



Retention Mechanism of Proteins in Hydroxyapatite Chromatography – Multimodal Interaction Based Protein Separations: A Model Study



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Abstract: Background: Retention mechanism of proteins in hydroxyapatite chromatography (HAC) was investigated by linear gradient elution experiments (LGE).

Materials and Methods: Several mobile phase (buffer) solution strategies and solutes were evaluated in order to probe the relative contributions of two adsorption sites of hydroxyapatite (HA) particles, C-site due to Ca (metal affinity) and P-site due to PO₄ (cation-exchange). When P-site was blocked, two basic proteins, lysozyme (Lys) and ribonuclease A (RNase), were not retained whereas cytochrome C (Cyt C) and lactoferrin (LF) were retained and also retention of acidic proteins became stronger as the repulsion due to P-site was eliminated. The number of the binding site *B* values determined from LGE also increased, which also showed reduction of repulsion forces.

Conclusion: The selectivity (retention) of four basic proteins (RNase, Lys, Cyt C, LF) in HAC was different from that in ion-exchange chromatography. Moreover, it was possible to tune the selectivity by using NaCl gradient.

Keywords: Hydroxyapatite chromatography, linear gradient elution, protein retention, binding site, LGE, LF.

1. INTRODUCTION

Protein separation by chromatography is quite important for purification of biologics (protein-based drugs) [1-5]. Hydroxyapatite (HA) chromatography (HAC) is one of the oldest chromatography methods in the field of biomolecule separations [6, 7], and has been used for separation of proteins and DNAs [8-25]. It is regarded as a mixed-mode or multi-modal chromatography. Most mixed-mode chromatography has hydrophobic, ion-exchange, and hydrogen-bonding ligands [26-28]. HAC is unique in the exploitation of electrostatic interaction (ion-exchange) and metal affinity. Many studies have shown that HAC enabled good separation performance of difficult separation such as removal of aggregates from monoclonal antibodies [14, 15], which cannot be achieved by a single mode chromatography such as ion-exchange chromatography (IEC) or hydrophobic interaction chromatography (HIC).

HA is a calcium-phosphate complex Ca₁₀(PO₄)₆(OH)₂, composed of two sites, C-site due to Ca and P-site due to PO₄. Previous studies [8, 11-13, 20] have shown the following general principles.

i) P-site works as a cation-exchange ligand whereas C-site has a metal affinity as well as an anion-exchange ligand function.

ii) At neutral pH regions basic proteins are retained on HAC based on the electrostatic interaction with P-site and eluted with NaCl as well as sodium phosphate. This is similar to cation exchange chromatography (CIEC).

iii) Acidic proteins are retained due to a strong interaction (metal affinity) with C-site, and are not eluted with NaCl, requiring sodium phosphate for their elution. Therefore, the elution or retention behavior is different from that for anion-exchange chromatography (AIEC).

In this study, we performed linear gradient elution (LGE) of proteins on ceramic HAC columns at various conditions such as gradient slope and mobile phase pH. Several mobile phase (buffer) solution strategies and solutes were evaluated in order to probe the relative contributions of two adsorption sites of hydroxyapatite (HA) particles, C-site due to Ca (metal affinity) and P-site due to PO₄ (cation-exchange). Binding site value and elution salt concentrations determined from LGE experiments based on our method [5, 25] were examined in order to elucidate the retention (binding) mechanism of HAC in comparison with the data obtained with ion-exchange chromatography (IEC).

2. MATERIALS AND METHODS

2.1. Materials

Model protein samples used in this study are shown in Table 1. The pI values were cited from the literature [29, 30]. Acidic, neutral and basic proteins of different molecular weights were chosen. Bovine serum albumin (BSA) and

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Table 1. Proteins employed in this study.

Sample (abbreviation)	pI ^{a)}	MW ^{b)}	Supplier ^{c)} (cat.no)
α -Lactalbumin (LA)	4.2-4.5	14000	SA(L-6010)
Ovalbumin (OA)	4.7	44300	SA(A-5378)
Bovine serum albumin (BSA)	4.7-4.9	68000	SA(A-1900)
β -Lactoglobulin (LG)	5.1-5.3	36000	SA(L-0130)
Myoglobin (Mb)	7.2	17800	SA(M-0630)
Lactoferrin (LF)	8.7	83000	M
Ribonuclease A (RNase)	9.5-9.6	13700	SA(R-4875)
Cytochrome C (Cyt C)	10	12400	SA(C-2506)
Lysozyme (Lys)	11.0-11.4	14300	SK(100940)

^{a)}pI isoelectric point [29, 30], ^{b)}MW, molecular weight (kg/kg-mol) [29, 30],

^{c)}SA; Sigma-Aldrich (St. Louis, MO), M; donation from Megmilk Snow Brand (Tokyo, Japan), SK; 6-time crystalized from hen egg white, Seikagaku Kogyo (Tokyo, Japan).

lysozyme (Lys) are most widely used acidic and basic model proteins, respectively. β -lactoglobulin (LG) is a good probe for the retention study as it has two genetic variants A and B forms, and the surface charge distribution is very asymmetrical [31, 32]. Other reagents were of analytical grade.

2.2. Chromatography Experiments

Ceramic hydroxyapatite particles (CHT Type I, nominal particle diameter = 40 μ m, Bio-Rad, USA) were self-packed into a stainless steel empty column (column inner diameter d_c = 4 mm, column length Z = 100 mm, the total column bed volume V_t = 1.257 mL, the column void fraction = 0.33). The void fraction ϵ was determined from the retention volume of high molecular weight dextran (molecular weight >2,000,000) solution pulse response curves. For comparison ion-exchange monolithic disks CIM SO₃ (BIA Separations, Ajdovščina, Slovenia) were employed. They are methacrylate-based monolithic (continuous-phase) disks, in which strong cation exchange ligands (SO₃) were immobilized onto flow-through pores (ca. 1.5 μ m) [33]. The disks (d_c = 12 mm, Z = 3 mm, V_t = 0.34 mL, void fraction = 0.62) were placed into the manufacturer's holder and used as a column.

Linear gradient elution (LGE) experiments were performed on a fully automated liquid chromatography system (AKTA explorer or purifier, GE Healthcare, Uppsala, Sweden) at 298 \pm 1K. Table 2 summarizes the buffer systems employed in this study. The column was equilibrated with the buffer solution until the pH and the conductivity values at the column outlet become the same as those of the buffer solution (20 to 50 column volumes). After loading the sample, the salt concentration of the mobile phase was changed with time linearly. The column was washed with the buffer solution of high salt concentrations after the sample was eluted. The column was also washed with 0.1 M NaOH once per day. The sample volume was 0.1 - 0.5 mL and the sample concentration was 5-10 mg/mL. The volumetric flow-rate F was 1.0 mL/min for the CHT column and 2.0 mL/min for the CIM disks unless otherwise noted. The salt concentration at the peak retention time (or volume) I_R was measured for different gradient slopes g and plotted against normalized gradient, GH . For the precise determination of I_R , the dead volume between the UV detector cell and the conductivity cell was measured. The calibration curve (conductivity vs. salt concentration) was prepared for each buffer solution.

The system dead volume was reduced as much as possible. The experiments were repeated twice and the average I_R value was used.

From the $GH-I_R$ curve, the number of binding sites (charges) involved in electrostatic interaction, B value, was determined according to the following equation [5, 25, 31, 32, 34]. The B value can provide valuable information on the interaction between the chromatography ligand and the solute [5, 25, 31, 32, 34]. A is the parameter including the equilibrium coefficient, the binding site and the ion-exchange capacity [5, 25, 31, 32, 34].

$$GH = I_R^{(B+1)}[A(B+1)] \quad (1)$$

$GH = g(V_t - V_o)$, $G = gV_o$, H is the volumetric phase ratio = $(1 - \epsilon)/\epsilon$ and ϵ is the column void fraction = V_o/V_t , and gradient slope, $g = (I_f - I_0)/V_g$, is the gradient slope of the salt (I_f : final salt concentration, I_0 : initial salt concentration, V_g : gradient volume). V_t is the total column bed volume, V_o is the column void (interstitial space) volume.

3. RESULTS

3.1. Retention Behavior as a Function of pI with Phosphate Buffers when the Two Sites are Active

Peak salt concentration I_R values were measured from linear gradient elution (LGE) curves of model proteins with commonly employed phosphate buffer solutions (mobile phase A in Table 2). Relationships between I_R and pI are shown in Fig. (1). It has been reported that the retention increases with pI at pH 6.8 [8]. The I_R values except for β -lactoglobulin, lactoferrin and cytochrome C show such a trend as shown in Fig. (1b). The I_R decreased with increasing mobile phase pH, which was similar to our previous finding as shown in (Fig. 1) [25].

As already mentioned it has been reported that basic proteins are retained on P-site, which basically works as a cation-exchange ligand. Therefore, basic proteins can be eluted with NaCl as well as sodium phosphate as a gradient (modulator) component. On the other hand, acidic proteins are said to be bound to C-site based on metal affinity or coordinate binding. Acidic proteins cannot be eluted with NaCl because of its strong (affinity-based) interaction. In addition, with increasing pH the dissociation at C-site decreases and the repulsion by P-site increases.

Table 2. Mobile phases employed in this study.

	Base buffer	Gradient
A	10 mM sodium phosphate + 0.1 mM CaCl ₂ (pH 6.0, 6.8, 8.0, 9.0)	Sodium phosphate gradient from 0.01 to 0.4 M
B	10 mM sodium phosphate + 0.1 mM CaCl ₂ (pH 6.8)	NaCl gradient from 0.03 M to 1.0 M
C	10 mM HEPES + 3 mM CaCl ₂ (pH 6.8)	Sodium Sulfate gradient from 0.01 to 0.6 M
D	10 mM sodium phosphate + 0.1 mM CaCl ₂ (pH 6.8) + 1.0 M boric acid	Sodium phosphate gradient from 0.01 to 0.4 M

Note: Buffer C suppresses P-site function whereas buffer D blocks C-site.

The base buffer component concentration is the same for the initial and the final mobile phase solution. Namely, during the gradient elution base buffer component concentrations do not change.

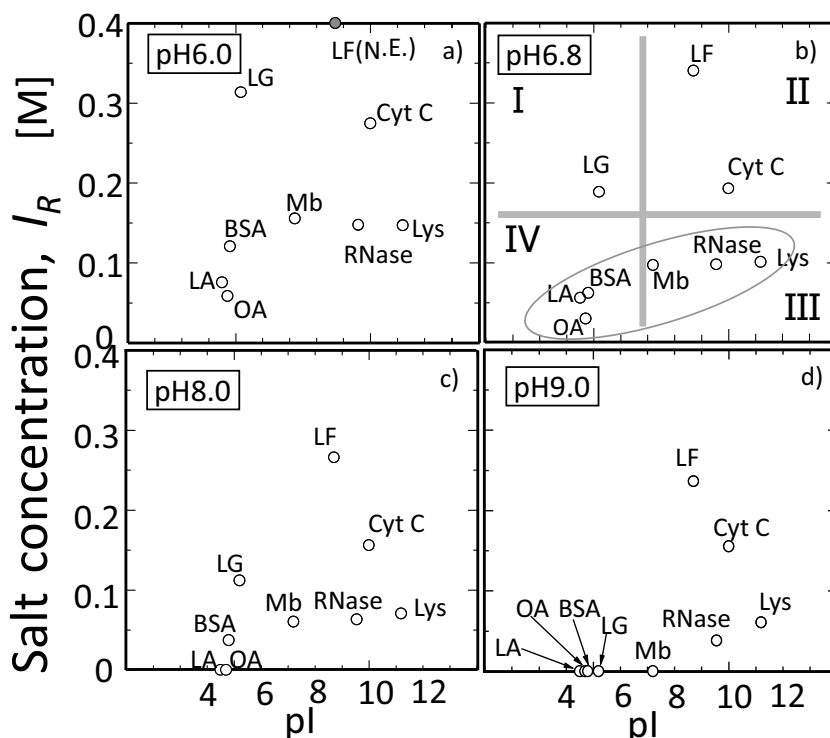


Fig. (1). Peak salt concentration of proteins I_R vs. pI at various mobile phase pH values with the phosphate buffer (Mobile phase A). $GH = 0.0082$ M. N.E. = not eluted. BSA; Bovine serum albumin, Cyt C; Cytochrome C, LA; α -Lactalbumin, LF; Lactoferrin, LG; β -Lactoglobulin, OA; ovalbumin, Lys; Lysozyme, Mb; Myoglobin, RNase; Ribonuclease A.

3.2. Retention Behavior when P-site was Blocked or when NaCl was used as a Gradient Modulator

In order to examine the contribution of the two sites to the retention, LGE experiments were carried out with two different mobile phases (mobile phase B and C, Table 2). Mobile phase B uses NaCl as a gradient modulator (component) whereas Mobile phase C can suppress the P-site function by the addition of Ca ions [13]. The results with four proteins (BSA, lysozyme, lactoferrin, β -lactoglobulin) are shown in Fig. (2). BSA and lysozyme (Lys) are typical acidic and basic proteins, respectively (Fig. 1a).

Lysozyme was retained both by Mobile phase A and B although it was not retained with Mobile phase C containing 30-fold higher Ca concentration than the former mobile phases. This is understandable as basic proteins are mainly retained on the P-site based cation exchange reaction that is blocked by Ca ions. Elution of basic proteins is possible with

phosphate (Mobile phase A) or NaCl (Mobile phase B). When P-site was blocked (Mobile phase C), the retention was no longer possible.

BSA was not eluted with Mobile phase B (NaCl) due to the strong C-site based interaction. When P-site was blocked, the retention volume increased (I_R increased). This is because the repulsion due to P-site was suppressed. Similar results were obtained with ovalbumin (OA).

The I_R values for cytochrome C (Cyt C) and especially for lactoferrin (LF) were much higher than those for other basic proteins. Strong retention of LF was reported in the literature [35]. Similar to Cyt C and LF, β -lactoglobulin (LG) was retained much more strongly compared with other acidic proteins with Mobile phase A.

Lactoferrin (LF) showed different retention behavior compared with lysozyme. Although it was eluted with NaCl (Mobile phase B), much higher NaCl concentration was

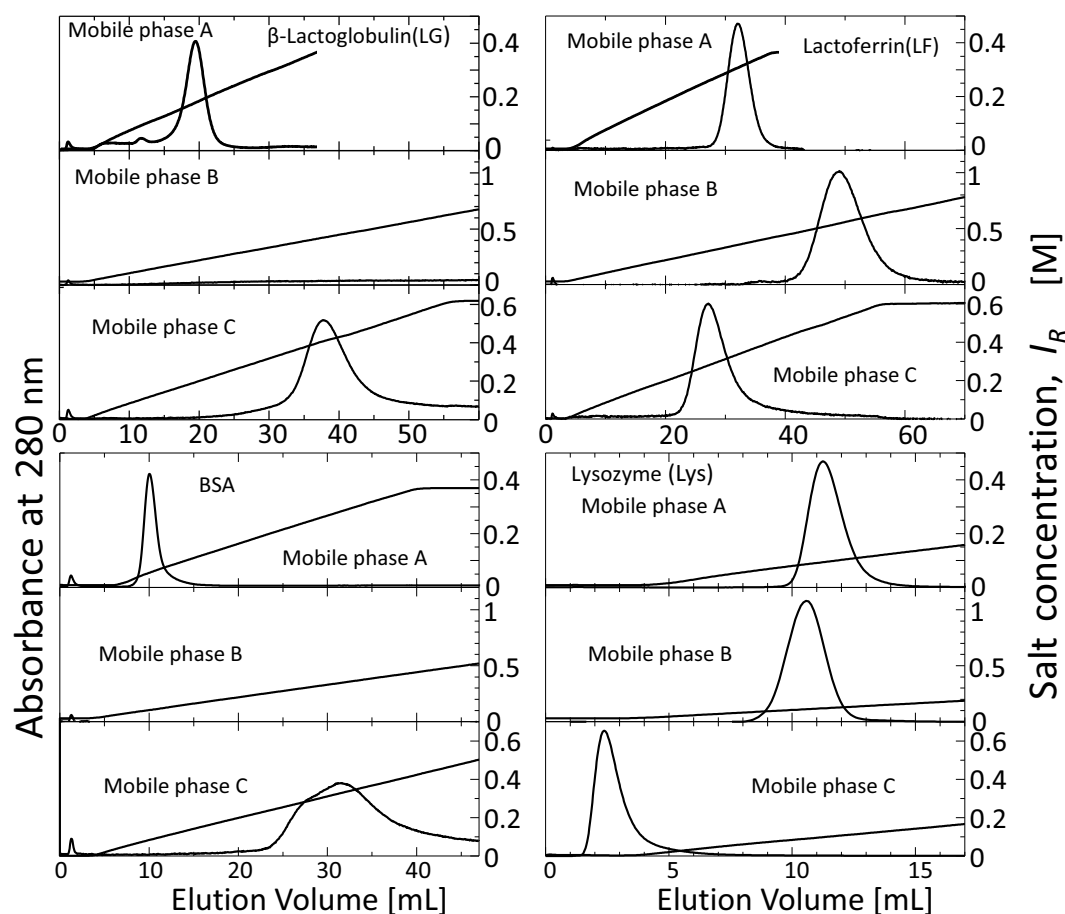


Fig. (2). Linear gradient elution curves of proteins with different mobile phases at pH 6.8 $GH = 0.0082$ M. Mobile phase A: Phosphate buffer (Sodium phosphate gradient), Mobile phase B: Phosphate buffer (NaCl gradient), Mobile phase C: HEPES + 3 mM CaCl_2 (Sodium sulfate gradient).

needed. It was also retained with Mobile phase C. This indicates that LF is retained on HAC with both P- and C-sites. Cytochrome C showed similar retention behavior.

The number of binding sites (effective charges) B was determined from $GH-I_R$ curves for the three mobile phases at pH 6.8. The results are shown in (Fig. 3) along with the I_R values. The B values increased for acidic proteins when Mobile phase C was employed. For two basic proteins (Lys, RNaseA), the B values decreased slightly with Mobile phase B. For another basic protein, LF the B value decreased markedly with Mobile phase B. However, the B value did not drop for Mobile phase C. Similarly, for Cyt C, the B value for Mobile C did not decrease.

3.3. Retention Behavior when C-site was Blocked

A buffer solution containing borate (1M) was proposed by Gagnon [36] to suppress C-site function although this solution was rather difficult to prepare compared with Mobile phase C. With this C-site blocking buffer (Mobile phase D, Table 2) acidic proteins (BSA, OA) and neutral protein (Mb) were not retained whereas a basic protein lysozyme was retained. However, β -lactoglobulin (LG) was retained and eluted by both NaCl and sodium phosphate gradient. LG may have also dual interactions with C- and P-sites.

4. DISCUSSION

The present study has shown generally accepted retention behavior of several model proteins in HAC. Namely, basic proteins were retained due to P-site based cation exchange reaction and acidic proteins were strongly retained due to C-site. The retention became weaker with increasing mobile phase pH. However, several proteins showed different retention behavior.

Repulsion due to P-site for negatively charged biomolecules were already pointed out. For example, DNA binding is favored at high salt concentrations, which can suppress the repulsion due to P-site [20]. By suppressing the P-site function with Mobile phase C, it was possible to elucidate the binding (retention) behavior of proteins.

Four possible binding mechanisms have been proposed [19]; I) Strong affinity due to C-site, II) dual interactions with both C- and P-sites, III) C-site interaction and IV) P-site interaction. The data shown in (Fig. 1b) were tentatively classified according to these four categories.

Cytochrome C (Cyt C) and lactoferrin (LF) were retained even with Mobile phase C. It is therefore likely that both C- and P-site interactions are involved for these two proteins (category II in Fig. 1b).

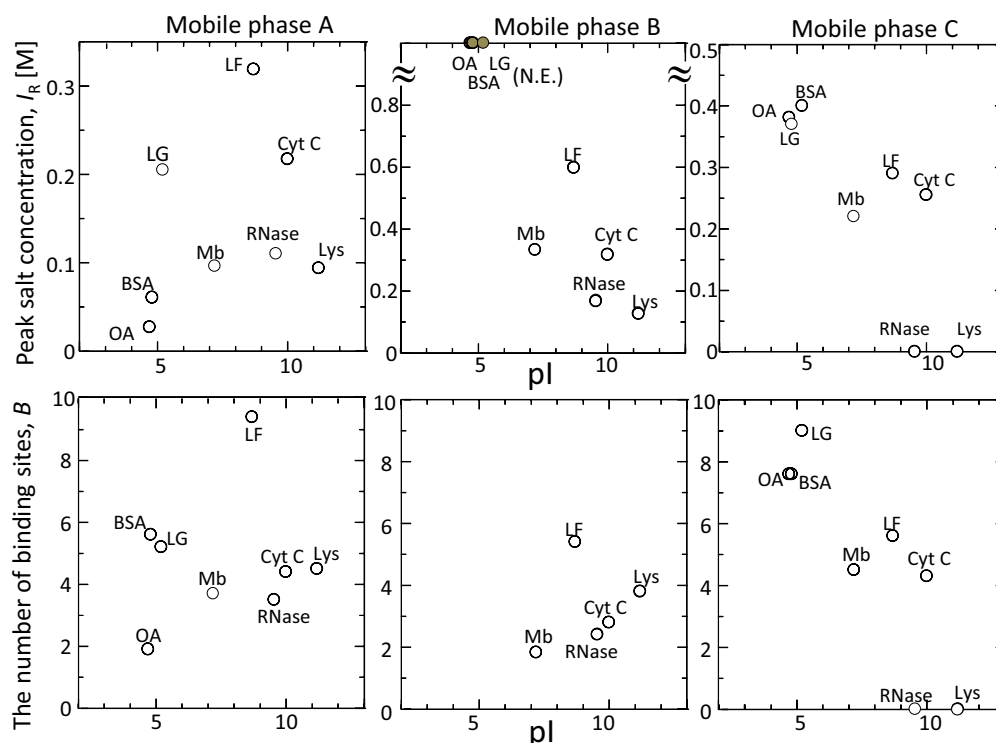


Fig. (3). Peak salt concentration I_R and the number of binding site B for different mobile phases at pH 6.8. B values were obtained from GH - I_R curves ($GH = ca. 0.004$ - 0.05 M). N.E. = not eluted.

BSA; Bovine serum albumin, Cyt C; Cytochrome C, LF; Lactoferrin, LG; β -Lactoglobulin, OA; ovalbumin, Lys; Lysozyme, Mb; Myoglobin, RNase; Ribonuclease A. Mobile phase A: Phosphate buffer (Sodium phosphate gradient), Mobile phase B: Phosphate buffer (NaCl gradient), Mobile phase C: HEPES + 3 mM $CaCl_2$ (Sodium sulfate gradient).

β -lactoglobulin (LG) was another strongly retained protein even with phosphate buffer solutions (Mobile phase A). LG was known to be retained near the pI in both anion- and cation- exchange chromatography [31, 32] due to its asymmetrical charge distribution. Arakawa and Timasheff [37] also reported this unique surface charge distribution of LG based on the solubility data. The contribution of P-site in addition to the strong interaction to the C-site might also explain the strong retention of LG.

Compared with ion-exchange chromatography (IEC), I_R values are relatively low for HAC when phosphate buffer solutions are employed. At pH 6.8 I_R values are below 0.1 M for most proteins. Selectivity and consequently the elution order in HAC are different from those in IEC. Moreover, in some cases as shown in (Fig. 4) the selectivity for basic proteins can be improved by using NaCl gradient instead of sodium phosphate gradient as pointed out in the literature [13-16, 20-22].

Retention of proteins in (pure) electrostatic interaction-based chromatography (EIC) commonly called ion-exchange chromatography (IEC) is rather easy to understand. Both I_R and B values increase with increasing mobile phase pH above the pI of a protein for anion-exchange chromatography (AIEC) [25, 31, 32]. Similarly, both I_R and B values increase with decreasing mobile phase pH below the pI of a protein for cation-exchange chromatography (CIEC). Elution order of proteins basically does not change when a different IEC column is used.

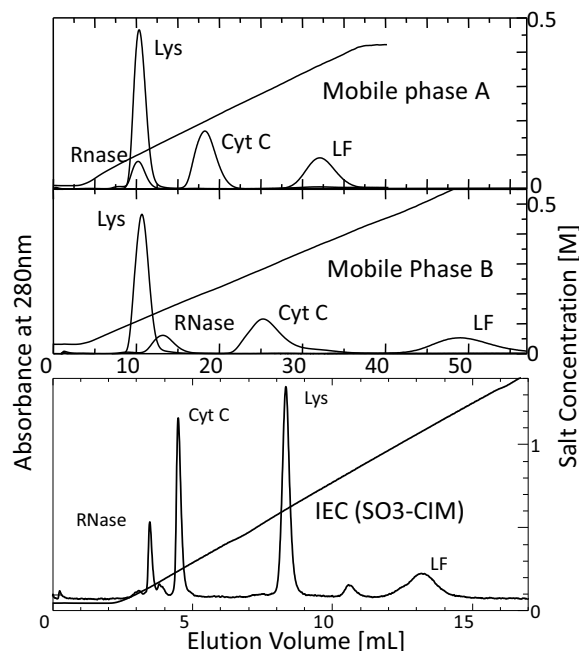


Fig. (4). Linear gradient elution curves of basic proteins in HAC with Mobile phase A and B in comparison with those in IEC. Cyt C; Cytochrome C, LF; Lactoferrin, Lys; Lysozyme, RNase; Ribonuclease A.

HAC, $GH = 0.0082$ M; Mobile phase A: Phosphate buffer (Sodium phosphate gradient), Mobile phase B: Phosphate buffer (NaCl gradient) IEC, 10 mM sodium phosphate buffer (pH 7.0), NaCl gradient from 0.03 M to 2 M, $GH = 0.0127$.

As shown in (Fig. 4), the elution order of four basic proteins in HAC is different from that in IEC. Moreover, the retention can be tuned by changing the mobile phase from Mobile phase A to B. In this case much better resolution can be obtained with Mobile phase B. This is one of the advantages of HAC as the retention and the resolution can be tuned by choosing the right buffer solutions or the gradient substance.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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