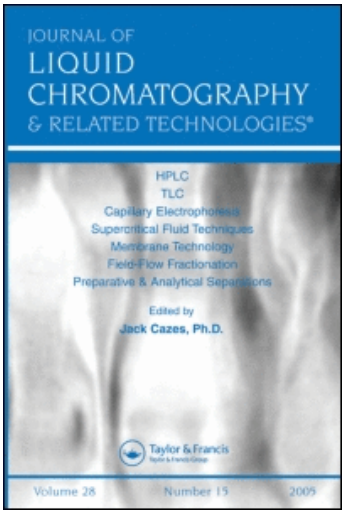

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Studies on the Thermodynamics During the Biopolymer Retention Process in Reversed-Phase Liquid Chromatography. I. The Free Energy Change and Its Fractions, Adsorption, and Desorption Free Energy Changes

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ABSTRACT

Based on the stoichiometric displacement theory for retention of solute (SDT-R), the changes of free energy of biopolymers during retention process in reversed-phase high performance liquid chromatography (RPLC) can be divided into two independent fractions, the adsorption and desorption free energy changes. The totally net free energy change and the two fractions of seven kinds of proteins in RPLC were accurately measured. The standard deviation between experimental values and theoretical expectation was less than ±2%. In addition, from the point of view of

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thermodynamics, with the comparisons of the adsorption and desorption free energy changes between small solutes and biopolymers in RPLC, the following experimental facts were first explained quantitatively: [Robbat, A., Jr.; Liu, T.Y. J. Chromatagr. **1990**, *513*, 117] The elution range of organic solvent for small solutes in the mobile phase in RPLC is usually broad, while that for biopolymers is very narrow. [Tan, L.C.; Carr, P.W. J. Chromatogr. **1993**, *656*, 521] Small solutes in RPLC can be eluted with isocratic elution, but biopolymers only with gradient elution. [Tchapla, A.; Heron, S.; Colin, H.; Guiochon, G. Anal. Chem. **1988**, *60*, 1443] The resolution of small solutes in RPLC depends on column length, while that of biopolymers is almost independent of it.

Key Words: Reversed-phase liquid chromatography; Chromatographic thermodynamic; Retention mechanism; Stoichiometric displacement theory; Fractions of free energy change.

INTRODUCTION

Although many studies were carried out on the thermodynamic behavior of small solutes in reversed-phase liquid chromatography (RPLC)[1–4] and hydrophobic interaction chromatography (HIC) with linear, or non-linear plot by van't Hoff plot,[5–7] none have been found for biopolymers. Due to many different characteristics between biopolymers and small solutes, both show varied chromatographic behaviors and different mechanisms in RPLC. Here are some examples. Firstly, organic solvent concentration in mobile phase can be changed in a wide range when small solutes are separated in RPLC, but only in a very narrow one for biopolymers. Secondly, small solutes in RPLC can usually be separated with isocratic elution, while biopolymers can only be separated with gradient elution. Thirdly, the resolution of small solutes in RPLC is dependent on a column length, on the contrary, that of biopolymers is almost independent of a column length. Therefore, studies on the chromatographic thermodynamics of biopolymers in RPLC should be helpful to explain those phenomena and to understand intensively the retention mechanisms of small solutes and biopolymers.

However, since the molecular conformation of proteins is changing during retention process due to the temperature variation in RPLC, the plot of $\log k'$ (k' is the capacity factor) of biopolymers usually appears nonlinear to $1/T$ (T is absolute temperature). The capacity factor (k') of proteins is so sensitive to temperature that it is difficult to obtain all the required k' values at a fixed concentration of organic solvent when the temperature changes in the range from 0°C to 50°C, which is necessary for most studies in chromatographic thermodynamics. As a result, the van't Hoff plot of biopolymers cannot even

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be obtained at all.[8] In addition, because the column phase ratio of biopolymers in RPLC is hard to be determined accurately, it is impossible to measure the Gibbs free energy changes (ΔG) and entropy changes (ΔS) of biopolymers in RPLC. Because of the three difficulties mentioned above, the investigation of the chromatographic thermodynamics of biopolymers becomes much more difficult.

According to the stoichiometric displacement theory for retention of solute (SDT-R) in liquid chromatography (LC), we have previously proposed a new definition of column phase ratio for solutes including small solute and biopolymers from the point of view of thermodynamics, that is the k' value of a solute when the partition coefficient of the solute in two phases equals one, or as the free energy change of the solute is zero.[9–11] Based on the new definition, the column phase ratio can be accurately determined. The authors reported that, ΔG, ΔH, and ΔS of small solutes can be also divided into two independent fractions, the net adsorption and desorption fractions.[11–13] A theoretical model and a new experimental methodology for determining those thermodynamic functions and their fractions were established, respectively.[11–13] Based on the linear parameters $\log I$ (the affinity of one mole of the solute to the stationary phase) and Z (the moles of solvent released from the interface between stationary phase and solute as one mole of the solute is absorbed) of the SDT-R having thermodynamic characters (the linear relationship between $\log I$ and Z vs. $1/T$),[8–14] in this paper, ΔG and its fractions, the adsorption and desorption free energy changes of biopolymers in RPLC have been determined accurately. Based on the experimental data obtained, the different chromatographic behaviors between small solutes and biopolymers in RPLC were explained quantitatively by thermodynamics.

Theory

The relationship among the solute capacity factor, k', distribution coefficient of solute between two phases, P_a, and column phase ratio, ϕ in RPLC can be shown in Eq. (1):

$$\log k' = \log P_a + \log \phi \tag{1}$$

Because solute retention in RPLC is a spontaneous process, it can be defined that the total free energy changes of solute, $\Delta G_{(P_a)}$, in its retention process is negative, and can be expressed as:

$$-\Delta G_{(P_a)} = 2.303RT \log P_a = 2.303RT(\log k' - \log \phi) \tag{2}$$

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where, R is the gas constant and T is the absolute temperature. In order to determine the $\Delta G_{(P_a)}$ of the solute in RPLC, as shown in Eq. (2), the ϕ must be determined accurately.

Two of the key equations of the SDT-R in RPLC are shown as:[15–17]

$$\log k' = \log I - Z\log a_{\mathrm{D}} \tag{3}$$

$$\log I = Zj + \log \phi \tag{4}$$

where

$$Z = n + q \tag{5}$$

$$\log I = \log K_a + n\log a_{\mathrm{LD}} + \log \phi \tag{6}$$

In these equations, a_{D} represents the activity of organic solvent or displacer in mobile phase employed. The Z denotes the moles of solvent released from the interface between stationary phase and solute as one mole of the solute is absorbed. The term $\log I$ stands for the affinity of one mole of the solute to the stationary phase. K_a is the general equilibrium constant of the stoichoimetric displacement process for solute displacing the solvent. The terms n and q are the moles of the displacer released separately from both sides of the solvated stationary phase and solvated solute at the contact surface region, respectively, when one mole of the solvated solute is adsorbed. Both a_{LD} and a_{D} separately represent the activities of the displacer in the adsorbed layer and mobile phase. The term j denotes the affinity of one mole of the displacer to the stationary phase and is a constant, independent to solutes. The column phase ratio, ϕ, is defined as above. With Eq. (4), $\log \phi$ for both of small solute and biopolymers can be determined accurately.[9–11]

In the SDT-R, Eq. (3) is a linear equation, indicating that the larger the $\log I$, the stronger the adsorption between the solute and the stationary phase is. On the contrary, the larger the a_{D}, the more easily the solute desorbs from the stationary phase in RPLC. So, $\log I$ and $Z\log a_{\mathrm{D}}$ in Eq. (3) can represent both the abilities of the adsorption and desorption of the solute, respectively. In addition, it has been proven that both $\log I$ and Z have the properties of thermodynamic equilibrium constants, indicating that the plots of both to $1/T$ to be linear, respectively.[8,11,14] Thus, they can be written as:[10]

$$\log I = \frac{A_{(I)}}{T} + C_{(I)} \tag{7}$$

$$Z = \frac{A_{(Z)}}{T} + C_{(Z)} \tag{8}$$

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where, $A_{(I)}$, $A_{(Z)}$, $C_{(I)}$, and $C_{(Z)}$ are all constants.

The relationship between $\log I$ and P_a has been reported as:[9,14]

$$\log I = \frac{j}{j - \log a_{\mathrm{D}}} \log P_a + \log \phi \tag{9}$$

Therefore, the adsorption free energy ($\Delta G_{(I)}$) and the desorption free energy ($\Delta G_{(\mathrm{Z,D})}$) can be obtained from $\log I$ and $Z \log a_{\mathrm{D}}$ and shown as Eqs. (10) and (11), respectively.[3]

$$-\Delta G_{(I)} = 2.303RT \log I \tag{10}$$

$$\Delta G_{(\mathrm{Z,D})} = 2.303RTZ \log a_{\mathrm{D}} \tag{11}$$

Because $\Delta G_{(I)}$ of solute is the change of total adsorption free energy, including the contribution of $\log \phi$, rather than the net adsorbed free energy change ($\Delta G_{(I,a)}$), it is necessary to eliminate the former and then Eq. (6) becomes Eq. (12).

$$\log I_a = \log K_a + n \log a_{\mathrm{LD}} \tag{12}$$

where

$$\log I_a = \log I - \log \phi \tag{13}$$

Because the difference between $\log I_a$ and $\log I$ is only the constant term $\log \phi$, the physical meaning of $\log I_a$ is the same as $\log I$. Since the free energy has a summarized character, $\Delta G_{(P_a)}$ in Eq. (2) can be expressed as the sum of the adsorption free energy change $\Delta G_{(I,a)}$ and desorption free energy change $\Delta G_{(\mathrm{Z,D})}$ with Eq. (14):

$$-\Delta G_{(P_a)} = -\Delta G_{(I,a)} + \Delta G_{(\mathrm{Z,D})} \tag{14}$$

where

$$-\Delta G_{(I,a)} = 2.303RT \log I_a \tag{15}$$

$\Delta G_{(I,a)}$ represents the net adsorption free energy change of one mole solute to the stationary phase. It is defined, that the $\Delta G_{(I,a)}$ at 20°C is the standard affinity potential of one mole solute to the stationary phase, and the $\Delta G_{(\mathrm{Z,D})}$ denotes the corresponding desorption free energy change of one mole of the solute, being a function of both Z and a_{D}. A conclusion is thus drawn that the total free energy change shown in Eq. (14), $\Delta G_{(P_a)}$ consists of two

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fractions, the net adsorbed free energy changes $\Delta G_{(I,a)}$ and the desorbed free energy changes, $\Delta G_{(Z,D)}$ can be easily determined.

However, it should be pointed out that $\Delta G_{(I,a)}$, shown in Eq. (15), is independent of the activity (a_D) of the displacing agent employed in the mobile phase, while $\Delta G_{(Z,D)}$ in Eq. (11) depends on it. Both of them for small solutes not only have different physical meanings, but also may be divided into their own adsorption and desorption fractions of enthalpy and entropy changes, the same as $\Delta G_{(I,a)}$ in Eq. (15) and $\Delta G_{(Z,D)}$ in Eq. (11), respectively. This will be investigated later.

EXPERIMENTAL

Reagents

Methanol (AG) was obtained from Xi'an Chemical Regent Company (Xi'an China). The proteins and trifluroacetic acid (TFA) were purchased from Sigma Co. (St. Louis, MO). The biological origin of proteins is as follows: insulin (Ins, bovine pancreas), myoglobin (Myo, horse heart), ferritin (Fer, horse heart), cytochrome-C (Cyt-C, horse heart), lysozyme (Lys, chicken egg white). carbonic anhydrase (CAB, bovine erythrocyte), and α-amylase (α-Amy, Bacillus anthracis, Type II). All of these biopolymers were bought from Sigma Co. (St. Louis, MO).

Equipment

Shimadzu LC-6A including two pumps, gradient elution system, and UV detector, 4×100 mm ID ODS reversed-phase column was used.

Method

The required composition of mobile phase was obtained from the solutions A (100% water and 0.1% TFA) and B (100% methanol and 0.1% TFA) by the solvent programmer of the liquid chromatography. The RPLC column was equilibrated with 40 mL of mobile phase after each changing methanol concentration. The flow-rate was 1.0 mL/min and the chart paper speed was 4 mm/min. All detections were done at wavelength 280 nm. The variation of the column temperature was controlled within $\pm 0.2^{\circ}$C (by a super stable water bath with a refrigeration). The protein concentration was prepared at 4.0 mg/mL. The injected sample size was 20 μg.

The column dead time for each protein was determined by $NaNO_2$.

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RESULTS AND DISCUSSION

Methanol–water solution was reported as an ideal solution,[18] indicating the activity of methanol. a_D in Eq. (3) in mobile phase can be replaced by concentration, [D]. Thus, Eq. (3) can be written as:

$$\log k' = \log I - Z\log[\mathrm{D}] \tag{16}$$

Log *I*, *Z*, *j*, and Log ϕ of Seven Proteins at Different Temperatures

According to Eq. (16), the plot of $\log k'$ vs. log[D] of seven proteins, as shown in Fig. 1, are all straight lines with slope Z and intercept $\log I$. The values of $\log I$ and Z of the seven standard proteins, insulin, myoglobin, ferritin, cytochrome-C, lyszoyme, carbonic anhydrase, and α-amylase, were determined at the temperature ranging from the interval of 0–50°C with 10°C interval and listed in Table 1. Because this plot for ferritin almost overlaps with that of myoglybin, that of ferritin was not put in Fig. 1 for a better view. From

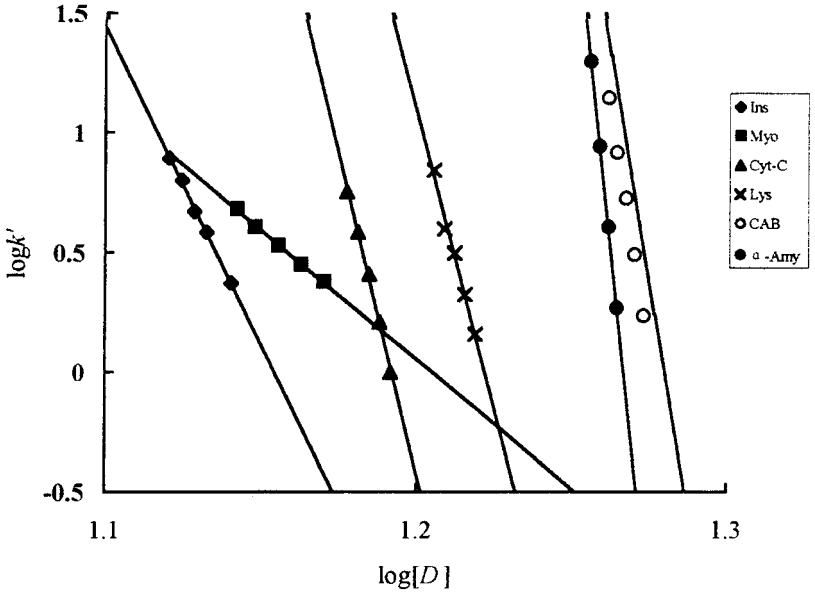

Figure 1. The plots of $\log k'$ of six kinds of proteins to log[D] at 30°C.

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Table 1. Log I and Z of seven proteins at different temperatures.[a]

Proteins	Linear parameters	$1000/T/K^{-1}$ 3.66	3.53	3.41	3.30	3.19	3.09
Insulin	log I	32.56	27.24	29.98	32.21	28.92	27.99
	Z	27.59	23.32	25.95	28.12	25.58	25.00
	R	0.9996	0.9997	0.9995	0.9990	0.9998	0.9967
Myoglobin	log I	18.05	15.00	12.37	12.87	10.59	10.80
	Z	14.74	12.36	10.25	10.81	8.93	9.29
	R	0.9993	0.9997	0.9997	0.9999	0.9995	0.9995
Ferritin	log I	16.70	15.64	12.07	12.71	10.63	10.85
	Z	13.61	12.91	9.98	10.67	9.00	9.34
	R	0.9988	0.9983	0.9995	0.9998	1.000	0.9986
Cytochrome-C	log I	77.32	69.81	64.68	59.65	55.59	51.51
	Z	63.28	57.93	54.26	50.90	48.03	45.43
	R	0.9961	0.9989	0.9979	0.9996	0.9998	0.9998
Lyszoyme	log I	74.03	66.54	63.28	58.33	54.03	48.91
	Z	59.40	53.93	51.80	48.49	45.66	41.97
	R	0.9987	0.9995	1.000	0.9999	0.9980	0.9995
Carbonic anhydrase	log I	113.86	107.69	98.57	92.04	80.19	71.65
	Z	88.42	83.82	77.22	72.68	64.06	57.94
	R	0.9955	0.9998	1.000	0.9990	0.9987	0.9996
α-Amylase	log I	184.98	164.49	147.85	134.67	119.60	102.79
	Z	144.13	128.91	116.70	107.65	96.73	84.29
	R	0.9998	0.9998	1.0000	0.9990	0.9987	0.9996

[a]Stationary phase: ODS; mobile phase: CH_3OH–H_2O—0.10% TFA.

Fig. 1 and Table 1, it is shown that the linear correlation coefficients R are all over 0.995. Such good linearity indicates that the biopolymers follow the SDT-R as the same as the small solutes.

According to Eq. (4), the linear plots of $\log I$ to Z of the seven proteins at the different temperatures are shown in Fig. 2. The slope, j, intercept, $\log \phi$, and linear correlation coefficient of 0.9997 indicate that all of the three terms can be determined accurately. The obtained average j and $\log \phi$ values are 1.27 ± 0.03 and -1.99 ± 0.16, respectively. In addition, it was reported in references[8,14] that the plots of $\log I$ and Z of the seven proteins to $1/T$ were linear, indicating that both $\log I$ and Z have the properties of thermodynamic equilibrium constants.

$\Delta G_{(P_{a,e})}$, $\Delta G_{(P_{a,c})}$, $\Delta G_{(I,a)}$, and $\Delta G_{(Z,D)}$ of Seven Proteins in RPLC

As pointed out in the theoretical section, the $\log \phi$ of biopolymers can be determined accurately, so too the total free energy change and their fractions of biopolymers ($\Delta G_{(P_a)}$, $\Delta G_{(I,a)}$, and $\Delta G_{(Z,D)}$) by the Eqs. (2), (15), and (11), respectively. The values of k', the obtained $\Delta G_{(P_a,e)}$ and $\Delta G_{(Z,D)}$ of the seven proteins under various methanol concentrations in the mobile phase employed at 20°C, were listed in Table 2 when $\log \phi$ was taken as -1.99.

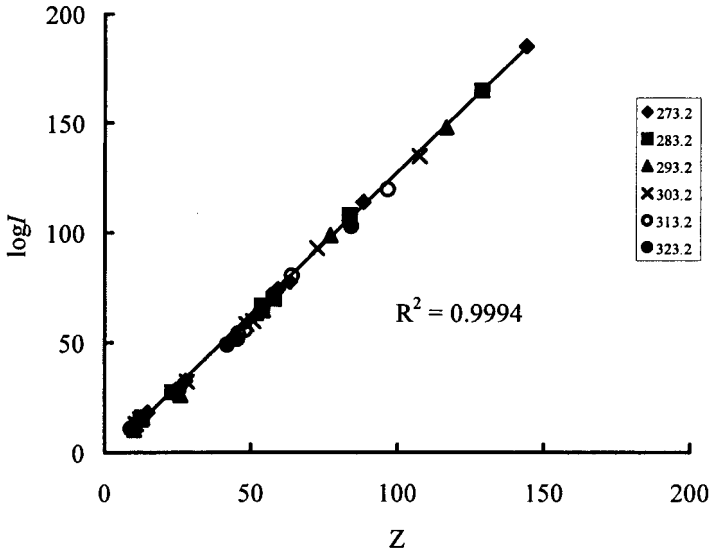

Figure 2. The plots $\log I$ vs. Z of seven proteins at different temperatures.

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Table 2. The values of k', $\Delta G_{(P_a)}$, $\Delta G_{(I,a)}$, and $\Delta G_{(Z,D)}$ of the seven proteins in different [D] at 20°C (kJ mol^{-1}).[a]

$\varphi_{\text{methanol}}$[b] (v/v %)	k'	$-\Delta G_{(P_a,e)}$	$-\Delta G_{(P_a,c)}$	$\Delta G_{(Z,\text{D})}$
		Insulin		
53.5	7.79	16.46	16.48	163.28
54	6.31	15.94	15.88	163.88
54.5	4.70	15.22	15.30	164.46
55	3.84	14.73	14.73	165.03
56	2.40	13.54	13.58	166.18
		Ferritin		
56	4.60	15.17	15.13	63.91
57	4.05	14.86	14.70	64.34
58	3.40	14.43	14.28	64.76
59	2.82	13.98	13.86	65.18
60	2.44	13.63	13.45	65.59
		Lysozyme		
65	6.94	16.18	16.17	350.53
65.5	3.96	14.81	15.21	351.49
66	3.14	14.24	14.25	352.45
66.5	2.11	13.27	13.29	353.41
67	1.44	12.35	12.33	354.37
		α-Amylase		
73	19.75	18.73	18.73	822.74
73.5	8.73	16.74	16.77	824.70
74	4.02	14.84	14.87	826.60
74.5	1.85	12.96	12.97	826.50
		Myoglobin		
56	4.80	15.28	15.26	65.64
57	4.03	14.85	14.82	66.08
58	3.38	14.42	14.39	66.51
59	2.81	13.97	13.96	66.94
60	2.38	13.57	13.54	67.36
		Cytochrome-C		
61	5.65	15.68	15.79	358.77
61.5	3.85	14.74	14.69	359.87
62	2.56	13.74	13.63	360.93
62.5	1.62	12.63	12.56	362.00
63	0.98	11.45	11.53	363.03

(*continued*)

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Table 2. Continued.

$\varphi_{\text{methanol}}$[b] (v/v %)	k'	$-\Delta G_{(P_a,e)}$	$-\Delta G_{(P_a,c)}$	$\Delta G_{(Z,\text{D})}$
		Carbonic anhydrase		
74	13.85	17.86	17.86	546.96
74.5	8.22	16.60	16.60	548.22
75	5.30	15.52	15.35	549.47
75.5	3.09	14.20	14.09	550.73
76	1.72	12.77	12.83	551.99

[a]$\Delta G_{(I,a)}$ of insulin, myoglobin, ferritin, cytochrome-C, lysozyme, carbonic anhydrase, and α-amylase were calculated as −198.84, −80.90, −79.04, −374.56, −366.70, −564.82, and −841.47 kJ mol^{-1}, respectively. $\log \phi$ was taken as -1.99 ± 0.16. $\Delta G_{(P_a,c)}$ denotes the calculated free energy change.
[b]φ is the volume fraction (v/v, %) of displacer in mobile phase.

Independent of the methanol concentration, the $\Delta G_{(I,a)}$ values of the seven proteins were calculated with Eq. (15) as −198.84, −80.90, −79.04, −374.56, −366.70, −564.82, and −841.47 kJ mol^{-1}, respectively.

In addition, the calculated total free energy changes $\Delta G_{(P_a,c)}$ of the seven proteins are also shown in Table 2, which should be equal to the sum of $\Delta G_{(I,a)}$ and $\Delta G_{(Z,\text{D})}$ at the corresponding methanol concentration. Comparing the experimental and the calculated free energy changes, i.e., $\Delta G_{(P_a,e)}$ and $\Delta G_{(P_a,c)}$, the standard deviation was found to be less than ±2%.

Comparison of $\Delta G_{(I,a)}$ Between Small Solutes and Biopolymers

According to the SDT-R, the linear parameter $\log I$ represents the affinity of one mole solute to the stationary phase. From Eq. (15), $\Delta G_{(I,a)}$ of the solute is original from $\log I$. In addition, it has been proven that $\log I$ depends on the distribution coefficient of solute between two phases (P_a).[14] In other words, the larger $\Delta G_{(I,a)}$ of the solute, the greater the P_a will be. The values of P_a shown in Table 3 are calculated by Eq. (9) with $\Delta G_{(I,a)}$ of four non-polar small solutes, biphenyl, 4-phenyltoluene, bibenzyl, and 3,3′-dimethylphenyl, as well as four proteins, insulin, lysozyme, carbonic anhydrase, and α-amylase in the same methanol concentration of 55%, 65%, and 75% at 20°C in RPLC. In the calculation, the values of j and $\log \phi$ for small solutes were taken as 1.40 and −1.02,[11] while for biopolymers, as mentioned above, were 1.27 and −1.99,[4] respectively.

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Table 3. Comparisons of $\log I$, $\Delta G_{(I,a)}$, and P_a between the small solutes and proteins with different [D] at 20°C.[a]

Analyte	$\log I$	$\Delta G_{(I,a)}$ (kJ mol^{-1})	P_a^2		
			$\varphi_{\text{methanol}}$[b](v/v) 55%	$\varphi_{\text{methanol}}$(v/v) 65%	$\varphi_{\text{methanol}}$(v/v) 75%
Solutes					
Biphenyl	7.29	−43.4	37.14	13.74	5.87
4-Phenyltoluene	8.49	−50.1	64.12	20.57	7.78
Bibenzyl	9.35	−53.1	83.04	23.86	8.21
3,3′-Dimethylphenyl	9.67	−56.2	108.5	30.25	10.15
Proteins					
Insulin	29.98	−198.8	42.06	5.49	0.19
Lysozyme	63.28	−366.7	4.37×10^6	759.1	0.92
Carbonic anhydrase	98.57	−564.8	1.37×10^{13}	3.38×10^7	541.7
α-Amylase	147.90	−841.5	4.92×10^{17}	1.66×10^9	446.3

[a]The values of j and $\log \phi$ were taken as 1.40 and −1.02 for small solutes, 1.27 and −1.99 for proteins, respectively. The values of P_a were calculated with Eq. (7).
[b]φ is the volume fraction (v/v, %) of displacer in mobile phase.

Comparing the $\Delta G_{(I,a)}$ of small solutes with that of biopolyers shown in Table 2, it can be seen that $\Delta G_{(I,a)}$ of small solutes varies in the range from 30 to 50 kJ mol^{-1}, while that of proteins changes from 300 to 800 kJ mol^{-1}. The latter is more than 10 fold of the former. As shown in Table 3, the P_a values of small solutes are in the order of tens, but those of biopolymers at the same experimental conditions are in the order of 10^3 to 10^8. With the methanol concentration of 65% in the mobile phase in RPLC at 20°C, the four small solutes and lysozyme can be retained in RPLC. However, under the same condition, the P_a value of lysozyme is 100 times greater than that of the small solutes.

Additionally, the values of P_a of the four small solutes shown in Table 3 vary from 10 to 30 and increase with the concentration of methanol. Because their P_a values are very close to each other, they, like the immiscible liquid–liquid extraction, can be separated with isocratic elution in RPLC. In the circumstance of small differences of P_a among small solutes, the usual extraction method must be carried out many times to separate them completely, and the less of this difference of the P_a among solutes, the more times the extraction are required. In order to make small solutes having similar P_a separate completely by RPLC, a long enough column must be employed.

On the contrary, as shown in Table 3, the values of P_a of biopolymers are between 10 and 10^9. If the separation of the four proteins is accomplished with the liquid–liquid extraction mentioned above, then these proteins can be separated completely from each other. With 65% methanol in the mobile phase at 20°C, insulin cannot be retained, while lysozyme can be retained and eluted, but neither the carbonic anhydrase and α-amylase can. The P_a of lysozyme is 100 times of that of insulin, but only 10^{-2} and 10^{-5} that of carbonic anhydrase and α-amylase, respectively. Thus, under the above conditions, carbonic anhydrase and α-amylase can be adsorbed tightly on the stationary phase, while insulin cannot be retained and go through the column directly with the same concentration of the mobile phase. A conclusion is, thus, drawn that the difference of P_a between biopolymers is so large that it is impossible to separate them by isocratic elution condition, but requires the gradient elution.

In order to separate the four proteins shown in Table 3, when the methanol concentration in the mobile phase increases with the linear gradient and approaches a certain concentration range, insulin can be firstly eluted because of the smallest P_a. However, the others are still adsorbed tightly on the stationary phase. They can be referred to as not moving, or moving very slowly. With the delay of the linear gradient, when the methanol concentration increases to another certain value, lysozyme can be eluted, but not so for carbonic anhydrase and α-amylase. In the same way, with the delay of the linear gradient elution, both carbonic anhydrase and α-amylase can be eluted

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Table 4. The values of k', $\Delta G_{(P_a)}$, $\Delta G_{(I,a)}$, and $\Delta G_{(Z,\mathrm{D})}$ of four non-polar solutes with different [D] at 30°C (kJ mol^{-1}).[a]

$\varphi_{\mathrm{methanol}}$[b] (v/v %)	k'	$-\Delta G_{(P_a,e)}$	$-\Delta G_{(P_a,c)}$	$\Delta G_{(Z,\mathrm{D})}$
		Biphenyl		
68	0.82	5.40	5.40	39.5
65	1.00	5.92	6.10	38.8
62	1.32	6.62	6.71	38.2
59	1.79	7.39	7.40	37.5
56	2.52	8.24	8.21	36.7
53	3.47	9.05	8.91	36.0
50	4.88	9.91	9.71	35.2
$-\Delta G_{(I,a)}$	44.9			
		Bibenzyl		
68	1.21	6.39	6.60	48.3
65	1.74	7.31	7.30	47.6
62	2.53	8.26	8.10	46.8
59	3.65	9.18	9.00	45.9
56	5.28	10.1	9.90	45.0
53	7.78	11.1	10.8	44.1
50	11.4	12.1	11.8	43.1
$\Delta G_{(I,a)}$	54.9			
		4-Phenyltoluene		
68	1.06	6.06	6.20	45.7
65	1.47	6.89	6.90	45.0
62	2.09	7.77	7.71	44.2
59	2.94	8.04	8.51	43.4
56	4.15	9.50	9.30	42.6
53	6.04	10.5	10.2	41.7
50	8.71	11.4	11.2	40.7
$\Delta G_{(I,a)}$	51.9			
		3,3′-Dimethylbiphenyl		
68	1.59	7.08	7.10	51.0
65	2.22	7.93	7.91	50.2
62	3.18	8.83	8.70	49.4
59	4.65	9.80	9.61	48.5
56	5.79	10.8	10.6	47.5
53	10.1	11.8	11.6	46.5
50	15.1	12.8	12.6	45.5
$\Delta G_{(I,a)}$	58.1			

[a]The table was obtained from Table 2 in Ref.[11].
[b]φ is the volume fraction (v/v, %) of displacer in mobile phase.

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one by one. Therefore, the separation efficiency of biopolymers with RPLC is basically independent with the column length.

Comparison of $\Delta G_{(Z,D)}$ Between Small Solutes and Biopolymers

Comparing $\Delta G_{(Z,D)}$ with small solutes as shown in Table 4 and biopolymers in Table 2, it can be seen that the magnitudes of their $\Delta G_{(Z,D)}$ have a comparable change range as do their $\Delta G_{(I,a)}$. If the methanol concentration in the mobile phase employed increases 1%, the $\Delta G_{(Z,D)}$ of small solutes averagely increases 0.2 kJ mol^{-1}, but $\Delta G_{(Z,D)}$ of biopolymers increases about 2.0 kJ mol^{-1}, which is 10 fold that of small solutes. Therefore, with a comparable change in $\Delta G_{(Z,D)}$, the concentration range of displacer for small solutes in the mobile phase changes more broadly, while that for biopolymers only changes very narrowly. It is also shown that small solutes in RPLC can be eluted with isocratic mode, but biopolymers only with gradient mode. This can also explain the "on–off" mechanism reported by M. B. Tennkniko et al.[19] and "all or nothing principle" suggested by B. G. Belenkii et al.[20] With either of the "mechanism" or the "principle," these authors tried to explain the reason why the biopolymer can be separated to obtain good resolution with a very short column.

CONCLUSIONS

Based on SDT-R of solute, the free energy change, $\Delta G_{(P_a)}$, of biopolymers in RPLC can be divided into two independent fractions, net adsorbed free energy change, $\Delta G_{(I,a)}$, and net desorbed free energy change, $\Delta G_{(Z,D)}$, respectively.

The $\Delta G_{(P_a)}$, $\Delta G_{(I,a)}$, and $\Delta G_{(Z,D)}$ of biopolymers in RPLC could be determined accurately. Comparing the experimental with the calculated values of the $G_{(P_a)}$, the standard deviation was found to be less than $\pm 2\%$.

From the point of view of thermodynamics, with the comparisons between the $\Delta G_{(I,a)}$ and $\Delta G_{(Z,D)}$ of small solutes and biopolymers in RPLC, the following experimental facts are first explained in a quantitative manner. (1) The elution range of organic solvent for small solutes in the mobile phase in RPLC is usually broad, while that for biopolymers is very narrow; (2) Small solutes in RPLC can be eluted with isocratic elution, but biopolymers only with gradient elution; (3) The resolution of small solutes in RPLC is dependent on column length, while that of biopolymers is almost independent of it.

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