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Article

Isotherm Type Shift of Hydrophobic Interaction Adsorption and its Effect on Chromatographic Behavior

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Adsorption isotherm type is a key factor in simulating chromatographic profiles. In this study, the adsorption isotherm type of pure bovine serum albumin (BSA) and immunoglobulin G (IgG) on hydrophobic interaction chromatography (HIC) media was found to shift from Freundlich to Langmuir with the increase of salt concentration. For BSA on butyl-, phenyl- and octyl-sepharose, the isotherm type shift occurred when the salt concentrations were higher than 1.8, 1.5 and 1.4 mol/L, respectively. For IgG, the turning points were 1.0 mol/L on phenyl-sepharose and 0.9 mol/L on octyl-sepharose. Circular dichroism spectra and high-performance size-exclusion chromatography found no obvious conformational change or oligomer formation for the proteins in the solutions with different salt concentrations. HIC profiles of BSA and IgG revealed that the isotherm type shift greatly affected the chromatographic behavior, because the original single peak was coincidentally split into two peaks at the salt concentrations over which the isotherm type shift occurred. Combining both the isotherm type shift and peak-splitting phenomena, it was possible that the change of protein–protein repulsion among adsorbed protein molecules under different salt concentrations caused the abnormal behavior of adsorption isotherm and chromatographic profiles.

Introduction

Hydrophobic interaction chromatography (HIC) has been extensively used in laboratories and industries for the separation of proteins (1). Its capacity, although lower than ion exchange, is much higher than affinity or gel filtration chromatography. The interaction between the hydrophobic patches of proteins and the surface ligands of HIC media provides vast selectivity to distinguish one protein from the others. Many proteins have been purified with HIC. However, HIC must be carefully designed and controlled to achieve satisfactory purification and recovery.

Several types of isotherm have been reported for HIC, among which Langmuir and Freundlich (2, 3) are most typical. The Langmuir model assumes that all adsorption sites are monolayer adsorption, have homogeneous energy, and have no interactions between the adsorbed molecules. Based on these assumptions, the Langmuir isotherm can be written as:

$$q^* = \frac{q_m C^*}{K_d + C^*} \quad (1)$$

where q^* is the equilibrium adsorption of protein; C^* is the equilibrium concentration of protein; q_m is the ultimate adsorption of proteins and K_d is the dissociation constant. Freundlich is given as:

$$q^* = K(C^*)^n \quad (2)$$

where K is the adsorption constant and n is the adsorption exponent. Freundlich has long been regarded as an empirical formula, for which the physical meanings of parameters n and K have not been clearly illuminated. A widely accepted opinion considers that the only difference between the Freundlich and Langmuir models is that the adsorption sites have heterogeneous energy in the Freundlich model. Based on this assumption, the derived isotherm can be written as (4):

$$\frac{q^*}{q_m} = \frac{A}{\alpha}\frac{k_a}{k_d}\left(\frac{C^*}{C_s}\right)^{\alpha RT} \quad (3)$$

where q_m is the maximum adsorption capacity; A and α are the coefficients of distribution function of adsorption energy; k_a and k_d are adsorption and desorption rate constant, respectively, and C_s is the solubility of the adsorbate in liquid. By comparing Eq. (2) with Eq. (3), the following relationships for Freundlich coefficients are obtained:

$$n = \alpha RT \quad (4)$$

$$K = q_m \frac{A}{\alpha}\frac{k_a}{k_d}\left(\frac{1}{C_s}\right)^n \quad (5)$$

Eqs. (4) and (5) indicate that K and n relate to some properties of adsorbate and adsorbent.

Although the adsorption state of proteins on HIC media has been profoundly researched, it is difficult to explain why different proteins have different isotherm types, even on the same HIC medium. For multi-point adsorption like protein HIC adsorption, Freundlich and Langmuir adsorptions were thought to be exchangeable under certain conditions. Kan *et al.* (5) studied the relationship between the irregularity of surface structure in adsorbents and the isotherm type, and concluded that if the binding sites are regularly arranged and the molecules can be adsorbed through the same number of binding sites, the adsorption is Langmuir type. On the contrary, if the binding sites are randomly distributed and the molecules are adsorbed through different numbers of binding sites, the adsorption is Freundlich type. Jennissen *et al.* (2) attributed the Freundlich isotherm to the sequential adsorption of protein molecules, in which the initially adsorbed molecules interact with the maximum ligand groups, and the later adsorbed molecules interact with fewer

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ligand groups, because some ligand groups are occupied by the previously adsorbed molecules. The sequential adsorption may be modified by the competition of the protein molecules for the ligand groups. Inevitably, the difference in the number of interacting ligand groups results in a difference in adsorption energy for the adsorbed molecules, thus causing the Freundlich isotherm. Jennissen further explained that the Langmuir isotherm, on the other hand, is caused by the interaction between protein and media with single, independent and identical binding sites. We believe that the reason lies in the differences in the distribution of hydrophobic patches on the protein surface, the conformation of protein, or the protein–protein interaction among absorbed molecules. For a single protein, its distribution of hydrophobic patches, conformation and intermolecular interaction are often influenced by salt concentration. If the isotherm type of a single protein shifts from one type to another in a salt concentration range, it may be caused by a change of distribution of the hydrophobic patches on the protein surface, the conformation of the protein, or the intermolecular interaction.

In this research, the adsorption of three proteins [bovine serum albumin (BSA), immunoglobulin G (IgG) and lysozyme] on butyl-, phenyl- and octyl-sepharose was studied in a range of salt concentrations wider than that described in the literature (usually low than 1 mol/L) to investigate whether there was an isotherm type change for a single protein. Circular dichroism (CD) and high-performance size-exclusion chromatography (HP-SEC) were employed to characterize the conformational change and protein–protein association in solutions. HIC was also performed to study the effect of isotherm type on the chromatographic behavior.

Experimental

Materials

All chemical reagents were of analytical grade. The salt used in this study was $(NH_4)_2SO_4$, supplied by Beijing Chemistry (Beijing, China). BSA and lysozyme were purchased from Amresco (Solon, OH) and Sigma (St. Louis, MO), respectively. IgG was provided by Hualan, Inc. (China). Various HIC adsorbents, including butyl-sepharose fast flow, phenyl-sepharose fast flow and octyl-sephrose fast flow were purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden).

Adsorption isotherms

Adsorption isotherms were measured in given $(NH_4)_2SO_4$ concentrations at 25°C using a batch method. Suction-dried HIC medium, 0.3 g (butyl-, octyl- or bentyl-sepharose fast flow, pretreated with corresponding buffer) was weighed into a 50-mL conical flask, and then a 10-mL protein solution of a given salt concentration was added. The conical flasks were then placed in a shaker maintained at room temperature and agitated for 3 h. After equilibration, the suspension was filtered. The supernatant was measured at 280 nm with an ultraviolet (UV) spectrophotometer (Ultrospec 2100 pro, GE Healthcare Bio-Sciences) to obtain the equilibrium solution concentration. The amount of protein adsorbed was calibrated with the amount eluted from the gel.

The Langmuir and Freundlich isotherm models were employed to fit the data in this study. The values of q_m, K_d, n and K were obtained by fitting the isotherms with nonlinear least-squares fitting (NLSF) of the software Origin. The value of R2 is the criterion for the model-based fitting results, indicating which isotherm model is obeyed by the data from adsorption experiments at selected salt concentration.

CD spectra assay for conformational change

The conformational change of proteins under the given condition was characterized with CD. The CD spectra were measured with a Jasco J-810 recording spectropolarimeter at a protein concentration of 200 μg/mL in sodium phosphate with various $(NH_4)_2SO_4$. The measurement was conducted at room temperature using a 0.1-cm cylindrical quartz cell.

HP-SEC characterization for protein association

The association of proteins under the given conditions was determined by HP-SEC. The analysis was performed on an AKTÄ Purifier 100 working station (GE Healthcare Bio-Sciences), with an isocratic mobile phase of 0.05 mol/L sodium phosphate, pH 7.0, plus 0.1 mol/L Na_2SO_4, on a SuperdexTM 75 column (GE Healthcare Bio-Sciences). The flow rate was 1 mL/min. One hundred microliters of sample were loaded for each run.

HIC of model proteins under various $(NH_4)2SO_4$ concentrations

An HIC column (100 × 26 mm i.d.) was packed with butyl- or octyl- sepharose fast flow. The column was initially equilibrated in 20 mmol/L phosphate, pH 7.0, containing various concentrations of $(NH_4)_2SO_4$, and eluted with 20 mmol/L phosphate, pH 7.0. All chromatography tests were performed on an AKTÄ Purifier 100 working station (GE Healthcare Bio-Sciences). Fifty milligrams of protein were loaded for each run. The flow rate for all steps was 1 mL/min. The elution profile was monitored at 280 nm. The whole operation was conducted at room temperature.

Results and Discussion

Isotherms of the three proteins and the isotherm type shift

The static adsorption isotherms of the three proteins and the related parameters are shown in Figures 1 and 2 and the Supplementary Table I.

For BSA adsorbed on butyl-sepharose (Figure 1A), the adsorption data fits to the Freundlich isotherm but not to the Langmuir at the salt concentration range of 1.4 ~ 1.8 mol/L. However, when the salt concentration was increased to 1.9 ~ 2.1 mol/L, the adsorption data fits to the Langmuir isotherm. This means that the isotherm type shift occurred with the increase of salt concentration. Adsorption of BSA on the other two media, phenyl-sepharose (Figure 1B) and octyl-sepharose (Figure 1C), were similar to that of butyl-sepharose, and the isotherm type shifted when the salt concentration increased to 1.6 mol/L for phenyl-sephrose, and to 1.5 mol/L for octyl-sepharose.

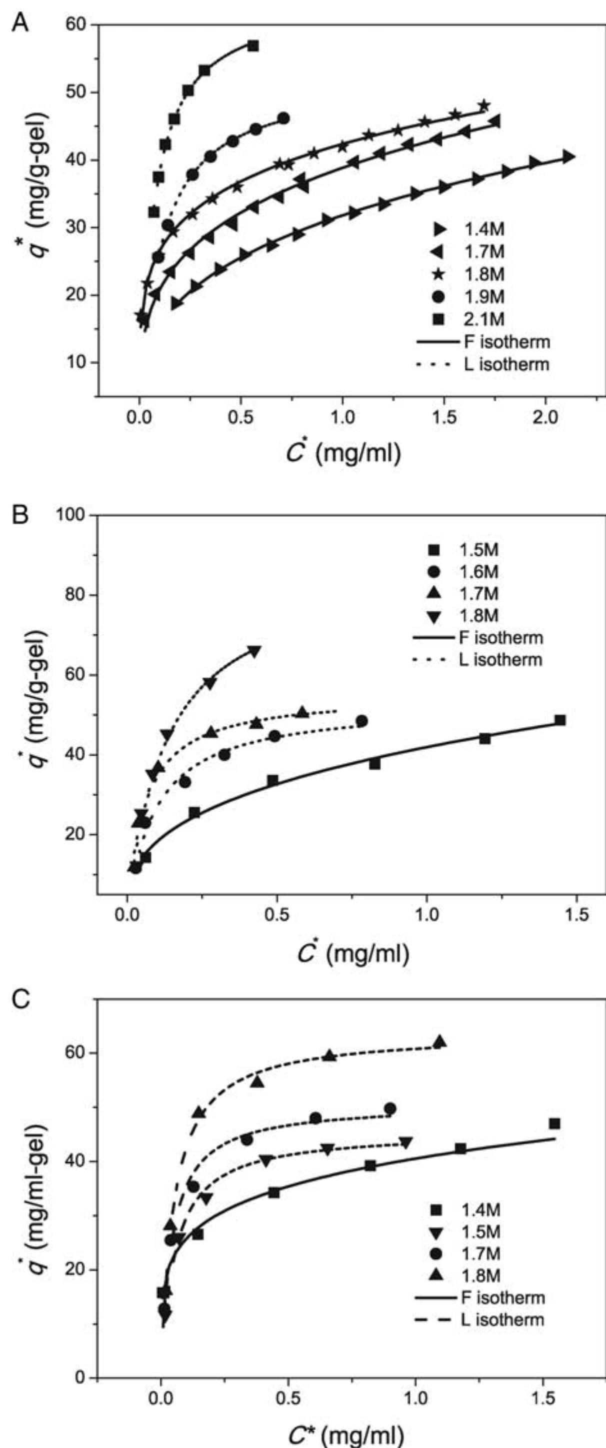

Figure 1. Comparison between isotherm models and experimental data for BSA absorbed on: butyl-sepharose (A); phenyl-sepharose (B); octyl-sepharose (C).

In the case of IgG, the same isotherm type shift was also observed at high salt concentration. The turning point of salt concentration was 1.1 mol/L for phenyl-sepharose (Figure 2A) and 1.0 mol/L for octyl-sepharorse (Figure 2B).

Unlike BSA and IgG, no obvious change of isotherm type was observed for lysozyme. The results summarized in

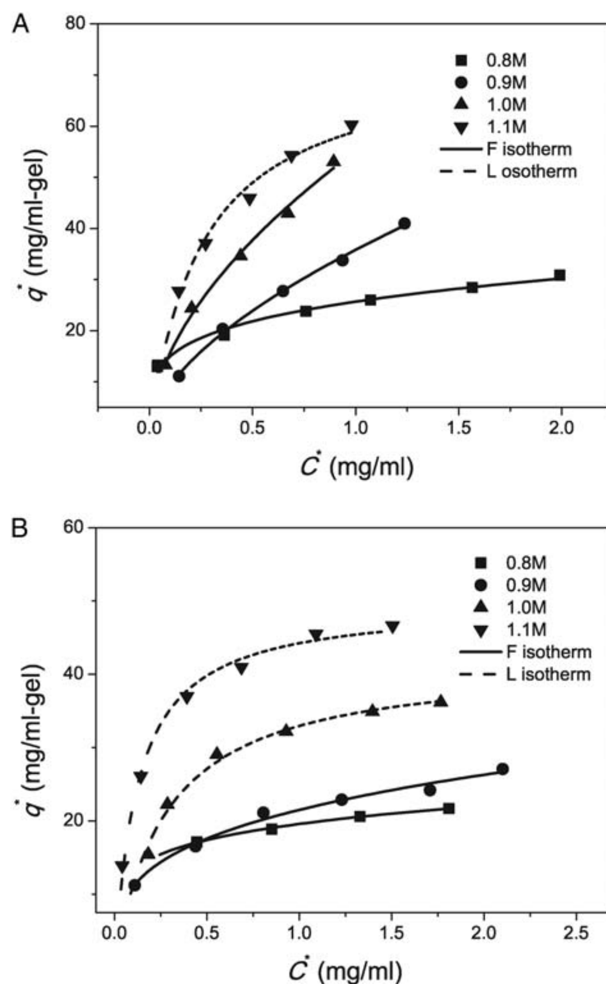

Figure 2. Comparison between isotherm models and experimental data for IgG absorbed on: phenyl-sepharose (A); octyl-sepharose (B).

Supplementary Table I indicate that the experimental data fit both the Langmuir and Freundlich isotherms.

CD spectra and HP-SEC profiles

The CD spectra of the protein at three salt concentrations (zero, low and high across the turning point found in the isotherm experiments) are shown in Figure 3. The similarity of the three spectra indicates that the increase of salt concentration did not change the secondary structure of BSA (Figure 3A). Similar results were observed for IgG (Figure 3B) and lysozyme (Figure 3C).

Table I

Parameters Used to Calculate Coverage

Protein	M_w (g/mol)	R_η^* (nm)	$R_h^\dagger$ (nm)	$A_{ac}^\ddagger$ (m^2/g-gel)
Lysozyme	14,300	1.85	1.95	42.2
BSA	68,000	3.62	3.80	36.4
IgG	150,000	5.23	5.5	32.0

*Viscosity radius of protein.
†Hydrodynamic radius, used as the real radius of protein molecules to calculate coverage.
‡Accessible area, determined according to R_η.

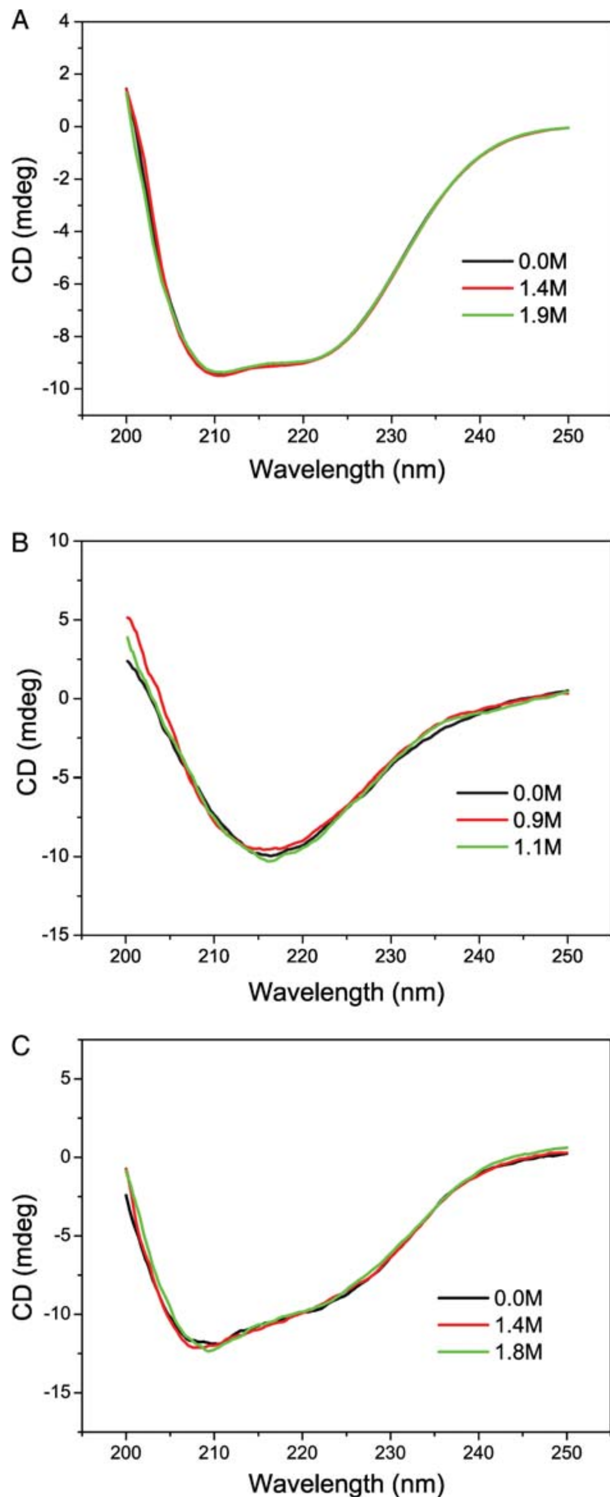

Figure 3. CD profiles: BSA (A); IgG (B); lysozyme (C).

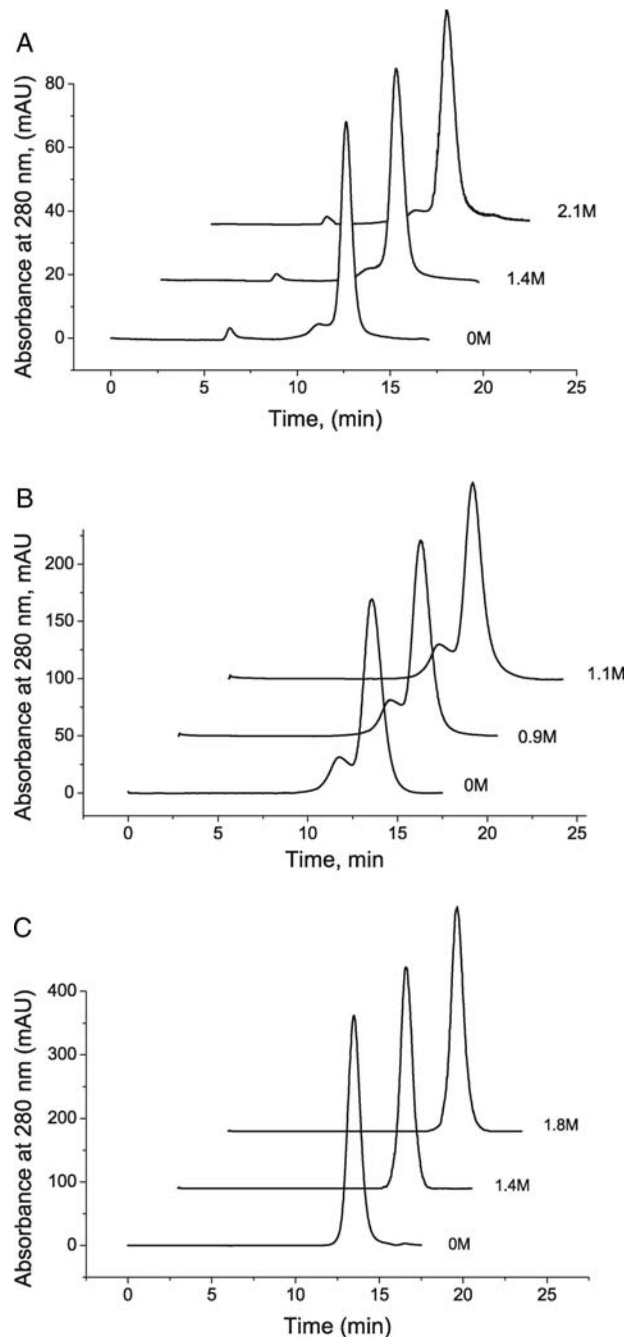

Figure 4. SEC profiles: BSA (A); IgG (B); lysozyme (C).

Figure 4 shows the HP-SEC profiles obtained by using a Superdex 75 column for BSA, IgG and lysozyme solution at the salt concentrations corresponding to the preceding CD spectra determination. The identity of the three HP-SEC profiles demonstrates that the increase of salt concentration did not cause association of BSA (Figure 4A), IgG (Figure 4B and lysozyme (Figure 4C).

Therefore, no obvious conformational change or association was induced by the increase of salt concentration in the protein solutions.

Proposed reasons causing such isotherm type shift

Conformational change

Although CD found no conformational change in both high- and low-salt-concentration solutions, the conformational change induced by strong hydrophobic interaction upon adsorption

cannot be excluded as a reason causing such an isotherm type shift. Many *in situ* experiments have observed an adsorption-induced conformational change of protein in HIC, which is difficult to monitor by eluting the protein because a fast refolding process occurs after elution (6). Judging from the isotherm type shift, it is possible that a partial conformational change occurred at low salt concentration, and an entire conformational change occurred at high salt concentration, because the Langmuir isotherm adsorption has homogeneous energy, and any conformational diversity causes heterogeneous adsorption energy, which results in the Freundlich isotherm.

Change of distribution of hydrophobic patches

The second possible reason causing such an isotherm type shift is the distribution change of hydrophobic patches of protein surfaces, which might result from high-salt-concentration solutions.

It was widely accepted that HIC adsorption is multi-point, in which one protein molecule interacts with $4 \sim 8$ ligand groups (7). The studies of Jennissen (2) and Kan (5) indicated that the isotherm type for adsorption of proteins on HIC media depends on whether or not the number of ligand groups through which the protein molecules are absorbed is variable. Adsorption of each protein molecule through a variable number of ligand groups results in the Fruendlich isotherm, otherwise, the adsorption is Langmuir type.

It is well known that $48 \sim 70\%$ of the solvent-accessible surface of protein consists of hydrophobic patches. Large hydrophobic patches are often dissected by the hydrogen-bonded water molecules that bridge polar sites (8). However, these water molecules are dynamically similar to bulk water molecules and usually have no localized sites (9). Salt, which is hydrated in water, has the ability to remove these bound water molecules. In low-salt-concentration solutions, the hydration of ions is fulfilled by the bulk water. When the salt concentration is increased, the hydration requires an increasing amount of water molecules, which will deprive the hydrophobic patches of the weakly bound water molecules and reduce the solubility of proteins, sometimes even causing salting-out. Therefore, the small hydrophobic patches that are connected by the weakly-bound water will become big patches. It is possible for IgG and BSA to experience the change of distribution of hydrophobic patches when the salt concentration is increased.

According to the preferential interaction theory, the protein is preferentially hydrated in solutions, and the hydrophobic patches are covered by the ordered water molecules (10). The introduction of salting-out salt into protein solution results in the positive charge of the chemical potential of the protein. When the salt concentration is increased to a critical value, the chemical potential of some hydrophobic patches is high enough to interact weakly with ligand groups of HIC media. It is possible that these patches have the same or similar chemical potential, because they are in exactly the critical chemical potential state to interact with ligand groups. If the number of such hydrophobic patches is larger than one, the adsorption will follow the sequential adsorption hypothesis of Jennissen, and the isotherm will be the Freundlich type. When the salt concentration is further increased, with the loss of weakly bound water molecules, larger hydrophobic patches are formed and become dominant in adsorption, which interact tightly with HIC media and the small patches cannot compete with them for the ligand groups. Hence, the protein will be adsorbed by a fixed number of ligand groups, and the isotherm will be Langmuir.

In this article, molecular weights of IgG (M_w 150,000) and BSA (M_w 68,000) are far bigger than that of lysozyme (M_w 14,300), and the hydrophobic patch distributions of IgG and BSA are much more complicated than those of lysozyme. Accordingly, it is reasonable that the isotherm type of IgG and BSA changed with the increase of salt concentration, whereas lysozyme did not.

Change of intermolecular interaction

The third factor causing the isotherm type shift is believed to be the change of protein–protein interaction with the increase of salt concentration. Upon adsorption, the proteins are concentrated on media surface, and protein–protein interaction such as hydrophobic interaction, electrostatic repulsion or hydrogen bond interaction becomes dominant, which might result in the shift of isotherm type (11). In HIC, protein molecules are absorbed without overlapping (12), thus the maximum surface coverage (θ_m) of HIC in both low and high salt concentrations was calculated to qualify the protein–protein interaction.

Two theories of packing are related to the adsorption of protein molecules: random sequential adsorption (RSA) and hexagonal-close-packing (HCP). RSA models protein molecules and media as hyperspheres and two-dimensional (2D) surfaces, respectively. The basic model of RSA is: the molecules are placed at random in a 2D surface in a sequence order; if the last-placed molecule overlaps any other molecules, it is removed at once; once the molecule has been placed, its position is permanently fixed; when no more molecules can be placed, the jamming limit has been reached and the process stops. Simulations performed using this statistical method have shown that the surface coverage (θ) limit of RSA is 0.547 (13, 14). As for HCP, in which protein molecules are densely arranged to contact with each other (one molecule contact with six molecules), coverage limit has been determined to be 0.91 (15).

Although the position of protein molecules on the medium surface is not fixed, the early stage of protein adsorption can be described by the RSA model (14). During the following stage, the protein molecules diffuse on the surface of medium, and more protein molecules are adsorbed to the medium, until the equilibrium is reached. Therefore, the θ_m value is larger than 0.547 if there is no repulsion, or if there is interaction-enhancing adsorption among adsorbed molecules.

To calculate the coverage, protein molecules were treated as spheres. Accordingly, the surface area occupied by one molecule can be written as:

$$A = \pi \cdot R_{sp}^2$$

where A is the surface area occupied and R_{sp} is the radius of the sphere. Here, we use the hydrodynamic radius (R_h) as R_{sp},

and A can be calculated form the following formula:

$$A = \pi \cdot R_h^2$$

Protein molecules were absorbed without overlapping, thus the area occupied per milliliter of absorbent according to adsorption can be written as:

$$A_{occ} = \frac{q \cdot N_A \cdot \pi R_h^2}{1000 M_w}$$

where A_{occ} is the area occupied (m^2 mL/gel); N_A is Avogadro's number; M_w is the protein molecular weight (g/mol). The surface coverage can be calculated as:

$$\theta = \frac{A_{occ}}{A_{ac}}$$

where A_{ac} is the accessible surface area of the absorbent (m^2 mL/gel), which depends on the type of absorbent and the size of protein molecules. The values of A_{ac} has been determined for the media used in this article, which are listed in Table I. All other required parameters are taken from the studies of To, Briscaronscaronov, Jøssang, Kim, Su and Bai (16–21).

This study focuses more more on the maximum coverage (θ_m) corresponding to q_m to determine the type of arrangement of protein molecules. Supplementary Table I shows the calculated θ_m values. It is obvious that for BSA and IgG, all the θ_m values of Freundlich isotherms are lower than or close to 0.547; on the other hand, the θ_m values of Langmuir isotherms are always higher than 0.547. Any θ_m values of Freundlich isotherms that are lower than 0.547 indicates the existence of protein–protein repulsion in the Freundlich adsorption. With the increase of salt concentration, the θ_m values became larger, and the distance between adsorbed molecules became nearer, which means that the repulsion was weakened.

The protein–protein repulsion might be electrostatic repulsion. In our experiments, all pH values were 7.0, other than pI values of BSA (pI 4.9) and IgG (pI 6.5), and the proteins were negatively charged. Electrostatic repulsion among free protein molecules may be very weak in solutions because they are too far away from each other. However, when the distance is very close, the interactions among them become remarkable. In low salt concentrations, electrostatic repulsion exists between adsorbed protein molecules, which was proven by Tsai *et al.* with isotherm titration microcalorimetrics of the HIC adsorption (22). When free protein molecules closed up to the hydrophobic surface, the electrostatic repulsion between free protein molecules and adsorbed protein molecules prevented the free protein molecules from being adsorbed by hydrophobic media. Additionally, the repulsion made the adsorption energy heterogeneous for each protein molecule, resulting in the Freundlich isotherm. In high salt concentrations, the electrostatic repulsion between protein molecules was weakened, and the hydrophobic interaction between protein and HIC media was enhanced, which neglected the electrostatic repulsion be compared with the hydrophobic interaction, and thus the adsorption isotherm become Langmuir.

For lysozyme, whether isotherms are considered to be Freundlich or Langmuir, the q_m value is the same. As shown in Supplementary Table I, the θ_m values of lysozyme are higher than 0.547, in some cases close to 0.91, indicating HCP, which means that the repulsion among adsorbed lysozyme molecules was weak, irrespective of the high pI value. This may related to the shape or the polar group distribution of lysozyme.

Effect of isotherm type shift on HIC behaviors

Regarding chromatographic behavior, dynamic rather than static adsorption isotherm should be used because the equilibrium adsorption amount in dynamic adsorption is usually lower than in static adsorption at the same equilibrium adsorbate concentration. In this article, however, the dynamic adsorption isotherms are close to the static adsorption isotherm, which is illustrated by the adsorptions of the three proteins on phenyl-sepharose at the high salt concentration points as examples (Supplementary Figure 1). This may be because the adsorptions were fast (Supplementary Figure 2) and the flow rate was low. Therefore, the measured static adsorption isotherms were combined with chromatographic behavior here.

It is curious that some pure proteins present distorted or split peaks on HIC even at normal pH and temperature conditions, which might be misunderstood as impurities or poor column performance. As shown in Figure 5, on the butyl-sepharose fast flow (Figure 5A), BSA was eluted as a standard bell-shape peak when loaded at a relatively low salt concentration on butyl-

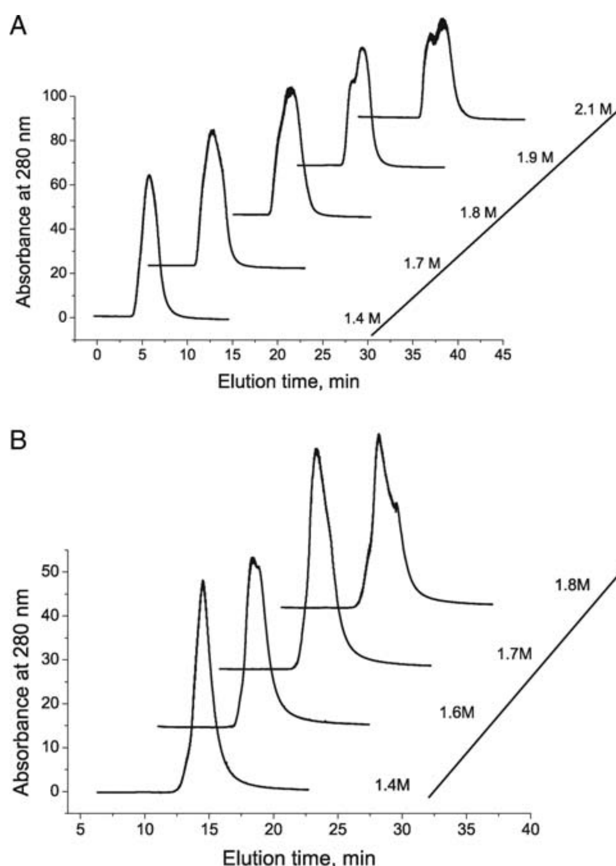

Figure 5. HIC elution profiles of BSA: BSA on butyl-sepharose (A); BSA on octyl-sephrose (B).

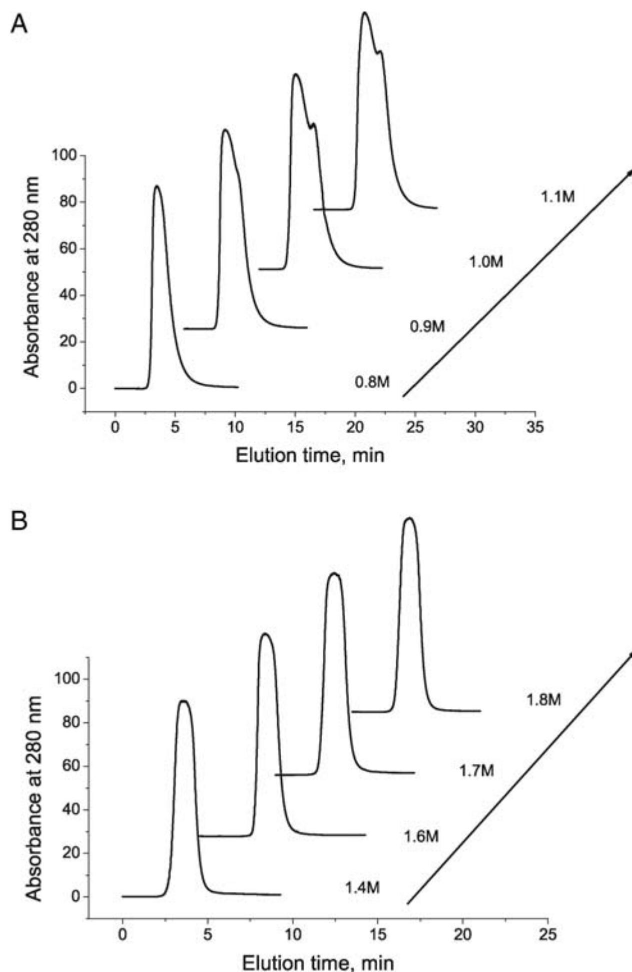

Figure 6. HIC elution profiles of IgG and lysozyme: IgG on octyl-sepharose (A); lysozyme on octyl-sepharose (B).

sepharose. However, when the salt concentration in the loading buffer increased gradually, the peak became distorted and finally split into two peaks. On the octyl-sepharose fast flow (Figure 5B), the single peak of BSA is also split into two at high salt concentrations. For IgG, a similar elution profile was observed, as shown in Figure 6A. However, no significant change of HIC behavior occurred for lysozyme (Figure 6B).

Peak-splitting has been reported on HIC for certain proteins. A pure protein sample, when loaded onto the HIC column, distributes in one distorted or two separated peaks instead of the typical bell-shaped peak (23–25). In 1984, Goheen and Engelhorn observed peak-splitting of albumin and β-lactoglobulin in a linear gradient elution from HIC columns (26). They speculated that it was caused by partial conformational changes. Oroszlan and Karger traced the intrinsic fluorescence of protein upon adsorption on HIC media by *in situ* analysis (27, 28). They packed HIC media into quartz flow cells, which were used for both fluorescence measurements and as a micro HIC column. The results revealed two peaks for α-lactalbumin, the first being native and the second conformation-changed. The rate of conformation change increased with increase of the medium hydrophobicity. Jones and his colleague used on-column hydrogen exchange (HX) followed by mass spectrometry (MS) to monitor the conformational difference between the two peaks of pure α-lactalbumin (29). They found that the early-eluting peak had the same solvent exposure degree as the native α-lactalbumin, while the following peak was more solvent-exposed. Jungbauer and Xiao developed models to qualitatively and quantitatively characterize the conformational change of proteins on HIC (6, 30).

More or less, the previously reported peak-splitting is related to the extreme pH and temperature (23, 25, 28–30). However, in this study, extreme pH and temperature were not used. Moreover, the salt concentrations for isotherm type shift on hydrophobic media were consistent with the turning points of peak-splitting in HIC experiments for BSA and IgG; no isotherm type shift on hydrophobic media was found for lysozyme, which had no peak-dividing phenomenon on HIC. Therefore, it is evident that the isotherm type shift correlated to the chromatographic behavior on HIC.

Taking peak-splitting into consideration, change of conformation and distribution of hydrophobic patches, although possibly occurring during adsorption, can be excluded as the primary reasons causing isotherm type shift in this article. Typically, the protein absorbed abiding by the Langmuir isotherm, hypothesizing no adsorbate–adsorbate interaction, is supposed to be eluted as a single peak, not split peaks. Therefore, a change of protein–protein repulsion is determined to be the reason behind isotherm type shift and peak-splitting.

In the presence of high salt concentrations, when the protein was loaded on the column and adsorbed by hydrophobic interaction, protein–protein repulsion was completely or almost shielded. Both hydrophobic interaction and protein–protein repulsion are closely related to salt conditions. In the cases of BSA and IgG, during elution, when the salt concentration decreased to a certain critical value, the interaction between the HIC surface and the protein molecule was weakened; on the other hand, the electrostatic repulsion became notable, which resulted in the elution of parts of molecules. The rest of the molecules were still absorbed, which needed further decreasing of the salt concentration to be eluted. Therefore, two peaks were eluted when the protein was loaded with a high salt concentration, whereas was only one peak eluted with a low salt concentration.

Conclusions

In this report, we have shown that the isotherm type shift occurred in HIC adsorption for single proteins when the salt concentration was changed. Further study of the HIC behavior of proteins revealed that the original single peak was split into two as the salt concentration increased to a critical value. Combining both isotherm type shift and peak-splitting phenomena, the most plausible reason was the change of protein–protein repulsion among adsorbed protein molecules, which existed under low salt concentrations but could be neglected under high salt concentrations. In essence, the difference in the way protein molecules were absorbed was caused by the different salt concentrations. Therefore, it can be concluded that the peak-splitting could be caused by high salt concentration.

Differences in protein–protein interaction can also be employed to explain why different proteins have different isotherm types even on the same HIC medium. If the protein–protein interaction is very weak and neglectful against hydrophobic interaction, the isotherm might be Langmiur. On the contrary, if the protein–protein interaction is strong enough to affect the adsorption, the isotherm would be changed.

Acknowledgments

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Supplementary material

Supplementary Material is available at *Journal of Chromatographic Science* online.

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