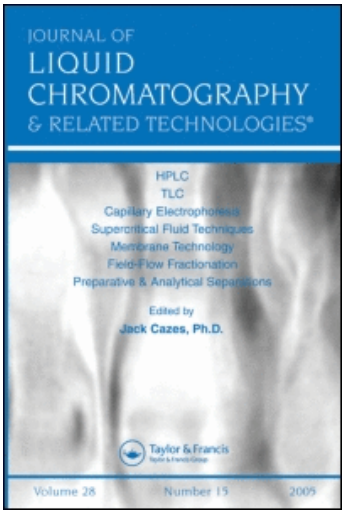

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Temperature Effects in Hydrophobic Interaction Chromatography of Proteins

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Temperature Effects in Hydrophobic Interaction Chromatography of Proteins

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Abstract: The thermal behavior of five proteins in hydrophobic interaction chromatography (HIC) were investigated in the temperature range from 0 to 50°C. The conformational change of protein was characterized by the parameter, Z, in the stoichiometric displacement model for retention (SDM-R). The thermodynamic parameters (ΔH°, ΔS°, ΔC_p° and ΔG°) of these proteins were determined. It was found that the retention process of protein in HIC is entropy driven, and enthalpy or entropy change of unfolding protein correlated linearly with temperature. The existence of enthalpy and entropy convergence with temperature was also confirmed. The differences of the isoentropic and isoenthalpic temperature for protein unfolding in HIC system from the traditional solution were elucidated.

Keywords: Column liquid chromatography, Hydrophobic interaction chromatography, Protein unfolding, Thermodynamic convergence

INTRODUCTION

Hydrophobic interaction chromatography (HIC) is widely used for the separation and purification of biopolymers in their native state, and now also becomes a new method to refold the denatured proteins.[1] This technique employs weakly hydrophobic stationary phase and the retention is modulated

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by varying the salt concentration in the aqueous mobile phase. The effect of salt on the protein interaction in aqueous solution as well as on the protein adsorption in HIC has been investigated in detail.[2–5] However, less progress has been made in elucidating the effect of temperature on retention in HIC. So far the only thermodynamic studies on the effect of temperature in HIC were carried out with dansyl derivatives of amino acids[6] and aromatic alcohol homologues.[7] For the retention of bovine serum albumin in HIC, Dias-Cabral et al.[8] determined the enthalpy and entropy in the process of the retention by nonlinear Van't Hoff equation and flow microcalorimetry. Flow microcalorimetry and adsorption isotherm measurements were also used to study the temperature effect on the adsorption enthalpy and entropy of protein. Hearn et al.[9] used isothermal titration microcalorimetry in connection with binding equilibrium to study the adsorption behaviors of three proteins with significantly different hydrophobicities, yet similar molecular weights on HIC supports.

Since the enthalpy and entropy convergent temperatures in protein unfolding were found,[10] many efforts were made to define the origin of this convergence phenomenon,[11] which was proposed to relate to the hydrophobic effect. If this was true, HIC should be used to investigate this question in protein unfolding studies. In this report, we try to analyze the unfolding thermodynamics of protein under conditions of HIC, and elucidate the stable reason for protein existence in their native state.

EXPERIMENTAL

Chemicals

Analytical reagent grade ammonium sulfate [$(NH_4)_2SO_4$], dibasic sodium phosphate, and 85% phosphoric acid were obtained from Xi'an Chemical Company (Xi'an, China). Cytochrome C (Cyt-C), myoglobin (Myo), Bovine serum albumin (BSA), lysozyme (Lys) and β-lactoglobulin (β-Lact) were purchased from Sigma (St. Louis, MO, USA).

Mobile Phase

Deionized HPLC grade water was used to prepare eluents having the desired molar concentration of $(NH_4)_2SO_4$ as well as dibasic sodium phosphate. The pH of the solution was adjusted to 7.0 with 85% phosphoric acid. The solution was filtered through a 0.45 μm filter prior to use.

Equipment

Two LC-10A pumps and a model LC-10 UV/VIS detector with a model CLASS-VP Chromatopac integrator (Shimadzu, Japan) were used in

chromatographic experiments. The HIC column was made by packing with the HIC packings (PEG-silica, silica linked to ligands consisting of a polyethylene glycol chain, made in our institute) in a 100 × 4.6 mm I.D. column. The column temperatures were controlled by enclosing the column in a glass jacket through which water was circulated from the thermostatted water bath (Chongqing Instrument Co., China) with the accuracy of $\pm 0.2^{\circ}$C. Prior to the entrance of Rheodyne valve, each mobile phase was preheated in a stainless steel capillary tube (0.5 mm I.D.) of 0.5 m coiled around the column inside the glass envelope. The column was equilibrated at each temperature for one hour before any measurement was made. Chromatographic experiments were carried out after thermal equilibrium was reached.

Procedures

Proteins were dissolved as a concentration of 5.0 mg mL^{-1} in the buffer of dibasic sodium phosphate. The sample volume varied between 5 and 10 μL. Proteins were eluted isocratically under temperature ranges from 0 to 50°C with intervals of 10°C and detection at 280 nm. The retention time of $NaNO_2$ was taken as t_0 (the dead time maker) and used to calculate the capacity factor defined as $k = (t_R - t_0)/t_0$, where t_R is the retention time of the solute. The retention times (t_R) of the proteins were measured at each mobile phase composition and the required temperature. The t_0 of the column was selected not to depend on the salt concentration in the mobile phase.

The concentration of water in mobile phase of HIC was measured according to the following equation:[5]

$$[H_2O] = \frac{W_m - W_s}{0.018} \ (\text{mol L}^{-1})$$

where W_m and W_s are the amount of the solution and salt (g L^{-1}) per liter in the mobile phase, respectively.

RESULTS AND DISCUSSION

Determination of the Unfolding Thermodynamic Parameters of Proteins

Isocratic elution of five proteins on PEG-silica, carried out with ammonium sulfate at 293.15 K showed that protein retention increased with increasing salt concentration in the mobile phase. This trend was observed at all temperatures investigated. The presence of salt has a great influence on the equilibrium behavior in HIC, which has been extensively investigated. Also, the retention factor, k, was found to increase with an increase in temperature, as is generally observed for HIC.[6] Generally, the capacity factor for each of the five proteins

did not change linearly with temperature (Figure 1), and, if it did, it was over a very small range. The nonlinearity is, in part,[12] due to a change in protein conformation. The curves of Van't Hoff plots for each protein at other salt concentrations in the mobile phase also showed a similar trend. Figure 2 only shows the Van't Hoff plots of lysozyme at different salt concentrations. It is well known that a protein in a folded state is less retained in HIC than in an unfolded state. This is because the area of contact of the protein with the adsorbent surface is generally larger in an unfolded state.[4] Thus, a nonlinear dependence of retention with temperature could be due to a conformational change in the protein, which results in an increase in the conformational entropy at higher temperature.

An important measurement, one that can reflect changes in contact area, is the change of protein retention with solvent composition. Capacity factors can be analyzed with the stoichiometric displacement model for retention (SDM-R) to obtain Z, which is a measure of protein conformation.[5] This model was given by:[5]

$$\ln k = \ln I - Z \ln[H_2O] \tag{1}$$

where k is the capacity factor, $[H_2O]$ is the molar concentration of water in the mobile phase, and Z and $\log I$ are constants. The physical meaning of $\log I$ is: the affinity of 1 mol of solute to the stationary phase. That of Z can be described as the total moles of solvent released at the contact region between the stationary phase and the solute interface when 1 mol of

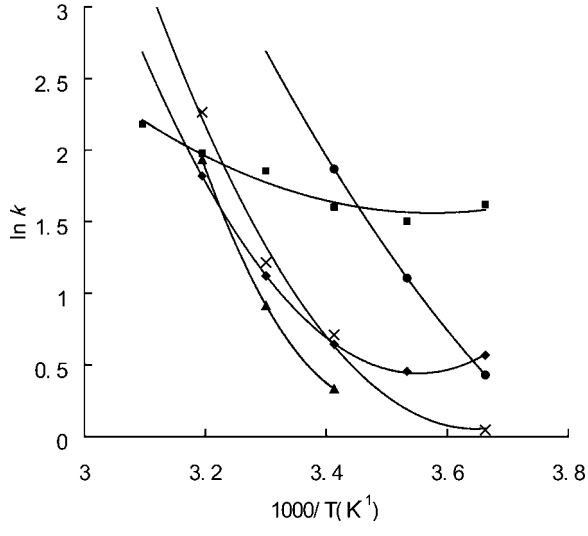

Figure 1. Van't Hoff plots of five proteins. Symbol: (■) Lys, 1.65; (□) Cyt-C, 2.58; (▲) β-Lact, 1.05; (●) Myo, 2.16; (×) BSA, 1.59 mol L^{-1} $(NH_4)_2SO_4$. Stationary phase: PEG-silica. Flow rate: 1.0 mL min^{-1}. Detector: 280 nm.

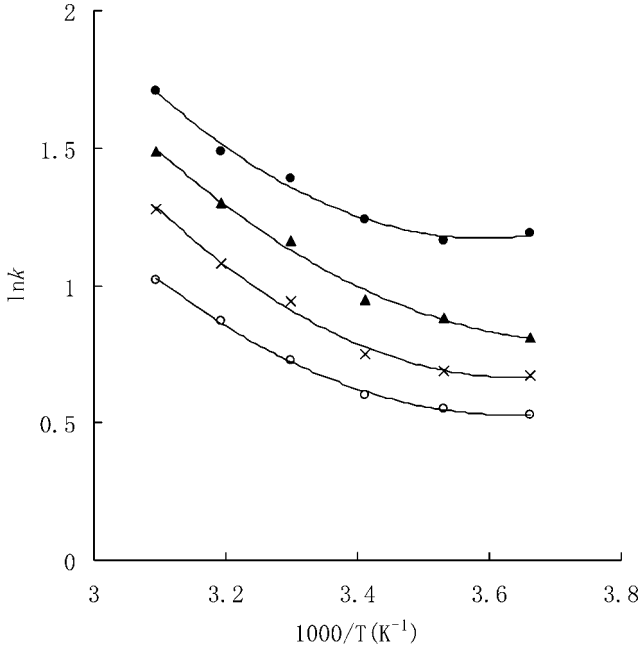

Figure 2. Van't Hoff plots of lysozyme. $(NH_4)_2SO_4$ concentrations: (●) 1.65; (▲) 1.62; (×) 1.59; (○) 1.56 mol L^{-1}. Stationary phase: PEG-silica. Flow rate: 1.0 mL min^{-1}. Detector: 280 nm.

solvated solute is absorbed. *Z* has been referred to be a new characterization parameter in LC.[13] When the salt, ligand, and temperature are fixed, *Z* is a characteristic constant related to protein conformation.[5]

Figure 3 shows the changes in *Z* value as a function of column temperature. For all five proteins investigated, the *Z* values increase with temperature.

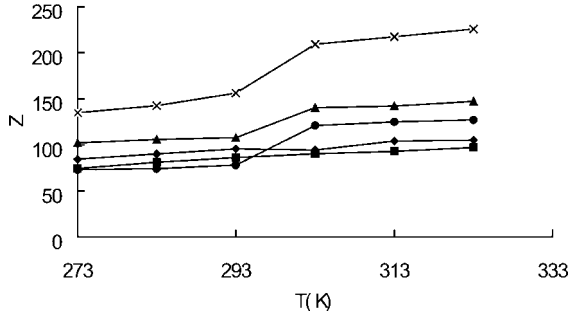

Figure 3. Plots of *Z* values of five proteins versus temperature. Symbol: (■) Lys; (□) Cyt-C; (▲) β-Lact; (●) Myo; (×) BSA. Other chromatographic conditions are the same as that shown in Figure 1.

Z values of BSA, OVA, and Myo show sharp changes at the temperature range from 293 to 303 K, indicating a conformational change in the proteins. In contrast, for Lys and Cyt-C no sharp change is evident, this may be related to the fact that these two proteins are more stable among the five proteins. In the temperature range investigated, the structures of these two proteins might produce small changes, so that Z does not show a sharp change but a continuing change trend. Conversely, the conformational change of proteins further resulted in the increase in hydrophobic contact area between the protein and the stationary phase. Therefore, it could be concluded that the increments in Z came from two parts, one is the changes in the number of water molecules surrounding the protein due to conformational changes of proteins, and the other is that resulting from an increase in hydrophobic contact area between the protein and the stationary phase. On the other hand, it is clear that Z values of five proteins are large at all the temperatures investigated, indicating that the adsorption is accompanied by the release of a large number of water molecules, this supports the expectation of an entropically driven process in which the release of a large number of ordered water molecules provides the driving force for adsorption.

Assuming the standard heat capacity, ΔC_p°, associated with the transfer of eluites from stationary to mobile phase is invariant with temperature; the thermodynamic parameters can be calculated by the following equations as:[6,14]

$$\ln k' = \frac{\Delta C_p^\circ}{R}\left(\frac{a}{T} - \ln\frac{b}{T} - 1\right) + \ln\phi \tag{2}$$

$$\Delta H^\circ = -R\frac{d\ln k'}{d(1/T)} = -\Delta C_p^\circ(a - T) \tag{3}$$

$$\Delta S^\circ = -\Delta C_p^\circ \ln\frac{b}{T}, \tag{4}$$

where R is the gas constant, T is thermodynamic temperature, ϕ is the phase ratio, ΔH°, and ΔS° are standard enthalpy and entropy changes, respectively, a and b are the temperatures at which the enthalpy and entropy changes for the same transfer process are zero. In order to simplify the treatment, the value of the phase ratio was set arbitrarily to unity. The data in Figure 1 and those at other salt concentrations for each protein were found to be well fitted to Eq. (2) with correlation coefficients greater than 0.98. Thus, the thermodynamic parameters were obtained with the above equations. Table 1 only lists the thermodynamic parameters of Lys, Cyt-C, Myo, BSA, and β-Lact at 1.65, 2.58, 2.16, 1.59, and 1.05 mol L^{-1} $(NH_4)_2SO_4$, respectively. The thermodynamic parameters of these proteins at other salt concentrations were not shown.

It can be seen in Table 1 that, for five proteins, the retention process is predicted to be endothermic, indicating an entropically driven process, is expected.[3,4,15] This reinforces the conclusion obtained with the Z value. Interestingly, entropy increases with temperature in the retention process. These observations are inconsistent with the expectation that the mechanism

Table 1. Thermodynamic parameters associated with the unfolding process of proteins at different temperatures in HIC system

Proteins	$(NH_4)_2SO_4$ (mol L^{-1})	Temperature (K)	ΔH° (kJ mol^{-1})	ΔS° (J K^{-1} mol^{-1})	ΔC_P° (kJ K^{-1} mol^{-1})	ΔG° (kJ mol^{-1})
Lys	1.65	273	2.11	17.46		−2.65
		283	4.50	26.05		−2.87
		293	6.72	33.77		−3.17
		303	8.80	40.73	0.20	−3.55
		313	10.74	47.04		−3.98
		323	12.56	52.77		−4.48
Cyt-C	2.58	283	3.35	15.54		−1.05
		293	24.95	90.57	2.09	−1.59
		303	45.13	158.29		−2.84
		313	64.01	219.63		−4.73
Myo	2.16	283	34.96	127.80		−1.21
		293	40.46	146.92	0.53	−2.59
		303	45.60	164.17		−4.14
BSA	1.59	293	27.55	94.40		−0.11
		303	68.90	233.21	4.00	−1.76
		313	107.61	358.94		−4.73
β-Lact	1.05	283	46.08	166.36		−1.00
		293	48.78	175.75	0.26	−2.71
		303	51.31	184.23		−4.51

driving HIC shifts in the direction of a decreasing entropic influence as the temperature is increasing.[6,9,14] This phenomenon is also due to conformational change of protein. In HIC of proteins, entropy changes are more complex than in HIC of small molecules, as observed in the retention process of densylamino acids,[6] and it was regarded to come from temperature induced conformational changes of proteins, reorganization of water molecules on the surface of protein molecules,[11] and the changes in the number of water molecules released at the interface between the stationary phase and proteins due to its conformational change as described above.

Relationship Between Thermodynamic Parameters and Temperature

As shown in Table 1, in the temperature range investigated, all of ΔH° and ΔS° are positive while ΔG° is negative, suggesting the enthalpy increase to be

compensated by entropy increase, therefore to make the free energy lower so as to favor the protein to be retained on the stationary phase, in other words, the protein retention in HIC was governed by entropy. This conclusion is different from the results that the retention process of densylamino acids[6] and aromatic alcohol homologues[7] are entropy driven at low temperatures and enthalpy driven at high temperature in HIC.

It could also be obtained from Table 1 that ΔH°, ΔS°, and ΔG° depend on the temperature, respectively, and plots of ΔH° or ΔS° of the five proteins versus temperature give good linearity with correlation coefficients (R^2) more than 0.990 (Table 2). For each protein at other salt concentrations investigated, the relationships between ΔH° or ΔS° and temperature were also found to be linear. In the protein unfolding study investigated with calorimetry, the similar phenomenon also exits.[16] On the other hand, Table 1 also shows that both ΔH° and ΔS° strongly depend on the temperature and increase with temperature, while ΔG° exhibits a much weaker dependence, suggesting some of the enthalpy and entropy changes compensate each other and lead free energy to change within a small range. This result is consistent with that obtained with calorimetry.[10,11] It is the very reason that although living beings endure the changes to the outside environment, especially temperature, they can keep themselves alive.

Isoentropic and Isoenthalpic Temperature

When ΔC_p° is constant for a process involving a structurally related substance, the enthalpy and entropy changes are expressed as:[6]

$$\Delta S^\circ = \Delta S^* + \Delta C_p^\circ \ln(T/T_s^*) \tag{5}$$

$$\Delta H^\circ = \Delta H^* + \Delta C_p^\circ (T - T_H^*) \tag{6}$$

Table 2. Linear parameters of plots of ΔH° or ΔS° of five proteins *vs*. the absolute temperature

	Lys	Cyt-C	Myo	BSA	β-Lact
$\Delta H^\circ \sim T$					
Slope	0.203	2.023	0.532	4.00	0.262
Intercept	52.9	568.5	115.5	1144.0	27.9
R^2	0.997	0.999	1.000	1.000	1.000
$\Delta S^\circ \sim T$					
Slope	0.672	6.80	1.82	13.2	0.894
Intercept	164.1	1905.4	386.5	3778.3	86.3
R^2	0.993	0.998	0.999	0.999	0.999

where T_s^* and T_H^* are the isoentropic and isoenthalpic temperature at which all participating substances attain a common ΔS^* and ΔH^*, respectively. The significance of T_s^* and T_H^* has been recognized in studies on protein folding, and Eqs. (5) and (6) have been used for mechanistic interpretation of calorimetric data.[10] Essentially, T_s^* and T_H^* mark common intersection of points of the respective plots of entropy and enthalpy versus temperature.[6] Figure 4 only illustrates the plots of ΔH° (B) or ΔS° (A) of Lys at different salt concentrations in the mobile phase. Four straight lines in Figure 4 (A) or (B) converged well. Plots of ΔH° or ΔS° of other four proteins versus temperature at different mobile phase concentrations were also found to converge well, T_s^* and T_H^*, thus, were obtained, respectively (Table 3). All T_s^* and T_H^* of the five

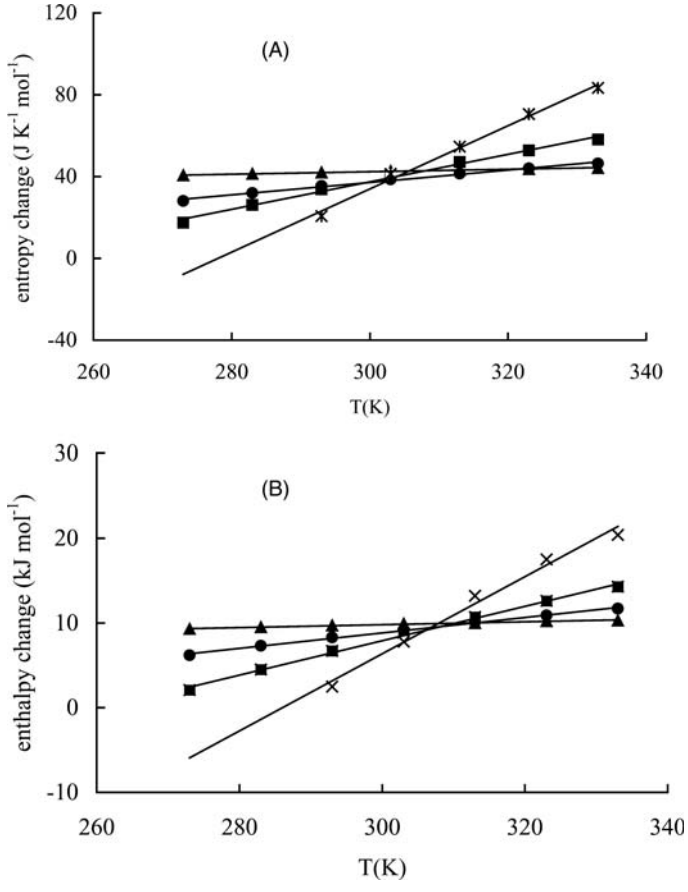

Figure 4. Plots of ΔS° (A) and ΔH° (B) of lysozyme versus temperature at different salt concentrations in the mobile phase. (×) 1.56; (●) 1.59; (▲) 1.62; (■) 1.65 mol L^{-1} $(NH_4)_2SO_4$. Other chromatographic conditions are the same as that shown in Figure 1.

Table 3. The isoentropic and isoenthalpic temperature in the process of protein unfolding in HIC

	Lys	Cyt-C	Myo	BSA	β-Lact
T_H^* (K)	305 ± 4	304 ± 6	306 ± 4	307 ± 2	306 ± 7
T_s^* (K)	305 ± 4	308 ± 6	310 ± 4	309 ± 2	316 ± 7

proteins are in the range of 309.6 ± 7 K and 304.6 ± 7 K, respectively, showing the similarity in the unfolding process of the five proteins in the HIC system. But these values are much different from the results that T_H^* was centered around 374 K, whereas T_s^* was located near 385 K for protein folding in the solution system investigated with the calorimetric method.[17] This could be attributed to the fact that protein unfolding was controlled by different interactions in solution from the HIC system. In solution, protein unfolding was mainly affected by the hydrophobic interaction within the interior of molecules itself, while in the HIC system, it is affected by hydrophobic and static electronic force between protein and stationary phase beside the force of the interior of the molecule as temperatures increased.[18]

CONCLUSION

The present study has extended the knowledge of unfolding thermodynamics of proteins in the hydrophobic interaction chromatography (HIC).

The influence of temperature on the retention of proteins in HIC was studied and the conformational change of proteins was characterized by the parameter, Z, in the stoichiometric displacement model for retention (SDM-R). The unfolding thermodynamic parameters derived were used to improve understanding of the thermodynamic driving forces of the retention process for proteins. The endothermic process indicates the protein unfolding and the retention process is entropically driven. The existence of enthalpy and entropy convergence with temperature was also confirmed. The differences of the isoentropic and isoenthalpic temperature for protein unfolding in HIC systems from the traditional solution were caused by the complex interaction between protein and the chromatographic system.

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