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Mixed retention mechanism of proteins in weak anion-exchange chromatography

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ABSTRACT

Using four commercial weak anion-exchange chromatography (WAX) columns and 11 kinds of different proteins, we experimentally examined the involvement of hydrophobic interaction chromatography (HIC) mechanism in protein retention on the WAX columns. The HIC mechanism was found to operate in all four WAX columns, and each of these columns had a better resolution in the HIC mode than in the corresponding WAX mode. Detailed analysis of the molecular interactions in a chromatographic system indicated that it is impossible to completely eliminate hydrophobic interactions from a WAX column. Based on these results, it may be possible to employ a single WAX column for protein separation by exploiting mixed modes (WAX and HIC) of retention. The stoichiometric displacement theory and two linear plots were used to show that mechanism of the mixed modes of retention in the system was a combination of two kinds of interactions, i.e., nonselective interactions in the HIC mode and selective interactions in the IEC mode. The obtained U-shaped elution curve of proteins could be distinguished into four different ranges of salt concentration, which also represent four retention regions.

1. Introduction

Many investigations about substance separation by stationary phase with mixed mode of hydrophobic interaction chromatography (HIC) and ion-exchange chromatography (IEC), reversed phase liquid chromatography (RPLC) and IEC, affinity chromatography (AFC) and IEC have been reported for inorganic anions [1], organic compounds [2–4], peptides [5] and proteins [6–11]. A fast native protein separation with an on-line two-dimensional liquid chromatography (LC) of weak cation-exchange chromatography (WCX) and HIC by a single column (2D-LC-1D) shows some special advantages and potential applications for protein separation in future [12]. Two review papers for the introducing the development of the mixed-mode stationary phase were recently published [13,14]. However, the mixed retention mechanism can also lead to some problems in protein separation. For example, hydrophobic interaction is a type of nonselective interaction [15] that causes nonselective protein adsorption. It is unfavorable for protein separation in the IEC mode. In contrast, electrostatic interactions are selective and facilitate protein separation in the HIC mode. The existence of other types of interactive forces may decrease the resolution and recovery of the amount and/or bioactivity of the proteins. To increase the advantages and decrease the disadvantages of protein separation by mixed-mode interactions, further investigation is required.

Yang et al. [2] and Neue et al. [4] investigated the mechanism of the mixed-mode of RPLC and IEC for small solutes. It has not been found any intensive investigation the mixed-mode for protein separation. Kennedy et al. first synthesized a type of weak anion-exchange chromatography (WAX) packing having the character of the retention mechanism of HIC. They named it as mixed-mode WAX packing [16]. Later, Rassi and Horvath [17] also reported a polymer-based WAX packing that operated with the same mixed-mode interactions. The former explained that the hydrophobic interactions originated from the chemical structure of the ligands bonded on the surface of the packings, while the latter attributed it to the polymer matrix. Gao et al. [6] recently also reported protein adsorption with a mixed-mode. All of these explanations are unexceptably attributed to the mixed-mode to the specially ligand structure of stationary phase [13,14]. Each of the mixed retention mechanism has been explained by an "U-shape" elution curve of proteins as that a half of this curve to causes from one mode corresponding to one kind LC and its rest half corresponding to other kind of LC [6,16–17]. A logical problem is raised that same protein is separated by same stationary phase and same mobile phase, why does the protein retention perform the special mixed character of two kinds of LC? When the salt concentration of the mobile phase changes, how does the protein change its retention mode from one kind of LC to another LC, is there a switch on the stationary phase? The mechanism by which a solute is retained in LC is a basic yet complicated theoretical problem, which has not been completely resolved even in the case of small solutes in RPLC [18]. In comparison to small solutes, protein molecules are very large and complicated and easily change

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their molecular conformation. Consequently, the mechanism by which they are retained in LC, especially if it is by mixed interactions, is much more complicated. Several retention models have been proposed for ion-exchange chromatography, including the stoichiometric displacement model [19–21] and nonstoichiometric model, which includes both the mechanistic model [22] and charge regulated slab model [23–25]. Bruch et al. recently evaluated protein retention using different retention models [26]. Although no correlation was observed between ligand hydrophobicity and retention, several groups investigated the retention mechanism by studying the hydrophobic groups on the spacer arms or the base matrix of the IEC stationary phase [27–29]. Many models have been proposed to explain retention in HIC. These are based on various theories, including the solvophobic theory propounded by Horvath's group [30–32], the preferential interaction theory outlined by Arakawa's group [33–35], and the stoichiometric displacement theory (SDT) of solute [36–38]. Other retention models in HIC have also been proposed [39]. The SDT is theoretically derived from different chemical equilibria that exist in chromatographic systems; therefore, it is independent of any particular molecular interactions. The validity of the SDT has been confirmed in both IEC and HIC and in other kinds of LC [13,40–45]. Snyder and Stadalius used the SDT as a general model of the behavior of biopolymers in high-performance liquid chromatography (HPLC) [46]. All these data indicate the possibility of using the SDT to explain the mixed modes (HIC and IEC) of retention on a single WAX column. In the previous report, the "U-shape" elution curve from mixed mode of WCX and HIC was actually explained and easily understood by the SDT, but it is also a qualitative one [12].

In this study, we tried to determine whether the mixed IEC and HIC modes of interaction occur not only in a specially designed ligands, but also in all commercial WAX columns. The SDT was used to explain and quantitatively characterize the mixed retention mechanism. In comparison with other mixed modes corresponding to different LCs, the mixed retention mechanism of proteins was first explained by the SDT alone as that when the salt concentration changes, the continuously changing of the orientation of protein molecules to stationary phase accomplishes the continuous changes in retention mechanism from one mode to other mode. The parameters of the SDT are also employed for quantitatively explaining the molecular interactions of the mixed mode, the retention prolongation, and the improvement of the selectivity of protein separation. Testing of four typical commercial WAX columns for protein separation revealed that the resolution of protein separation in the HIC mode was superior to that in the corresponding IEC mode; this suggested the possibility of employing a single commercial WAX column for 2D-LC in the IEC and HIC modes for protein separation. With deeply understanding the mixed retention mechanism of protein separation, it would be helpful to increase the selectivity and synthesize some excellent 2D-LC packings for on-line protein fast separation.

2. Experimental

2.1. Equipment

The chromatographic system consisted of two pumps (LC-10ATvp, Shimadzu, Kyoto, Japan), a system controller (SCL-10Avp, Shimadzu, Kyoto, Japan), a UV–Vis detector (SPD-10Avp, Shimadzu, Kyoto, Japan), and a Class VP 5.03 chromatographic workstation. The following four WAX columns were selected as typical polymer-based matrices: a TSKgel DEAE-5PW column (75 mm × 7.5 mm I.D.; Tosoh, Tokyo, Japan), Shim-pack PA-DEAE column (100 mm × 8.0 mm I.D.; Kyoto, Japan), silica-based PolyWAX LP coating column (150 mm × 4.6 mm I.D.; Polylaboratory, Columbia, MD, USA), and silica-based Aolan-WAX column (100 mm × 4.6 I.D.; Xi'an Aolan Scientific and Technology Company, Xi'an, China, http://www.aulanst.com). A TSKgel Ether-5PW column (75 mm × 7.5 mm I.D.) was used for protein separation in the HIC mode. All the silica-based matrices were packed under 500 bar.

2.2. Chemicals

The following proteins were used in this study: myoglobin (Myo, horse heart), α-amylase (α-Amy, bacillus species), conalbumin (Con, chicken egg white), insulin (Ins, bovine pancreas), bovine serum albumin (BSA, bovine serum), cytochrome *c* (Cyt *c*, horse heart), ribonuclease *A* (RNase *A*, bovine pancreas), lysozyme (Lys, chicken egg white), ovalbumin (OVA, chicken egg white), α-chymotrypsin (α-chy, bovine pancreas), and carbonic anhydrase (bovine erythrocytes). All proteins were purchased from Sigma (St. Louis, MO, USA) and dissolved in water to a concentration of 10 mg/mL. HPLC-grade water was prepared in a Barnstead E-Pure system (Barnstead Thermolyne, Dubuque, IA, USA). All other chemicals were of analytical grade.

The following mobile phases were used. In the WAX mode, solution A was 0.02 M Tris (pH 7.5), and solution B was 0.02 M Tris + 1.0 M sodium chloride (pH 7.5). In the HIC mode, solution C was 3.0 M ammonium sulfate + 0.05 M potassium dihydrogenphosphate (pH 7.0), and solution D was 0.05 M potassium dihydrogenphosphate (pH 7.0).

2.3. Chromatography conditions

Linear gradient elution for 30 min with a 10 min delay was employed in all protein separations; however, the mobile phases and gradient modes differed. In the WAX mode, the Shim-pack PA-DEAE WAX column was used with a gradient of 100% solution A to 30% solution B. The TSKgel DEAE-5PW, PolyWAX LP and Aolan-WAX columns were used with a gradient of 100% solution A to 100% solution B. In the HIC mode, the gradient was 100% solution C to 100% solution D. Prior to each chromatographic run, the columns were equilibrated with 20 column volumes of their corresponding mobile phase. The flow rate was 1.0 mL/min, and detection was carried out at 280 nm.

The capacity factor *k* of the protein was calculated as

$$k = \frac{t_R - t_0}{t_0} \quad (1)$$

The factor *k* is a dimensionless measure of protein retention under isocratic elution condition, where t_R is the retention time at a given salt concentration and t_0 is the retention time of the protein under nonadsorptive conditions [36,39].

2.4. Measurement of log *I* and *Z*

Both isocratic and gradient elution can be employed to measure log *I* and *Z* [41,42] or *S* [47,48]. The former is accurate, but requires more experiments, while the latter is simple and fast but its results are approximations. In this study, the log *I* and *Z* of each protein were measured by isocratic elution at a flow rate of 1.0 mL/min. Prior to each injection, the chromatography column was equilibrated with 20 column volumes of the mobile phase. For simplicity and convenience, the activity coefficient of water in both the stationary and mobile phases was taken as unity, i.e., a_{H_2O}, in this study can be represented in its concentration form, i.e. $[H_2O]$, in the mobile phase. The term $[H_2O]$ can be calculated by Eq. (2) [36] as

$$[H_2O] = \frac{d_A\Psi_A + d_B\Psi_B - W_s}{0.018} \quad (2)$$

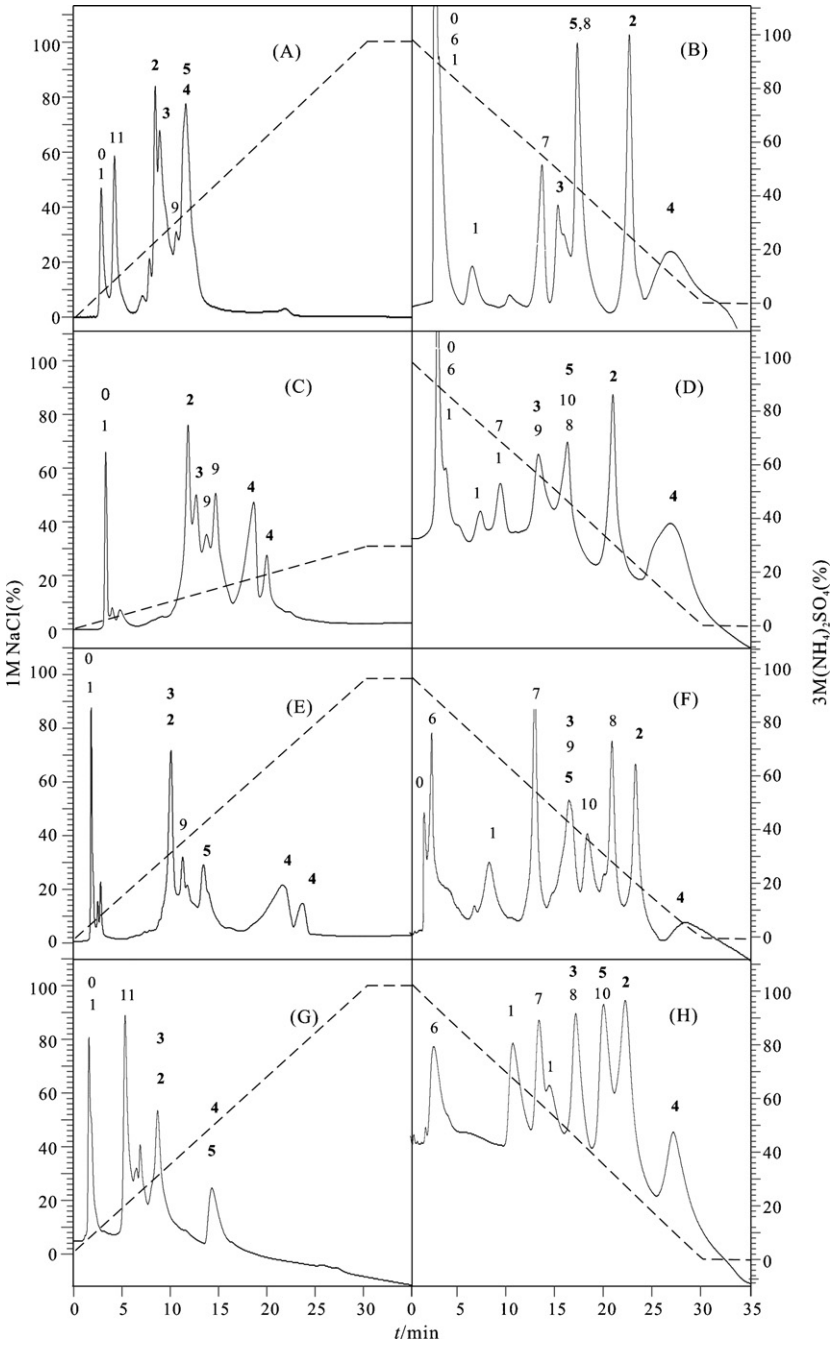

Fig. 1. Chromatogram of protein separation in the IEC and HIC modes on the four WAX columns. The following columns were used: (A and B), TSKgel DEAE-5PW column (75 mm × 7.5 mm I.D.); (C and D), Shim-pack PA-DEAE WAX column (100 mm × 8.0 mm I.D.); (E and F), PolyWAX LP column (150 mm × 4.6 mm I.D.); and (G and H), Aolan WAX column (100 mm × 4.6 mm I.D.). A, C, E, and G represent separation in the WAX mode, while B, D, F, and H represent separation in the HIC mode. The following mobile phases were used: solution A, 20 mM Tris (pH 7.5); solution B, 1 M sodium chloride + 20 mM Tris (pH 7.5); solution C, 50 mM potassium dihydrogenphosphate + 3 M ammonium sulfate (pH 7.0); and solution D, 50 mM potassium dihydrogenphosphate (pH 7.0). Gradient mode is indicated by dashed lines. flow rate, 1.0 mL/min; detection, 280 nm. Peaks: (0) solvent, (1) myoglobin, (2) α-amylase, (3) conalbumin, (4) insulin, (5) bovine serum albumin, (6) cytochrome *c*, (7) ribonuclease *A*, (8) lysozyme, (9) ovalbumin, (10) α-chymotrypsin, and (11) carbonic anhydrase. The bold Arabic numbers represent proteins that can be separated in both the IEC and HIC modes.

where d_A and d_B are the densities of solutions A and B (kg/L), respectively, and Ψ_A and Ψ_B are the volume fractions (v/v, %) of solutions A and B, respectively. W_s is the weight of salt per volume of the mixed solution (kg/L). The dimension of 0.018 is kg/mol.

3. Results and discussion

3.1. Protein separation in both the HIC and WAX modes

To investigate whether mixed interactions occur on WAX columns, we selected the following four typical commercial WAX columns: TSKgel DEAE-5PW and Shim-pack PA-DEAE (two kinds of polymer-based packings), Aolan WAX (a silica-based matrix), and PolyWAX LP (another kind of silica-based packing with a polymer coating). Fig. 1 shows the chromatogram of standard protein separation on the four columns in both the WAX and HIC modes. Chromatograms A, C, E, and G were obtained in the WAX mode, while B, D, F, and H were recorded in the HIC mode. A and B were from the TSKgel DEAE-5PW column; C and D, Shim-pack PA-DEAE column; E and F, PolyWAX LP column; and G and H, Aolan WAX column.

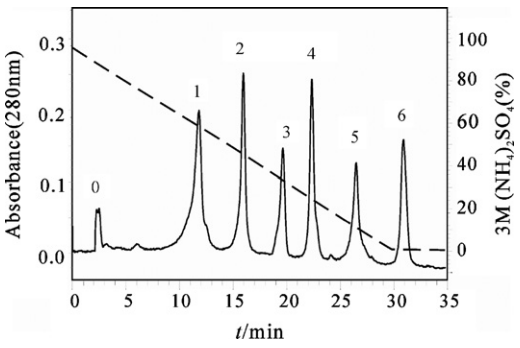

Fig. 2. Chromatogram of protein separation on the TSKgel Ether-5PW HIC column. Column: TSKgel Ether-5PW column (75 mm × 7.5 mm I.D.); Peaks: (0) solvent, (1) cytochrome *c*, (2) myoglobin, (3) ribonuclease *A*, (4) lysozyme, (5) α-amylase, and (6) insulin. Mobile phase: solutions C and D. Other experimental conditions were the same as those shown in Fig. 1.

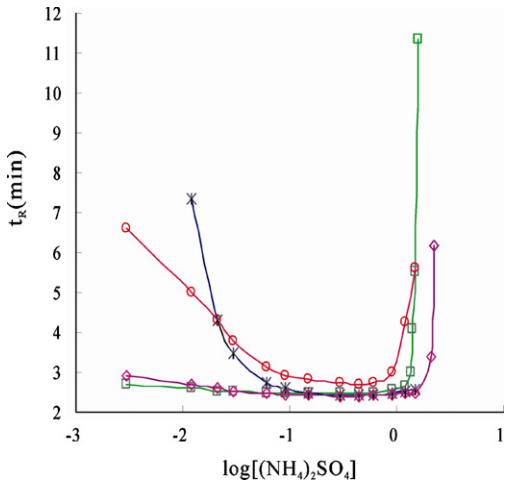

Fig. 3. U-shaped elution curves of four different proteins on a TSKgel DEAE-5PW column. Proteins: ○, insulin; ◊, conalbumin; *, bovine serum albumin; and □, α-amylase. Isocratic elution with a mixture of solutions C and D. The flow rate was 1.0 mL/min with detection at 280 nm.

Several observations can be made from Fig. 1. First, when the four typical columns are used with different mobile phases, both IEC and HIC retention mechanisms are operational, indicating that the HIC mode of interaction is a universal phenomenon in the WAX column. Second, the elution order of many of the 11 kinds of standard proteins tested changed not only in the WAX mode but also in the HIC mode, which changed the selectivity of protein separation. Third, the resolution of protein separation in the HIC mode was better than that in the corresponding WAX mode. A similar observation was made earlier where it was reported that the HIC mode is also operational in a WCX column but that the resolution of the protein separation process, for example, during separation on a PolyLC Poly CAT-WCX column, in the HIC mode was much worse than that in its corresponding WCX mode [12].

In comparison with the four chromatograms obtained in the HIC mode (shown in Fig. 1), the resolution of protein separation on a TSKgel Ether-5PW HIC column (shown in Fig. 2) was better, and the elution order of the proteins was quite different. These changes can be attributed to the interactive effects of the IEC and HIC modes of retention with each other. From a theoretical perspective, any IEC stationary phase should diminish the effects of nonselective interactions arising from the surface of the synthetic packing material and increase the hydrophilicity of the surface. The chromatograms in the HIC mode shown in Fig. 1 indicate that it is actually impossible to completely diminish the hydrophobicity on the surface of WAX packings. In contrast, any of the four WAX columns may be employed for protein separation by 2D-LC in the WAX-HIC mode. However, for most protein separations, a single column with mixed mode interactions cannot match the resolution achieved by two individual orthogonal columns.

3.2. U-shaped elution curve

We have to study the mixed mode interactions by which proteins are retained on a WAX column to understand why each of the four WAX columns has such a good resolution in the HIC mode. Except BSA exhibited several peak with Shim-pack PA-DEAE column in WAX mode, not showing in Fig. 1C, four proteins, which are represented by bold Arabic numbers in Fig. 1, were retarded and separated in both the HIC and IEC modes by the four columns. Fig. 3 shows a plot of the retention of the four proteins on the TSKgel DEAE-5PW WAX column in terms of t_R *vs*. the logarithm of the ammonium sulfate concentration in the mobile phase, i.e., $\log[C_{salt}]$. Each of the elution curves is U-shaped, although they have different asymmetries. The elution curves of Con and α-amylase almost coincide in the low salt concentration range in the IEC mode, but the two proteins can be completely separated in the high salt concentration range in the HIC mode. Kennedy et al. [16] and Rassi and Horvath [17] attributed the performance in the HIC mode to the chemical structure of the stationary phase. Since the chemical structure of the stationary phase is fixed and only the salt concentration of the mobile phase changes, a question arises as to how protein molecules recognize ionic groups on the stationary phase and react with these in the IEC mode or with the nonpolar groups in the HIC mode. The occurrence of other types of molecular interactions in the chromatographic system must therefore be further investigated.

3.3. Complicated molecular interactions in chromatographic systems

Any ligand on a WAX stationary phase consists of nonpolar and polar groups, which include ionic and hydrophilic groups. For example, the ligand on a TSKgel DEAE-5PW column is bonded hydroxylated methacrylic polymer. Apart from both the polar (—$N^+\equiv$) and nonpolar groups (—CH_2— and —CH_3) on the surface of the polymer matrix, some hydrophilic groups are also present. All three kinds of groups contribute to protein separation on the TSKgel DEAE-5PW column. The structure also indicates that the —$N^+\equiv$ group can interact with a negatively charged solute, and other nonpolar groups can interact with the nonpolar region of the same solute. A protein molecule contains both polar and nonpolar groups and has both positive and negative charges. By changing the environmental conditions, such as the salt concentration or pH value of the mobile phase, a protein molecule can selectively interact with any charged, hydrophobic, or hydrophilic group of the ligand on the stationary phase. The kind of molecular interactions is thus dominated by a series molecular interactions occurred in mobile phase. However, at a specific salt concentration, one kind of molecular interaction generally or mainly predominates, in the interactions with the mobile phase.

It is well known that solute solvation occurs, but hydration of a nonpolar solute in water is doubtful. Despa and Berry [15] presented a mechanism for hydrophobic aggregation in an aqueous solution based on basic molecular principles, direct atomic force microscopy measurements of a hydrophobic molecule tethered to a surface, and observation of the transverse relaxation rates in water proton magnetic resonance. They proposed that hydrated nonpolar solutes or two hydrophobes may attract each other at longer distances via dipole–dipole and induction-dispersion effects

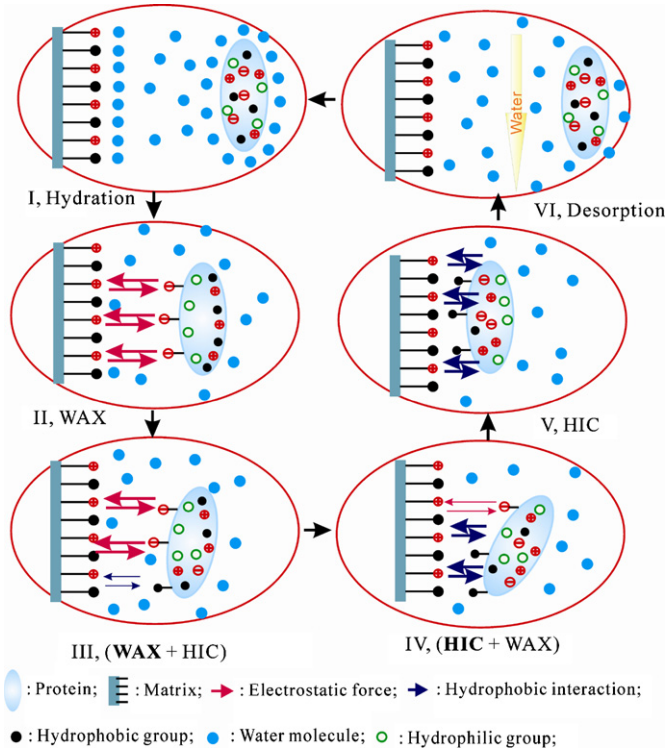

Fig. 4. Schematic diagram of the interactions between the protein and stationary phase on a WAX column. The bold letter denotes the predominant mode by which protein retention occurs.

generated by polarization fields of the water structured at the interface. Simultaneously, when the water-coated hydrophobic surfaces meet, direct contacts form between the surfaces, and short-range van der Waals attractive forces provide the driving force to squeeze out the water. Hydration also occurs on the surface of the stationary phase. The reverse process, i.e., dehydration, occurs during interaction between the nonpolar groups of the protein and stationary phase. If a stoichiometric mole of water in the contact region between the protein and the employed stationary phase is squeezed out when a mole of protein is adsorbed on the stationary phase, protein retention will occur in the HIC mode [13]. If an ion is adsorbed by the stationary phase and interacts with the ionic groups, the displacer that is squeezed out in the contact region is also a kind of ion but is not water. In this case, protein retention occurs in the IEC mode [20]. If the two processes occur simultaneously, both the HIC and IEC modes exist, which is termed the mixed retention mode. In all the three cases, the kind of stationary phase used, i.e., HIC or IEC, does not really matter.

3.4. Changes in molecular interactions with varying salt concentration

Based on the molecular mechanism, the electronic interaction force is a long-distance force that attracts protein molecules close to the WAX stationary phase. As shown in Fig. 4, when the salt concentration is very low, the negative electrostatic and hydrophilic groups of the protein molecules face the stationary phase and the nonpolar groups of the protein molecules face the mobile phase. Electrostatic interactions (red arrows) occur between the protein and stationary phase. In contrast, when the salt concentration in the mobile phase is very high, the protein molecules change their original orientation to contact the stationary phase with their hydrophobic groups (dark spots) and simultaneously squeeze out a stoichiometric mole of water. An intermediate situation is when both kinds of interactions occur simultaneously. The processes can occur continuously because in LC, the salt concentration continuously changes during linear gradient elution. The main feature of this process is that the orientation of protein molecules changes continuously with changes in the salt concentration of the mobile phase. The entire process can be divided into six steps, as shown in Fig. 4.

Step I involves the formation of the hydrated stationary phase and protein molecules. In step II, when the salt concentration in the mobile phase is very low, the protein is displaced by the sulfate groups, and electrostatic interactions dominate protein separation; in this case, protein retention is in the IEC mode. In step III, the salt concentration increases slightly, and a very small fraction of protein molecules is also displaced by water; in this case, protein retention is mainly in the IEC mode but the HIC mode is also involved to a small extent. In step IV, when the salt concentration is relatively high, protein retention is predominantly in the HIC mode but the IEC mode is also involved to a slight extent. In step V, at a very high salt concentration, the protein is completely displaced by water, and protein retention is dominated by the HIC mode. In step VI, when the salt concentration is gradually decreased, a reversal of the processes from steps VI to I occurs, and the adsorbed proteins are eluted.

In summary, changes in the salt concentration of the mobile phase simultaneously result in changes in the concentration of water in the mobile phase, which alters the retention mechanism. These results provide a molecular basis for quantitatively explaining the mixed mode interactions in protein separation using the SDT.

Table 1
Linear parameters of four proteins in the WAX mode.

Proteins	Z^a	$\log I$	R
α-Amy	2.10	3.249	0.991
Con	1.94	2.757	0.987
BSA	3.44	3.006	1.000
Ins	2.36	2.265	0.989

[a] Z value corresponding to univalent anion.

3.5. Verification of the mechanism of mixed mode interactions

The molecular mechanism supporting the SDT was only presented 2 years ago [15]; however, the SDT has been tested and validated by countless experiments. In 1984, the SDT was used to explain protein separation by RPLC [41], and in 2001, it was experimentally proven by directly measuring the amount of released methanol [49]. SDT was also used to explain HIC in 1990 [36], and tested by many investigations [13,37,38,40–45] and the amount of displaced water was measured in 1997 [50]. All of these investigations were only directed toward analyzing a specific retention mechanism. Therefore, to prove the existence of mixed mode interactions during protein separation on a single column, the SDT needs to be validated separately.

3.5.1. Linear plot of $\log k$ vs. $\log [C_{displacer}]$

SDT-R can be represented as [20,36]

$$\log k = \log I - Z \log[D] \quad (3)$$

In this case, k is the capacity factor of the protein; $\log I$ is a set of constants (five molecular interactions in HPLC) and relates to the affinity of a protein to the stationary phase; Z is the total moles of water released from stationary phase side and protein side in the HIC mode [36] and the total negative charges in WAX mode; and $[D]$ is the molar concentration of the displacer in the mobile phase, water in HIC mode and bivalent anion, sulfate in WAX mode. Table 1 shows the linear parameters from the plot in the IEC mode,

Table 2
Linear parameters of four proteins in the HIC mode.

Proteins	Z	log I	R
α-Amy	136	231	0.992
Con	50.3	83.9	0.987
BSA	37.9	62.9	0.995
Ins	83.6	142	0.999

and Table 2 shows those in the HIC mode. The R values listed in the two Tables indicate that the SDT can also be used to explain mixed mode interactions.

3.5.2. Linear plot of log I vs. Z

Both linear parameters Z (or S) and log I have been used to characterize the molecular structure parameter, character of mobile and stationary phases, and changes in the molecular conformation of proteins [47,48]. An excellent linear relationship exists between log I and Z of small solutes (nonpolar and polar) and proteins in their native (N) state [13,36,42,51]; however, it is only valid for solute retention that is dominated by nonspecific interactions, such as those in RPLC and HIC, and is not valid in IEC. This linear relationship can be expressed [51] by Eq. (4)

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

Physically, j denotes the affinity of one mole of water to the stationary phase of the WAX column, and φ is the column phase ratio defined by thermodynamics [13]. The usefulness of Eq. (4) is that as long as it is linear, the interaction between the protein and stationary phase in a given mobile phase composition will be nonselective, indicating that protein retention occurs in the HIC mode. Otherwise, the interaction will be selective, and protein retention will occur in the IEC mode [13,44,51]. Using the data from Tables 1 and 2, Eq. (4) is used to plot log I vs. Z. A linear relationship is observed in the HIC mode (Fig. 5A), and the linear dependent coefficient R^2 is 0.9999; however, such a relationship does not exist in the IEC mode (Fig. 5B). The j value thus obtained (1.71) is very close to the theoretical value of 1.74 [13,51], indicating that the SDT can be used to quantitatively assess mixed mode interactions in protein separation.

3.5.3. Protein separation by mixed mode interactions on a WAX column

Improving the selectivity of protein separation is important in LC; therefore, the U-shaped elution curve shown in Fig. 3 should be analyzed in greater detail. Fig. 6 shows a real U-shaped elution curve marked with dash line for insulin obtained from TSKgel DEAE-5PW column by plotting log k vs. log [C_{salt}]. For theoretical convenience, the elution curve is divided into four regions. Using Eq. (3), we can separately plot both log k vs. log [C_{salt}] for the IEC mode and log k vs. log[C_{H_2O}] for the HIC mode and only use the straight solid line parts, i.e., line AB on the left-hand side and line DE on the right-hand side of the curve. The two straight lines separately correspond to steps II and V shown in Fig. 4. One of the two middle parts, curve BC, represents protein retention by mixed mode interactions and

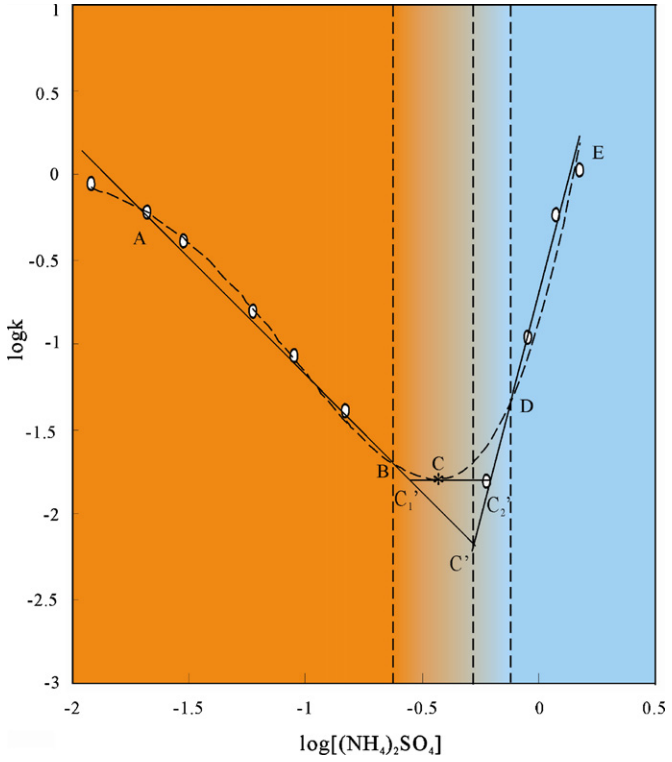

Fig. 6. Schematic diagram of the retention mechanism of insulin at different salt concentrations in the mobile phase.

also indicates the predominance of the WAX mode, corresponding to step III shown in Fig. 4. The other curve CD represents retention by mixed mode interactions but indicates predominance of the HIC mode, corresponding to step IV shown in Fig. 4.

Table 3 shows the four salt concentration ranges corresponding to the four different protein retention mechanisms. Additionally, changes in the selectivity of protein separation should be covered in the two ranges of the curve, i.e., BC for the IEC mode and CD for the HIC mode. When hydrophobic and electrostatic interactions offset each other, protein retention remains unaltered and has a minimum retention at C denoted by asterisk. This is termed iso-retention of the protein. Theoretically, the C point should coincide with the intersect point, C′ of the two extension lines of AB and ED because the asymmetry of the "U" shaped curve shown in Fig. 6. Although the corresponding salt concentration to the iso-retention of the protein has a deviation, the shown data in Table 3 still provides very useful information. Each protein would be expected to have its own iso-retention point; thus, the elution order of proteins may differ in both the pure IEC and HIC modes, thereby increasing the selectivity of protein separation.

The range of salt concentration BCD shown in Fig. 6 indicates a favorable factor for improving the selectivity of insulin separation.

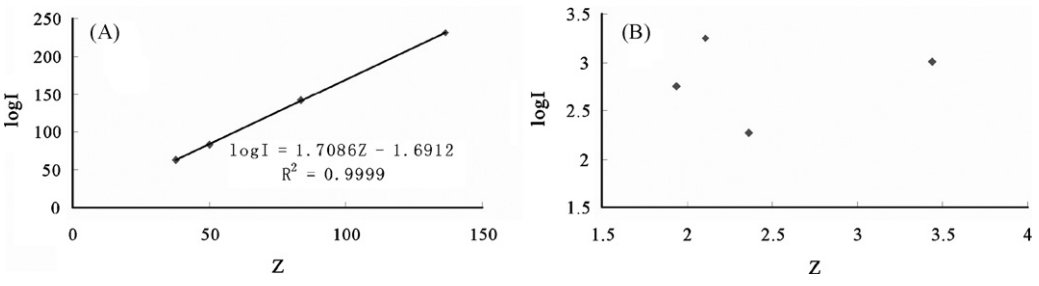

Fig. 5. Linear plot of log I vs. Z. Both of log I and Z were taken from Tables 1 and 2.

Table 3
C_{salt} range and mixed-mode of proteins.

Proteins	WAX A–B	**WAX**[a]-HIC B–C′	**HIC**[a]-WAX C′–D	HIC D–E
α-Amy	0.00–0.15	0.15–0.43	0.43–0.56	0.56–3.00
Con	0.00–0.12	0.12–0.57	0.57–0.75	0.75–3.00
Ins	0.00–0.24	0.24–0.53	0.53–0.75	0.75–3.00
BSA	0.00–0.27	0.27–0.55	0.55–0.90	0.90–3.00
Average	0.00–0.20	0.20–0.52	0.52–0.74	0.74–3.00

[a] Bold letter represents the mode mainly dominates protein retention.

Supposing insulin retention only follows either pure IEC model, i.e., the changes should along the straight solid line ABC'_1C', or pure HIC model to the straight solid line $ED\,C'_2C'$. However, the insulin retention actually follows to the dash line BCD. The same insulin retention, for example, C corresponds to the mixed mode, while C'_1 and C'_2 separately correspond to the insulin retention for pure WAX and pure HIC modes. Thus, the distances of between C and C'_1 and between C and C'_2 correspond to the difference of salt concentrations and also the prolonged retention of insulin. As a result, insulin retention is actually prolonged 1.8 min for WAX mode and 2.3 min for HIC interaction, resulting in the increases in the peak capacity for protein separation. This phenomenon for prolonged retention of small solutes with IEC column was also reported by Novič and Haddad [3].

3.6. Elution order of proteins with WAX mode

In the previous investigation, the selectivity of protein separation with HIC mode was found to change by WCX mode [12] and the same change also occurs by WAX mode in this study. In a contrary, protein retention originally dominated by electrostatic attraction also to be changed by hydrophobic interaction occurs in this study. It should thus be intensively investigated.

The protein retention on the WAX columns is dominated by three factors, electrostatic attraction, hydrophobic interaction and orientation of protein molecules to the WAX stationary phase. The former two bases on the intrinsic character of proteins, while the latter is dominated not only the three- or four-dimensional structure of protein molecules, but also the character of the employed stationary and mobile phases. Fausnaugh-Pollit et al. reported that the retention of protein on a HIC column is determined by amino acids on a single surface of the protein opposite to its catalytic left [52]. Karlsson and Carlsson also attributed the adsorption of the native human carbonic anhydrase II to be dominated by the orientation of its molecule to silica nanoparticles [53]. These factors indicate the orientation of protein molecular to play a significant rule for protein retention on a chromatographic column. However, scientists have not fully understood the rule yet.

The elution order of proteins on a WAX column theoretically should follow the iso-electrostatic point (*P*I). When protein separation with WAX mode with pH 7.5, all proteins having *P*I values to be higher than 7.5 should not be retarded and it is really true shown in Fig. 1, while proteins having *P*I value to be lower than 7.5 should be eluted out according to the rank of *P*I value (R_{WAX}) from high to low. However, as that shown in Fig. 1, the elution order of the retarded proteins on the four WAX columns totally does not follow to this rule and also appears a little different with each other. TSK gel DEAE-5PW column was taken as an example for this intensively investigating the competition between the two interactions shown in Fig. 6. If the electrostatic character of each protein is expressed by its *P*I value and its HIC strength is expressed by the percentage of hydrophobic amino acid residue of the proteins, this competition result may be related to the sum of the two parameters. Table 4 shows all of these parameters and that they are ranked from high to low values, R_{WAX} for *P*I and R_{HIC} for the HIC. The obtained elution order of the proteins from experiment data (due to some retentions are so closed that their peaks emerge together and thus their elution orders thus can not be seen from Fig. 1) on the TSK gel DEAE-5PW WAX column is also shown in Table 4. It is seen that the sum, R_{sum} ($R_{WAX} + R_{HIC}$) of these proteins coincides with the real elution order of proteins only except carbonic anhydrase. Although the elution order from the experiment in this study is the exactly same as that reported in other investigation with a Mono Q of an anion-exchange column by Hallgren [54], the author did not explain the reason why the elution order of carbonic anhydrase does not follow to the order of *P*I value. Based on the investigation by Karlsson and Carlsson employed native human carbonic anhydrase II [53], a reasonable deduction is that compared to the two close peaks of α-amylase and carbonic anhydrase shown in Fig. 1A, the orientation of carbonic anhydrase sourcing from bovine erythrocytes may be more sensitive to salt concentration, resulting in the changes in their elution order. For the same reason, the four WAX stationary phases having various ligand structures would have different interactions to these proteins, changing the molecular orientation of proteins to the stationary phase in different extents, resulting in a little different elution order of proteins of the four WAX columns.

Table 4
Elution order of proteins and the sum of the ranks of *P*I and percentage of hydrophobic interaction amino acid residues of protein molecules.

Proteins	*P*I[a]	R_{WAX}[a]	Hydrophobic amino acid residues (%)	R_{HIC}[a]	R_{sum}[a] ($R_{WAX} + R_{HIC}$)	Elution order[b] (WAX)	Expected elution order (WAX)
Myo	7.36	1	51.0	2	3	1	1
Car	6.40	3	49.3	4	7	2	3
α-Amy	5.74	4	51.6	1	5	3	2
Con	6.69	2	44.5	6	8	4	4
OVA	5.19	7	50.7	3	10	5	5
Ins	5.39	6	45.2	5	11	6	6
BSA	5.60	5	40.3	7	12	7	7

The value of *P*I and percentage hydrophobic amino acid residues (%) were taken from http://www.expasy.org/.

[a] *P*I, iso-electrostatic point, R_{PI}, rank of the *P*I from high to low; R_{HIC}, rank of the percentage of hydrophobic amino acid residues of proteins; R_{sum}, the sum of R_{WAX} and R_{HIC}.

[b] Because some retentions of two neighborhood is too close, such as the peaks (2) α-amylase and (3) conalbumin obtained from TSKgel DEAE-5PW column shown in Fig. 1, the two peaks coincides with each other and thus their elution ranks can not be seen from Figure, but they were taken from their real retentions.

4. Conclusions

The existence of hydrophobic interactions in the WAX column is a universal phenomenon although the strength of such hydrophobic interactions may vary across different columns. When the salt concentration gradually increases, the orientation of protein molecules toward the stationary phase and the nature of interacting forces, electrostatic or hydrophobic, simultaneously change. Protein retention on the WAX column by mixed mode interactions can be explained on the basis of the stoichiometric displacement theory (SDT). In terms of salt concentration, the U-shaped elution curve can be divided into four parts and evaluated by the SDT. It would be expected that a commercial WAX column can be employed to separate proteins in the HIC mode even when protein separation is performed by 2D-LC. The elution order of proteins with WAX mode on TSK gel DEAE-5PW WAX column was found to relate to the competition between the electrostatic attraction, hydrophobicity of proteins, and the orientation of protein molecules to stationary phase.

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