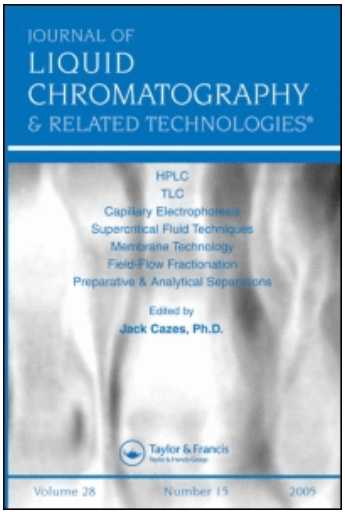

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ABSTRACT

This study investigated the effects of salt in a SynChropak column using peptides which are structurally simpler than proteins. Contrary to the established explanation of salt type by molal surface tension increment, the study attempted to explain salt effects by two retention parameters, S and log k_w. S was considered to be related to surface tension effect, log k_w to salting in effect. From the dependence of S and log k_w on temperature and the stationary phase ligand type, these parameters turned out to be useful in explaining the characteristics of hydrophobic interaction chromatography (HIC) which are different from those of reversed phase chromatography (RPC).

We examined enthalpy-entropy compensation because of the similar tendency of enthalpic and entropic values in the retention process. The uses and characteristics of five different salts were also discussed.

INTRODUCTION

Hydrophobic interaction chromatography (HIC) has been developed for the separation and purification of proteins. Reversed phase chromatography

(RPC) has been found effective in peptide and protein analysis due to its short analysis time, high resolution, and capacity of information about structure. However, it may lead to the denaturation of proteins due to its strong hydrophobic stationary phase, low pH, and high organic percentage mobile phase.[1,2]

HIC was developed to overcome this problem; recently, it has been commercialized for protein separation. It consists of a weak hydrophobic stationary phase and a mobile phase which contains high-concentration salt, and has the advantage of separating proteins, while maintaining its native structure at neutral pH. Therefore, its retention characteristics is different from that of RPC, which has more surface density of alkyl moiety. The manifestation of HIC mechanism can also provide structural information on peptides and proteins like RPC.

One of the most common methods of controlling solute retention in HIC is to change the salt type and concentration. The quantitative theory of salt effects has already been presented by Horváth *et al.*[3] In this theory, salting-in ions such as Mg^{2+}, Ca^{2+} were treated as exceptions and were not given much attention.

Our study started with the question concerning the salting-in effect that could be controlled and utilized for good separation in HIC. In addition, there have been some complex results by other researchers, which could not be explained by this theory.[4,5] Therefore, in this study we investigated salt effects by varying the salt types (especially for cations), as well as its concentrations and temperatures.

Also, by using peptides which are structurally simpler than proteins, we aimed to get more detailed information in controlling selectivity and resolution in HIC and to secure a more advanced explanation of salt effects.

EXPERIMENTAL

Instrumentation

The HPLC system was composed of a model 910 solvent delivery pump (Young-In Scientific Co., Seoul, Korea), a model 720 UV detector (Young-In Scientific Co., Seoul, Korea) and a model 7125 Rheodyne injection valve with a 20 μL sample loop (Rheodyne, Cotati, CA, USA).

The chromatograms were collected using a program PeakSimple (SRI Instrument, CA, USA) in an IBM PC compatible computer. Chromatography was performed on SynChropak pentyl columns (SynChrom, Linden, IN, USA) with dimensions of 25 x 4.6mm i.d. Column temperature was maintained using a RC-10V refrigerated bath circulator (JEIO Tech Co., Seoul, Korea).

Materials

Ammonium acetate, monobasic sodium phosphate, dibasic sodium phosphate, magnesium sulfate, sodium sulfate, and ammonium sulfate were all guaranteed or analytical reagents from Merck (Darmstadt, Germany), the lithium sulfate was from Junsei (Tokyo, Japan), while the potassium sulfate was from Sigma (MO, USA). The samples—seven angiotensin derivatives and two methionine enkephalin derivatives—were purchased from Sigma (St.Louis, MO, USA). Their characteristic values are listed in Table 1. Their pI values were calculated by a software, 2D tool kit1.

The molal surface tension increment values of five sulfates are listed in Table 2. Water was distilled and deionized in a Milli-Q system (Millipore, Bedford, MA, USA).

Procedures

Isocratic measurements were performed at five different salt concentrations ranging from 0.1M to 0.5M and at four different temperatures varying from 5°C to 35°C. UV detection was performed at 220nm. Each sulfate salt

Table 1

Molecular Weights, Amino Acid Sequences, and Isoelectric Point (pI) Values of Peptides Used in This Study

Peptide	Abbreviation	M. W.	Sequence	pI
Angiotensin I human	Ang 1	1296.5	DRVYIHPFHL	7.96
Angiotensin II human	Ang 2	1046.2	DRVYIHPF	7.81
[Val^5]-Angiotensin II	V-Ang 2	1032.2	DRVYVHPF	7.81
Ala-Pro-Gly-[Ile^3, Val^5]-Angiotensin II	APG-Ang 2	1271.4	APGDRVYIHPF	7.78
Angiotensin II 3-8 fragment	Ang 2 3-8	774.9	VY1HPF	7.74
[Val^4]-Angiotensin III	V-Ang 3	917.1	RVYVHPF	9.55
Angiotensinogen fragment 11-13	Ang 1-13	1645.9	DRVYIHPFHLVIH	8.06
Methionine enkephalin	Met	573.7	YGGFM	6.68
Methionine enkephalin-Arg-Gly-Leu	Met-RGL	900.1	YGGFMRGL	9.60

Table 2

Molal Surface Tension Increments of Selected Salts

Salt	(σ x 10^3 dyne-g/cm.mol)
$MgSO_4$	2.10
$(NH_4)_2SO_4$	2.16
K_2SO_4	2.58
Na_2SO_4	2.73
Li_2SO_4	2.78

was added to 0.05M sodium phosphate buffer, adjusted to pH 7.0 by mixing monobasic sodium phosphate and dibasic sodium phosphate, except magnesium sulfate. $MgSO_4$ was added to 0.05M ammonium acetate buffer because phosphate buffer is known to form precipitation with Mg^{2+}. Then the solution was filtered through a 0.45 μm Millipore filter and degassed with a Solid State/Ultrasonic FS-28 sonicator (Fisher, Fair Lawn, NJ, USA) for at least 20 minutes before use.

Samples were dissolved in 0.05M sodium phosphate buffer (pH 7.0) at a concentration of 0.5mg/mL. The injection volume was 10 or 15 μL. All the measurements were repeated at least twice and the average values were used.

RESULTS AND DISCUSSION

Investigation of Salt Effects Using S and log k_w

In HIC, the relationship between solute retention and molal concentration of salt in the mobile phase can be expressed as follow:[3]

$$\log k' = \log k_w + Sm \quad (1)$$

where m is the molality of the salt, S and log k_w are the slope and the intercept of the plot. Eq. 1 is similar to Eq. 2, which describes the relationship between solute retention and organic solvent in RPC.[6]

$$\log k' = \log k_0 - S'\Phi \quad (2)$$

In Eq. 1 and 2, S and S′ are proportional to the contact area between the solute and the stationary phase ligand. Log k_w and log k_0 are related to the affinity of the solute for the stationary phase in pure water. Studies were performed

by Purcell *et al*., which inferred conformational changes of peptides in column from the changes of S and log k_w.[7,8]

Rippel *et al*. also attempted to compare salt effects in HIC using these parameters.[9] We chose angiotensin II and methionine enkephalin for the representatives of angiotensin and methionine enkephalin derivatives. S and log k_w values were obtained from data using Na_2SO_4, $(NH_4)_2SO_4$, Li_2SO_4. Under the condition of using K_2SO_4, data were excluded because of low linearity in the log k′- m plots. To make things worse, column packings deteriorated during experiments using K_2SO_4. This problem could be due to the low solubility of K_2SO_4, especially at low temperatures. In the case of $MgSO_4$, abnormal retention behaviors were found, including low linearities and the decrease of retention according to increase of salt concentration.

Recently, similar phenomena in using $MgCl_2$ were also reported by Oscarsson.[6] Figure 1 describes the changes of these retention parameters with temperature. In Figure 1A, S values made little difference except for a few points in the investigated region, but in Figure 1B, log k_w values steeply increased in proportion to the rise of temperature. The above facts indicate that the retention increment with the rise of temperature depend on log k_w, rather than on S.

In Figure 2, changes of S and log k_w values with salt types were plotted. Salts on x axis were given by the order of molal surface tension increment. As shown, S mostly increased with the order of molal surface tension increment, but log k_w values increased in the order of Li_2SO_4, $(NH_4)_2SO_4$ and Na_2SO_4.

In the case of methionine enkephalin, the log k_w for Na_2SO_4 and Li_2SO_4 only made a little difference. And, in the case of angiotensin II, the log k_w for Li_2SO_4 was even smaller than that for $(NH_4)_2SO_4$. It is thought that this might be due to the salting-in (chaotropic) effect of Li^+ which increased the solubility of protein and peptide by the interaction between peptide bond dipole and the lithium ion.

Divalent cations such as Ca^{2+}, Mg^{2+} are known to have a strong salting-in effect for proteins, and this induces unusual retention behaviors in HIC. The retention behavior of $MgSO_4$ in the experiment of Fausnaugh *et al.* was similar to that of Li_2SO_4 in our study.[5] So, we attributed the peculiarity of Li_2SO_4 as a salting-in effect. The effects of $(NH_4)_2SO_4$ and Na_2SO_4 were correlated with the theory of Horváth *et al.,* and it was evident that they could be the general salts for HIC separation.

We investigated the effects of the stationary phase ligand type on S and log k_w in order to confirm S and log k_w as retention parameters, which can represent the overall retentions in HIC. In Figure 3, the changes of S and log k_w were

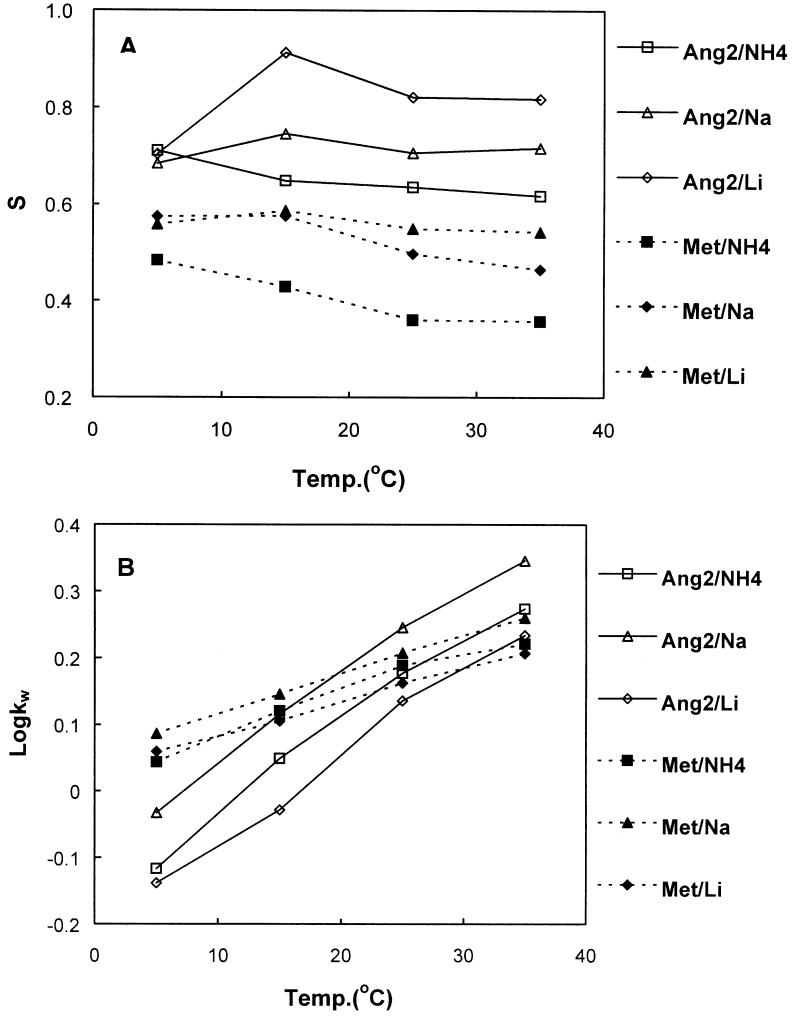

Figure 1. Dependence of (A) S and (B) log k_w on temperature for angiotensin II (solid line) in $(NH_4)_2SO_4$(square), Na_2SO_4(triangle), Li_2SO_4(diamond) solution and methionine enkephalin(dashed line) in $(NH_4)_2SO_4$(filled square), Na_2SO_4(filled triangle), Li_2SO_4(filled diamond) solution.

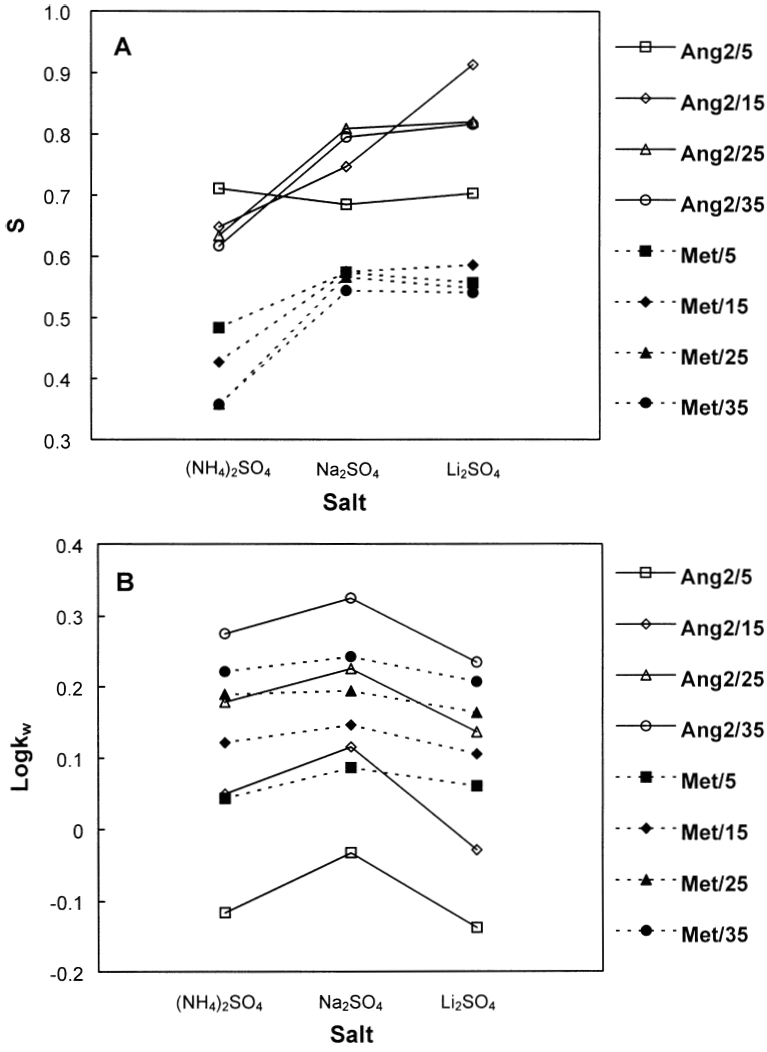

Figure 2. Dependence of (A) S and (B) log k_w on salt type for angiotensin II (solid line) at 5°C(square), 15°C(diamond), 25°C(triangle), 35°C(circle), and methionine enkephalin(dashed line) at 5°C(filled square), 15°C(filled diamond), 25°C(filled triangle), 35°C(filled circle).

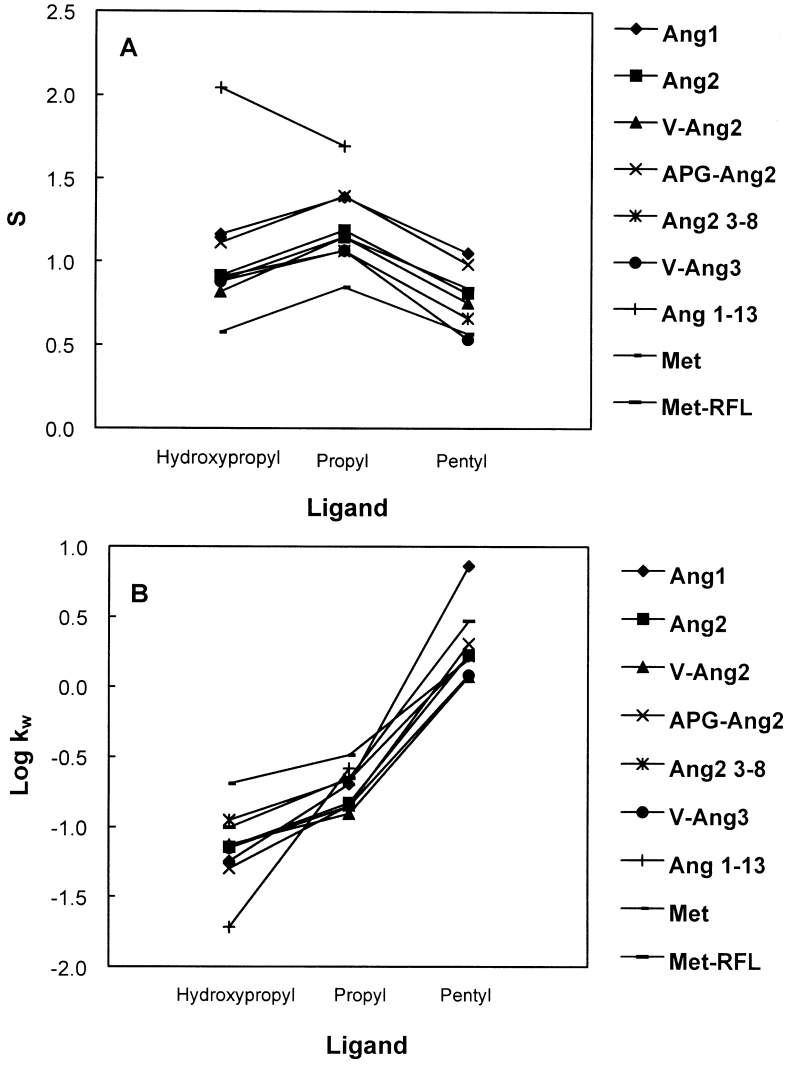

Figure 3. Dependence of (A) S and (B) log k_w on column ligand for 9 peptides at 25°C. (mobile phase : Na_2SO_4)

plotted on each column ligand at 25°C for 9 peptides. Log k_w values increased with the increase of ligand hydrophobicity (hydroxypropyl<propyl<pentyl) and matched well with the changing trends of retention.

S values were the largest in the propyl column, except in the angiotensin fragment 1-13. These trends could be explained by the increased contact area with the solute, due to the rigid structure of the propyl ligand, compared with the longer, loose pentyl ligand. These irregularities were also observed in the S and log k_w values of RPC, using peptides.[9]

Thermodynamic Investigation

As mentioned before, we attempted to explain salt effects by changes in the S and log k_w In order to understand salt effects, we investigated the changes of thermodynamic parameters such as enthalpy and entropy. In the chromatographic process, the relationship between retention and temperature can be given as follows;

$$\ln k' = - \Delta H/RT + \Delta S/R + \ln \varphi, \quad (3)$$

where R is the gas constant, T is the absolute temperature, and φ is the phase ratio which is the volume ratio of the stationary phase to the mobile phase. We calculated φ through the method of Jandera *et al.* They defined the phase ratio as follows;

$$\varphi = (V_G - V_M)/V_M, \text{ where} \quad (4)$$

V_G is the volume of an empty column and can be calculated by column dimensions, V_M is the volume of the mobile phase in column; the retention volume of the solute which has no interaction to the column stationary phase. We measured V_M values at each column using sodium nitrate. As a result, we got 0.572 for pentyl, 0.470 for propyl, and 0.545 for hydroxypropyl column as the phase ratio.

We calculated the ΔH and ΔS values from the slopes and the intercepts of the plot of ln k′ vs.1/T. The ΔH and ΔS values were plotted on salt concentration in Figure 4. But these parameters did not have any tendency to salt type or concentration. These values are thought to be more affected by the kinds of solutes or column ligands since the difference of ΔH and ΔS between angiotensinII and methionine enkephalin was almost constant.

Figure 5 describes the changes of ΔH and ΔS values with the column ligands. ΔH and ΔS values increased in the order of hydroxypropyl, pentyl, and propyl. In the case of methionine enkephalin, the ΔH and ΔS values were

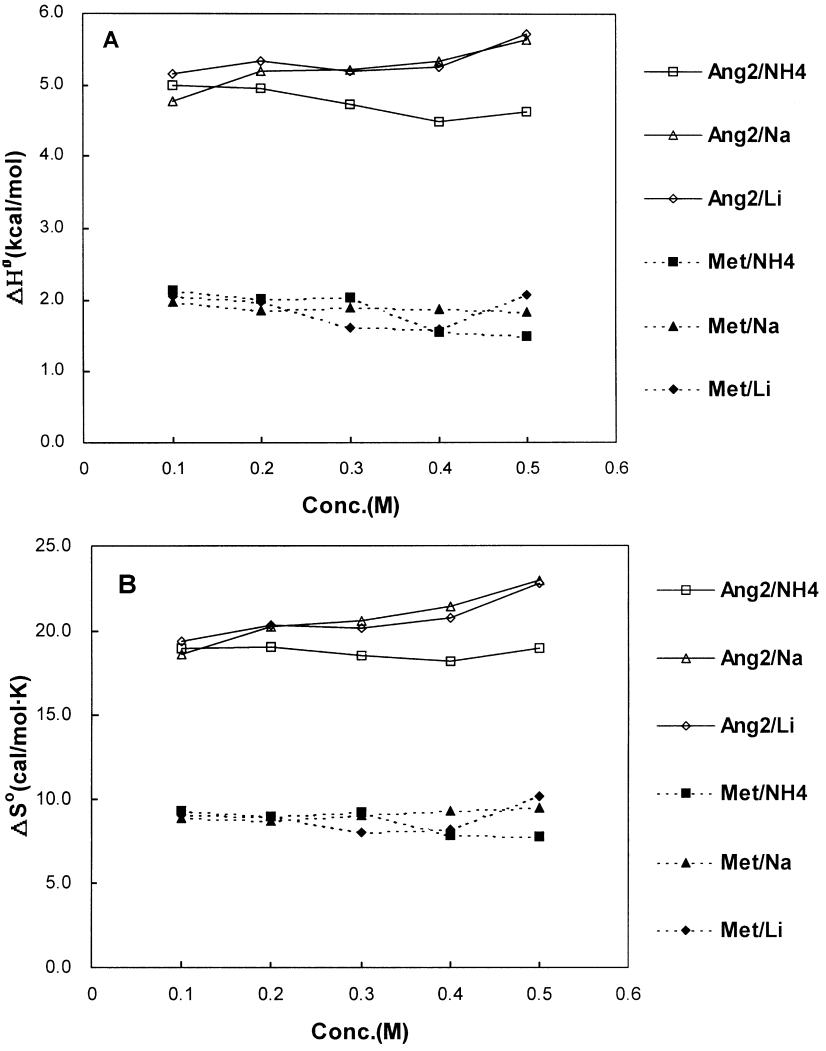

Figure 4. Dependence of (A) ΔH and (B) ΔS on salt concentration for angiotensin II in $(NH_4)_2SO_4$(square), Na_2SO_4(triangle), Li_2SO_4(diamond) solution and methionine enkephalin in $(NH_4)_2SO_4$(filled square), Na_2SO_4(filled triangle), Li_2SO_4(filled diamond) solution.

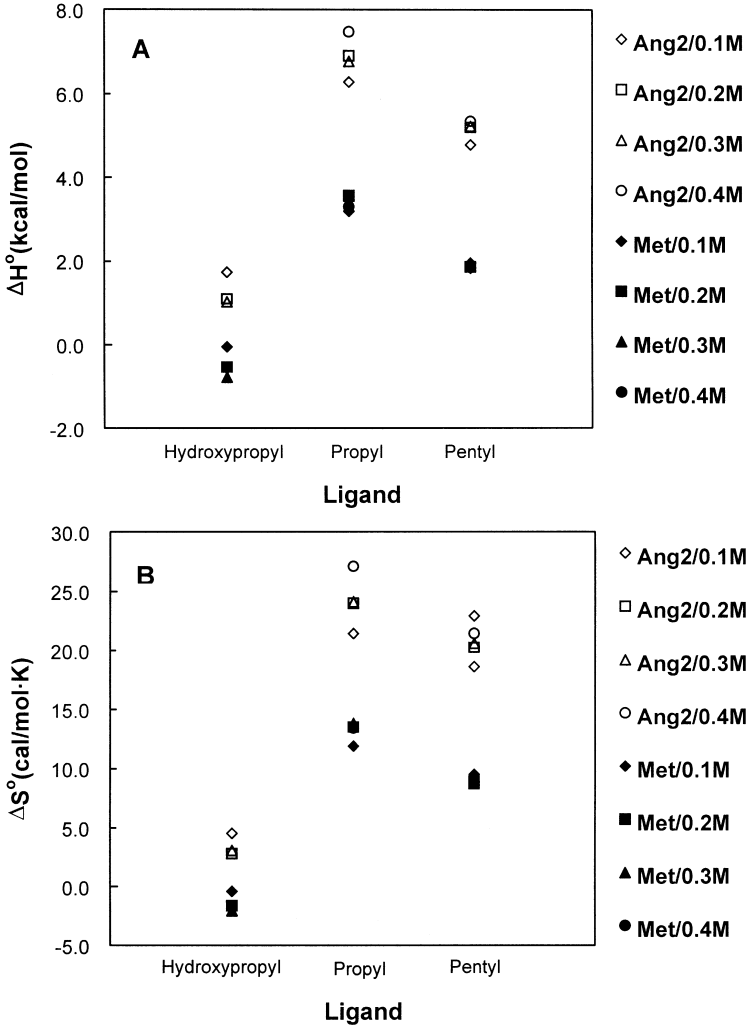

Figure 5. Dependence of (A) ΔH and (B) ΔS on column ligand for angiotensin II(diamond) and methionine enkephalin(filled diamond) at various salt concentrations.

slightly negative in the hydroxypropyl column. These trends are thought to be related with the structural change of stationary and mobile phase. The hydroxy group of the hydroxypropyl column is so hydrophilic that the attraction between hydroxypropyl ligand and water molecules is expected to be relatively stronger than the pentyl or propyl ligand. This is the reason why the entropy-stabilizing effect is unfavorable for hydroxypropyl ligand.

When hydrated peptides contacted at the propyl or pentyl ligand, lots of water molecules would be desorbed from the stationary phase ligands and peptides. The hydrophobic character of pentyl and propyl ligand is expected to give a stabilizing effect on entropy. Between them, pentyl ligand is more hydrophobic than propyl ligand, so that the water molecule layer located on the stationary phase surface in pentyl column would be relatively thinner than in the propyl column. This would detach less water molecules from pentyl ligand.

Since the mobile phase was in a structurally stable state, that is, surface tension increased by coexistence of water and salt, it would need energy for the transfer of water molecules from the column ligands or adsorbed peptides to the mobile phase. So, the ΔH values would be positive, and endothermic reaction would take place for propyl and pentyl column.

In a hydroxypropyl column, polar interaction between the -OH group of ligand and solute may have acted as an attractive force, leading to negative ΔH values and the exothermic retention process. These concepts are close to the displacement theory, one of the well-known retention mechanisms. In contrast to the solvophobic theory, the theory attaches importance to the effect of stationary phase and it was used as the explanation of the retention mechanism for proteins in HIC as well as in RPC by Geng *et al.*[10]

The similarity in the changing trends of ΔH and ΔS shows that there might be a compensation effect between these values. In many biological reactions or those in aqueous solutions, proportionalities between ΔH and ΔS have often been observed.[11-15] This phenomenon is called enthalpy-entropy compensation, and the compensation temperature (β) is the characteristic value in the plot of ΔH and ΔS as follows:

$$\Delta H = \beta \Delta S + \Delta G_{\beta}, \text{ where} \quad (5)$$

ΔG_{β} means the standard free energy change at the compensation temperature (β). If β is constant throughout the retention process using various conditions, it is believed that one universal reaction mechanism dominates the process.[16-19] So, comparison of β is used to investigate the maintenance of the retention mechanism of chromatography. If ΔS is eliminated by substituting Eq. 5 for Eq. 3, we obtain Eq. 6,

$$\ln k' = -\ \Delta H/R(1/T - 1/\beta) - \Delta G_{\beta}/R\beta + \ln \varphi \qquad (6)$$

Equation 6 is useful for calculating β from the slope of ln k′-ΔH plot. We calculated the compensation temperatures (βs) by the ΔH-ΔS plot. The results are listed on Table 3. As the cation size of the salt (except $(NH_4)_2SO_4$) was bigger and the ligand hydrophobicity was larger, β values were slightly diminished and coincided with the tendency of the retentions. The β values in Table 3 ranged from 248 to 294.4 K. This range was distinguished from 400 to 1800 K of RPC, but similar to the β range from 265 to 420K in biological reactions.[15]

So, it may prove that HIC could separate samples by maintaining conditions similar to biological reactions. The differences of β values in this study were not so large, but the trend of β values agreed with that of the retentions. The larger the β values, the shorter the retention at the condition because at the compensation temperature the retention of all solutes was universal, regardless of their types. However, the other method to calculate β using ln k′-ΔH plot did not show linearities so we could not get the β values.

As mentioned by Krug *et al.*, the ΔH and ΔS values from van't Hoff plots have fatal defects, because of the selection of a narrow temperature range.[20,21] So, when we use these values, there are possibilities to mistake the reactions of no compensation for compensation reactions and to get the harmonic mean of the selected temperature values as β. Though it is more useful to examine compensation using the ΔG and ΔH plots, most chromatographic papers have not intended to consider this fact.[22]

Table 3

Compensation Temperatures and Intercepts from ΔH-ΔS Plots in HIC

Salt	β(K)	Intercept
$MgSO_4$	284.3 ± 4.5	0.3410 ± 0.1338
$(NH_4)_2SO_4$	277.6 ± 2.0	0.0845 ± 0.0672
K_2SO_4	266.9 ± 7.8	0.2130 ± 0.1503
Na_2SO_4	258.4 ± 4.5	0.3547 ± 0.0945
Li_2SO_4	248.0 ± 5.5	0.5868 ± 0.1126
Column Ligand	**β(K)**	**Intercept**
Hydroxypropyl	294.3 ± 4.5	0.8337 ± 0.0428
Propyl	282.8 ± 3.3	0.5794 ± 0.0931
Pentyl	266.9 ± 7.8	0.2130 ± 0.1503

Enthalpy-entropy compensations in HIC have not been thoroughly studied yet. Although Vailaya *et al.* recently studied enthalpy-entropy compensation on dansyl amino acids, introducing new thermodynamic theories recently, they did not include peptides or proteins which are commonly used in HIC.[23] Therefore, this study is important in the investigation of enthalpy-entropy compensation for peptides in HIC.

CONCLUSIONS

We examined the salt effects in HIC by using peptides. The results are summarized in the following:

We explained salt effects by dividing two retention parameters, S and log k_w. S was thought to be related to surface tension, and log k_w to salting-in effect, so, we could understand the effect of a salting-in ion such as Li^+. The tendencies of change in S and log k_w were generally known to be similar to each other in RPC, but not in HIC. Log k_w was emerged as a major factor in increasing the retention with the rise of temperature in HIC. From the dependence of S and log k_w on the column ligand type, these parameters were also shown to be useful in explaining the retention characteristics in HIC which are different from those in RPC.

From the point of thermodynamics, the ΔH and ΔS values from van't Hoff plots changed little with salt type and concentration. Therefore, the ΔH and ΔS values were believed to be more dependent on the kinds of peptides and column ligand types.

Because of the similarity in the changing trends of the ΔH and ΔS values, we examined enthalpy-entropy compensation. As a result, we could explain salt effects by a change in the compensation temperature. We could infer the similarity between the retention and biological processes in HIC.

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