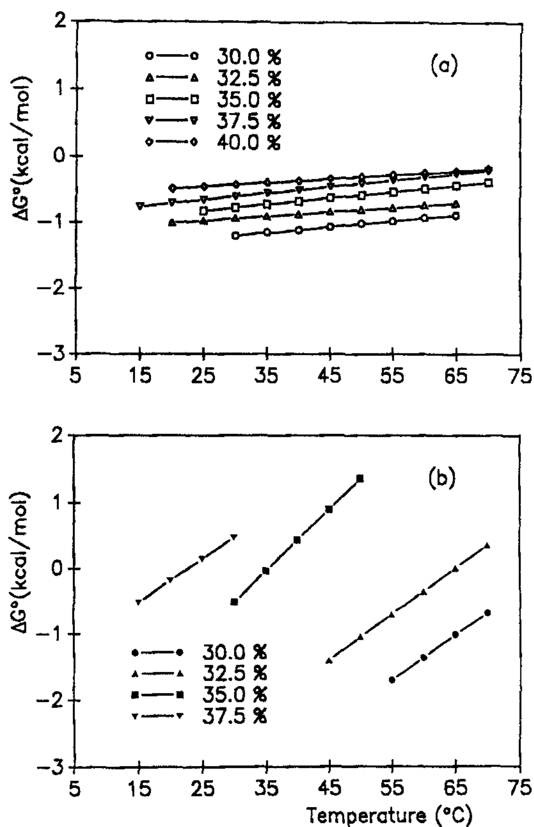

FIGURE 4. Plots of ΔG° vs. temperature for myoglobin (a) and apomyoglobin (b) under five different proportions of acetonitrile, 30.0, 32.5, 35.0, 37.5, and 40.0%.

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of myoglobin (b) and apomyoglobin (c) eluted with 0.1% aq. TFA containing 35% i-propanol at 30°C. The chromatograms of left and right side were obtained at 280nm and 405nm, respectively. At 280nm myoglobin showed two peaks, at 3.64 and 4.89 minutes, respectively. However, at 405nm only a peak containing heme group showed at 4.88 minutes. In case of eluting the apomyoglobin, one peak appeared at the sameretention time as the first peak of myoglobin at 280nm. Because this peak didn't contain heme group,

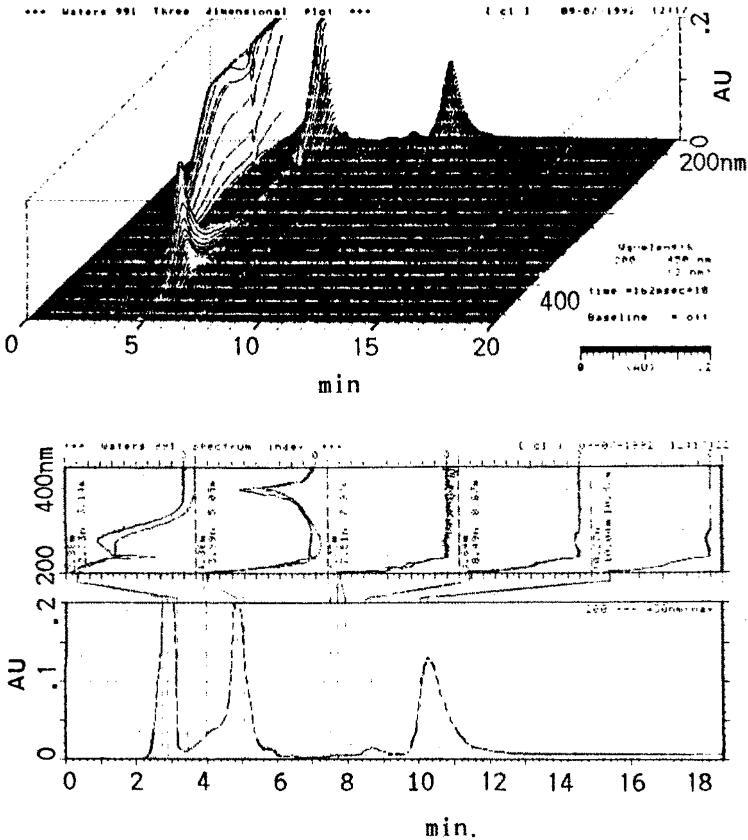

FIGURE 5. UV spectra with the photodiode array detector for cytochrome c eluted in 0.1% TFA containing 30% acetonitrile at 50° C.

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only solvent peak appeared at 405nm. By the comparison of the elution behavior between native protein and apoprotein, splitted peaks of hemeproteins can be identified as one for hemeprotein and the other for apoprotein.

Application of Size-Exclusion Chromatograpy

SEC provides information on the structural change of proteins by the change of molecular size. SEC technique offers

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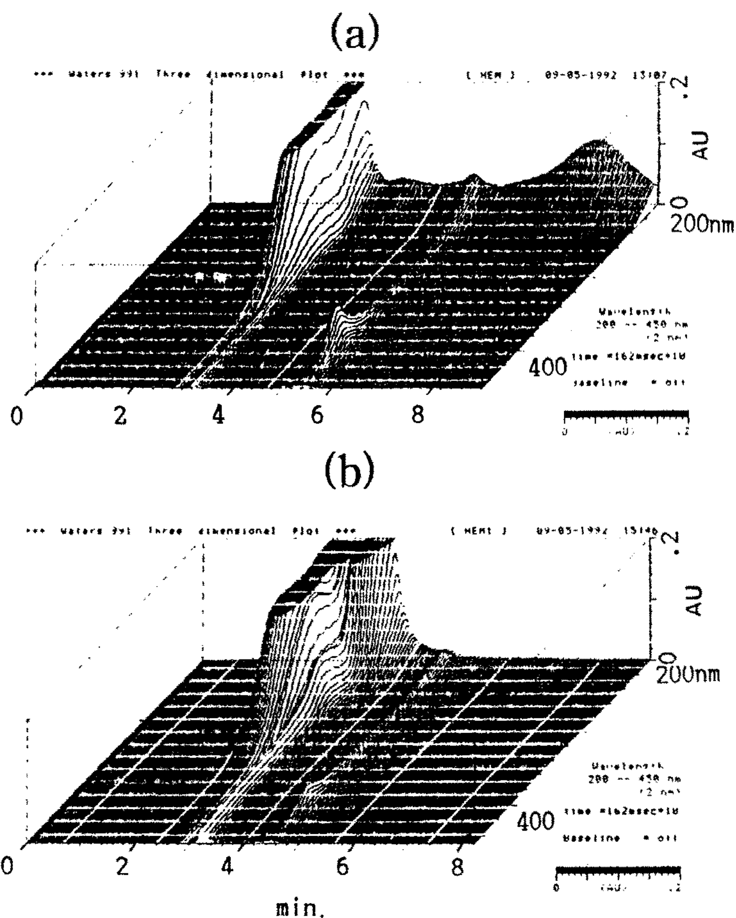

FIGURE 6. UV spectra with the photodiode array detector for hemoglobin eluted in 0.1% TFA containing 35% i-propanol at 30°C (a) and 50°C (b)

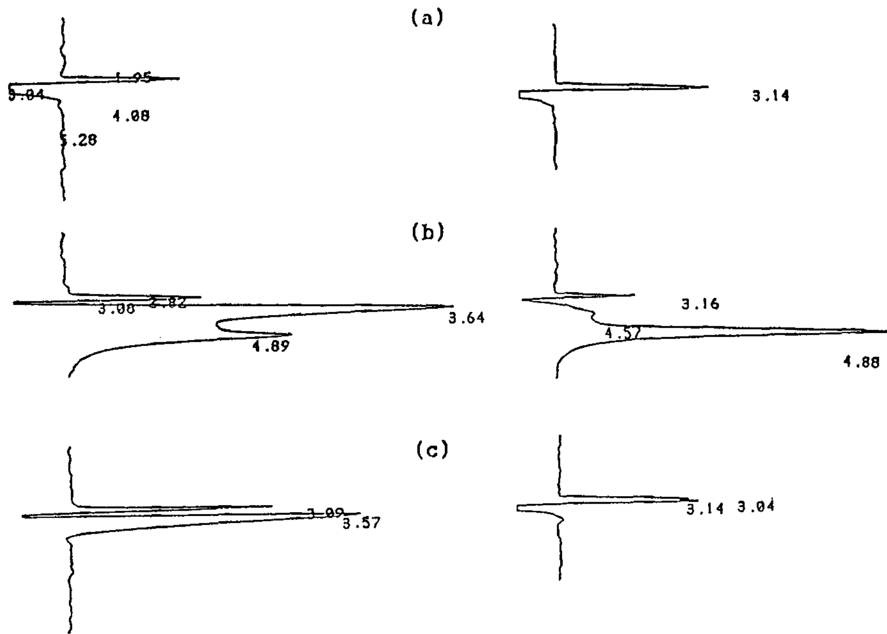

FIGURE 7. Chromatograms for water (a), myoglobin (b) and apomyoglobin (c) at 280nm (left), and 405nm (right).

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several advantages [20] : (1) It is possible to monitor relatively rapid folding-unfolding transitions by using SEC. (2) In addition to monitoring denaturation by urea, SEC may be used to monitor thermal denaturation as well as denaturation by acids and ionic denaturants such as guanidium chloride. (3) A variety of optical detection methods could potentially be used to study the properties of the eluted species. (4) Protein samples can be recorvered at the end of the experiment.

Figure 8 shows the plots of K_D vs. log M.W. for proteins eluted in the same solution condition as employed in RPC, 0.1% aq. TFA (pH 2.0) and 0.1% aq. TFA containing 30% i-propanol. Under the condition of 0.1% aq. TFA, all proteins were eluted with the similar retention time. The K_D values were independent on the size

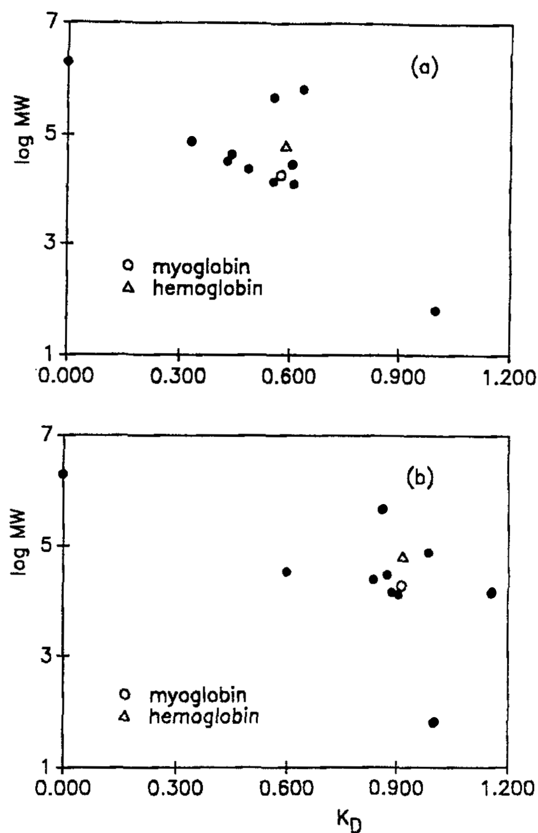

FIGURE 8. Plots of K_D vs. log M.W. for proteins eluted with 0.1% aq. TFA (a) and 0.1% aq. TFA containing 30% i-propanol (b).

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for each protein and smaller than the literature values for proteins. It seems that proteins were aggregated under the acidic condition. In 0.1% aq. TFA containing 30% i-propanol, K_D values were greater than the values in 0.1% aq. TFA for all proteins. The result suggests that the addition of i-propanol in mobile phase promoted ionic interaction between the proteins and the column material. The K_D values for hemoglobin and myoglobin are little different in the both cases. The result may be due to one of the

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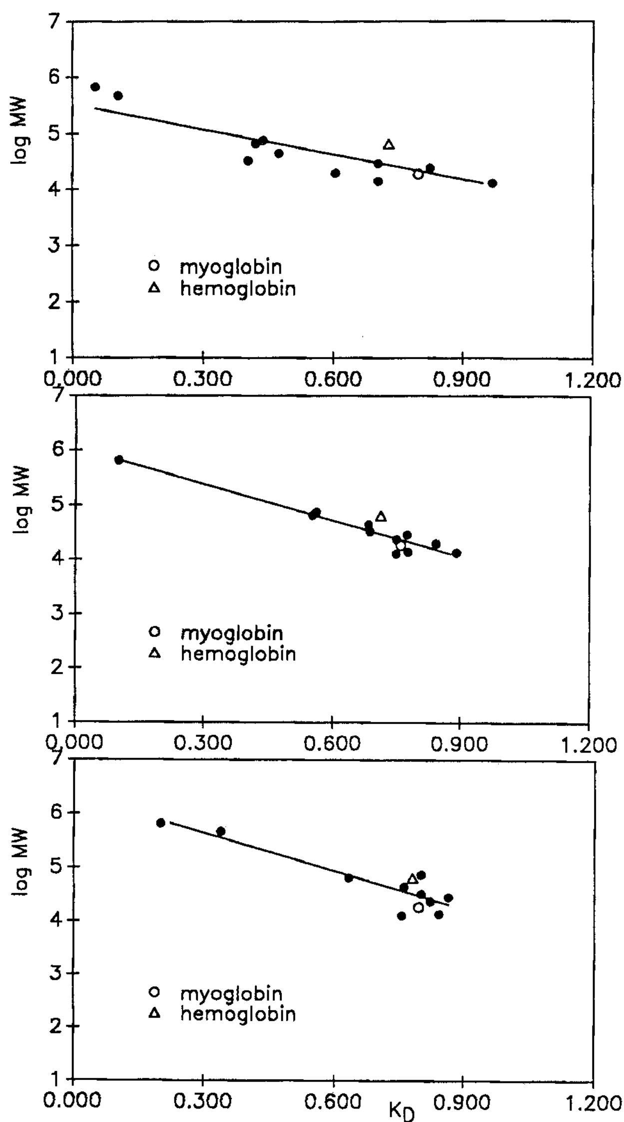

FIGURE 9. Plots of K_D vs. log M.W. for proteins eluted with 0.05M phosphate buffer (pH 7.0) containing 0M (a), 0.5M (b), and 0.85M (c) sodium sulfate.

reasons why two proteins had shown the same elution behavior in RPC[7]. *Figure 9 shows the plots of* K_D *vs. log M.W. for proteins* eluted with the same solution condition as employed in HIC [8], 0.05M phosphate buffer (pH 7.0) containing 0, 0.5, and 0.85M sodium sulfate. The correlation coefficients for each plot were 0.818 (n=10), 0.932 (n=10), and 0.605 (n=8), respectively. In the absence of a salt in buffer, electrostatic interaction between some proteins and column matrix may be occuring, which results in the poor relationship of the plot. In the buffer containing 0.85M sodium sulfate, K_D values increased for the most of proteins. The result demonstrates that increases in salt concentration facilitated hydrophobic interaction of proteins with the column material. In the buffer containing 0.5M sodium sulfate, the best relationship between log M. W. and K_D was obtained. It seems that this solution provides an approximative ideal SEC condition. In this condition, hemoglobin and myoglobin showed the difference in molecular size, in contrast to the result obtained in RPC. The difference in size may be due to one of the reason that two proteins showed the different elution behavior in HIC. However, K_D value for hemoglobin was larger than the value of the real size and hemoglobin deviated from the straight line. It may suggest that hemoglobin is divided into two subunits ($\alpha_1\beta_1$ and $\alpha_2\beta_2$). The precise information about molecular size may be obtained by using SEC/mass spctroscopy or SEC/LALLS.

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