Research Article

Calcium chloride and arginine show diametrically opposite effects on antibody elution in Protein A and Protein L chromatography

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Abstract

Protein A and Protein L affinity chromatographies are extensively used in mAb and bispecific antibody (bsAb) purification. In addition to product capture, they are both capable of separating certain product-related by-products and aggregates under appropriate conditions. For both types of chromatography, previous studies suggested that adding a salt additive to the mobile phase can significantly improve the resolution between product and by-products/aggregates. Nevertheless, the effects of different salt additives on antibody elution in Protein A and Protein L chromatography have not been compared. In the current study, we compared the effects of three salt additives, sodium chloride (NaCl), calcium chloride ($CaCl_2$), and arginine hydrochloride (Arg·HCl), on antibody elution in Protein A and Protein L chromatography. Interestingly, while NaCl suppressed antibody elution in both types of chromatography, $CaCl_2$, and Arg·HCl promoted antibody elution in Protein A chromatography but suppressed antibody elution in Protein L chromatography. In addition, we evaluated the effect of each salt gradient on aggregate removal by Protein L chromatography. The information provided by the current study should be useful to the selection of conditions/additives for improving by-product removal by Protein A and Protein L chromatography.

Keywords: Antibody, Arginine, Calcium chloride, Elution, Protein A, Protein L, Retention volume (time), Salt additive, Sodium chloride

1. INTRODUCTION

Protein A and Protein L affinity chromatographies are widely used in mAb and bispecific antibody (bsAb) purification (they are also routinely used for Fc-fusion protein and antibody fragment purification, respectively) [1-3]. While the Protein A ligand mainly binds to the Fc region of IgG1, IgG2, and IgG4, the Protein L ligand binds to the variable region of kappa light chains that belong to subtypes 1, 3, and 4 [4-9]. Antibody-Protein A and antibody-Protein L interactions around neutral pH are dominated by hydrophobic interactions and hydrogen bonding, respectively [4-6,8,9]. In addition to direct product capture, both types of chromatography are capable of separating various product-related impurities under appropriate conditions [10]. For example, both Protein A and Protein L chromatographies can effectively separate half-antibody from the intact bsAb [11,12]. Under optimized conditions, both chromatographies also showed great potential for removing aggregates [13-15]. In addition, Protein A and Protein L chromatographies have been shown to be able to separate the target heterodimer from homodimers in asymmetric bsAb purification [16,17].

In Protein A- and Protein L-mediated by-product removal, salt additives play a critical role in improving resolution. For example, Protein A chromatography provided limited resolution between half-antibody and the full-length bsAb under typical linear pH gradient elution without salt additive, and complete separation was achieved when 500 mM sodium chloride (NaCl) was added to the mobile phase [11]. Salt additives are also required for attaining good aggregate removal by Protein A and Protein L chromatography [13-15]. In the case of homodimer removal by differential Protein A chromatography (in such a scenario, one of the bsAb's heavy chains is mutated to ablate the

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Protein A binding capacity and hence results in asymmetric Protein A binding between the two heavy chains), salt additive significantly improved the resolution between heterodimer and homodimer [16]. According to previous studies, both kosmotropic and chaotropic salts, when used as mobile phase additives, could improve the resolution of Protein A and Protein L chromatography [11,14-16,18].

For both Protein A and Protein L chromatography, antibody elution is accomplished at low pH under which conditions disassociation is driven by strong electrostatic repulsions between positively charged residues of Protein A/Protein L ligands and the bound antibody [6,9,19]. In the current work, using a recombinant human IgG1 with a kappa light chain, we compared the effects of three salt additives, that is, NaCl, calcium chloride ($CaCl_2$), and arginine hydrochloride (Arg·HCl), on antibody elution in Protein A and Protein L chromatography. These three salts have been widely used as mobile phase additives in Protein A and Protein L chromatography to improve the resolution between product and by-products/aggregates [11,14-16,18]. Data from the current study suggested that while NaCl exerted the same effect in both types of chromatography, $CaCl_2$ and Arg·HCl had opposite effects on antibody elution in Protein A and Protein L chromatography. Specifically, $CaCl_2$ and Arg·HCl promoted antibody elution in Protein A chromatography, and they suppressed antibody elution in Protein L chromatography. This piece of information should be useful when selecting appropriate salt additive for improving resolution in Protein A and Protein L chromatography.

2. MATERIALS AND METHODS

2.1. Materials

Arginine hydrochloride, calcium chloride, sodium acetate trihydrate, sodium chloride, sodium hydroxide, and Tris(hydroxymethyl) aminomethane were purchased from Merck (Darmstadt, Germany). Acetic acid was procured from J. T. Baker (Phillipsburg, NJ, USA). MabSelect SuRe LX, MabSelect VL, and Tricorn 5/150 column (0.5 cm I.D.) were bought from Cytiva (Uppsala, Sweden). TSKgel G3000SWXL stainless steel column (5 μm, 7.8 × 300 mm) was from TOSOH (Tokyo, Japan). The human IgG1 used in the current study was expressed in stably transfected CHO-K1 cells grown in HyClone ActiPro culture medium supplemented with Cell Boost 7a and 7b (HyClone feeding supplements). The cell culture was allowed to grow for 14 days before harvest.

2.2. Equipment

An AKTA pure 150 system installed with Unicorn software version 7.8 (Cytiva, Uppsala, Sweden) was used for column chromatography. pH and conductivity were measured using the SevenExcelence S470 pH/Conductivity Meter (Mettler-Toledo, Columbus, OH, and USA). Protein concentration was measured on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). An Agilent 1260 liquid chromatography instrument (Agilent Technologies, Santa Clara, CA, USA) was employed for size-exclusion chromatography-high performance liquid chromatography (SEC-HPLC) analysis.

2.3. Protein A chromatography

MabSelect SuRe LX resin was packed in a 0.5 cm diameter column with a 15.1 cm bed height. The column volume (CV) was approximately 3.0 mL. The loading material was clarified cell culture harvest, and the column was loaded at 30 mg of protein per mL of resin. After loading, the column was washed consecutively with 50 mM Tris, HAc, 150 mM NaCl, pH 7.4, and buffer A used in the linear pH gradient elution, each for 3 CV. The column was eluted with a linear pH gradient (buffer A: 50 mM NaAc-HAc, pH 5.5; buffer B: 50 mM NaAc-HAc, pH 3.0; 0 – 100% B over 20 CV). A reference run was conducted without adding any salt additive to the two gradient generation buffers. For each of the three salt additives under study, different amounts (from 250 to 1000 mM at an interval of 250) were added to both buffer A and buffer B. The system was run at a flow rate of 180 cm/h (residence time: 5 min).

2.4. Protein L chromatography

MabSelect VL resin was packed in a 0.5 cm diameter column with a 15.2 cm bed height. The CV was approximately 3.0 mL. The loading material was clarified cell culture harvest, and the column was loaded at 30 mg of protein per mL of resin. On loading, the column was washed consecutively with 50 mM Tris, HAc, 150 mM NaCl, pH 7.4, and buffer A in the linear pH gradient elution, each for 3 CV. The column was eluted with a linear pH gradient (buffer A: 50 mM NaAc-HAc, pH 5.5; buffer B: 50 mM NaAc-HAc, pH 3.0; 0 – 100% B over 20 CV). A reference run was conducted without the addition of any salt additive to the two gradient generation buffers. For each of the three salt additives under study, different amounts (from 50 to 200 mM at an interval of 50) were added to both buffer A and buffer B. For runs conducted to demonstrate the effects of the three salts on aggregate removal, a linear salt gradient (100-0 mM) at pH 4.0 was first adopted. Next, based on the results from the linear conductivity gradient, stepwise gradient chromatography with each salt (13 mM NaCl, 10 mM $CaCl_2$, and 15 mM Arg·HCl) at the same pH was separately performed. The system was run at a flow rate of 180 cm/h (residence time: 5 min).

2.5. SEC-HPLC

SEC-HPLC was performed as previously described [20].

In brief, runs were conducted on an Agilent 1260 liquid chromatography instrument using a TOSOH TSKgel G3000SWXL stainless steel column (7.8 × 300 mm). 100 µg of sample was injected per run. The mobile phase consisted of 50 mM sodium phosphate and 300 mM sodium chloride at pH 6.8. Each sample was eluted isocratically for 20 min at a flow rate of 1.0 mL/min. Protein elution was monitored by ultraviolet (UV) absorbance at 280 nm.

3. RESULTS AND DISCUSSION

3.1. Effect of NaCl on antibody elution in Protein A and Protein L chromatography

As shown in Figure 1A, adding NaCl to the Protein A mobile phase increased the retention as reflected by the retention volume at the elution peak UV maximum. The observed effect of NaCl on antibody elution in Protein A chromatography was consistent with that reported in previous studies [18]. For the five runs conducted, NaCl concentration was increased from 0 to 1000 mM at an interval of 250. Although the increase in retention was not proportional to the rise in NaCl concentration, the effect of NaCl on antibody elution was clear. Antibody elution from the Protein A column at low pH is driven by electrostatic repulsion between positively charged residues of the Protein A ligand and bound antibody. The presence of NaCl shields electrostatic repulsion. In addition, antibody-Protein A interaction is dominated by hydrophobic interactions, and NaCl, as a kosmotropic salt, can strengthen non-polar interactions by increasing the surface tension of the solvent. These two effects of NaCl enhanced antibody-Protein A interaction and therefore resulted in the increased retention volume (time) as observed.

Adding NaCl to the Protein L mobile phase had the same effect as that observed with Protein A (Figure 1B). Nevertheless, the same concentration of salt had a greater impact on elution in Protein L chromatography. Therefore, in this study, the NaCl concentration was increased from 0 to 200 mM at an interval of

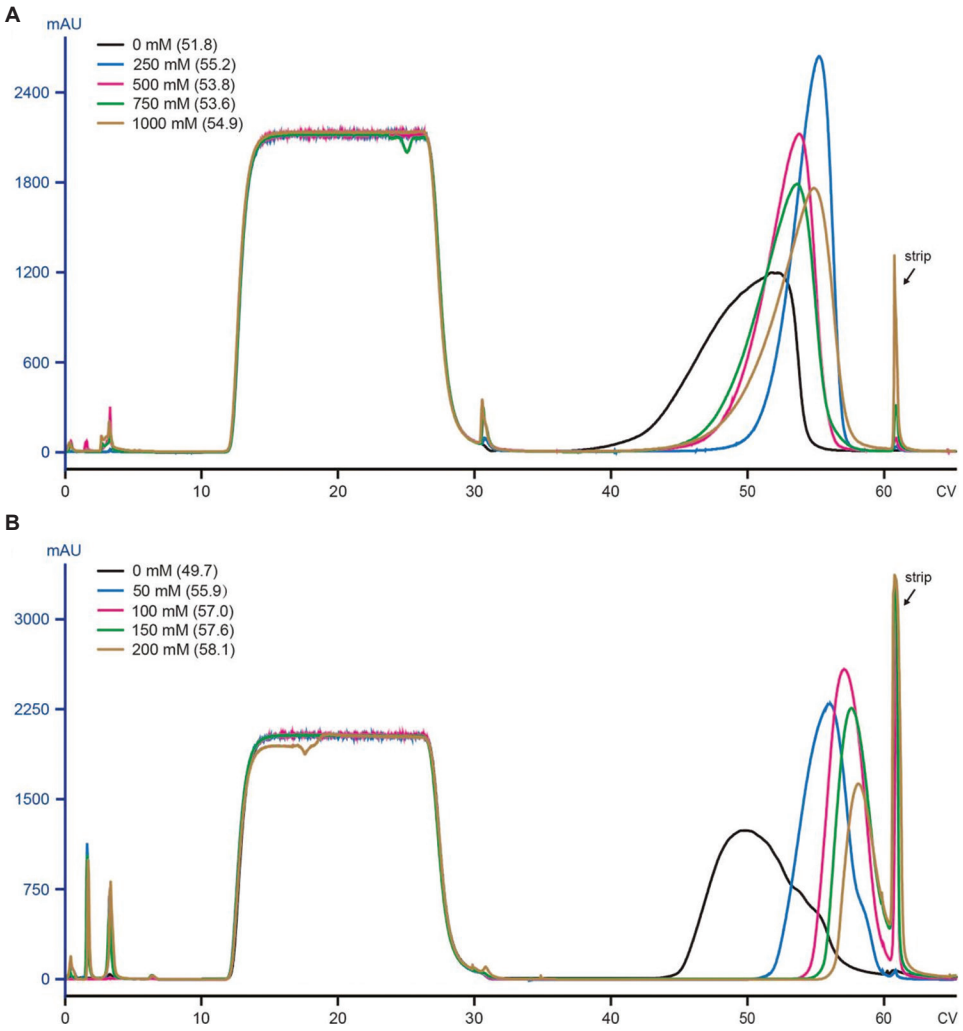

Figure 1. Overlay of (A) Protein A and (B) Protein L chromatograms. Runs were conducted under linear pH gradient elution with different amounts of NaCl being added to the mobile phase. The numbers in parentheses are retention volumes in terms of column volume at the elution peak ultraviolet maximum.

50. As can be seen from the chromatograms (Figure 1B), at the highest NaCl concentration tested (i.e., 200 mM), only a portion of the bound antibody was eluted. Similar to the situation in Protein A, the antibody and Protein L ligand are both positively charged at low elution pH, and disassociation is caused by electrostatic repulsion. The presence of NaCl masked repulsive interactions and exerted no effect on hydrogen bonding, the predominant force in antibody-Protein L interaction. Thus, the overall effect of NaCl is strengthening of antibody-Protein L interaction, leading to increased retention volume (time) and reduced antibody recovery from eluate.

3.2. Effect of $CaCl_2$ on antibody elution in Protein A and Protein L chromatography

In a prior study, we examined the effect of $CaCl_2$ on antibody elution in Protein A chromatography [13]. We tested four $CaCl_2$ concentrations (i.e., 250, 500, 750, and 1000 mM) in the mobile phase. For the first three concentrations, the antibody retention time was gradually reduced with increasing $CaCl_2$ concentrations. When $CaCl_2$ concentration was increased to 1000 mM, the antibody retention time was not further reduced and was instead roughly identical to that at 500 mM $CaCl_2$. Data from the current study largely confirmed the previous results (Figure 2A). The only difference was that, in the current study, retention volume (time) was further reduced when $CaCl_2$ concentration was increased to 1000 mM. This discrepancy observed at the highest $CaCl_2$ concentration tested might be ascribed to the difference in inherent properties of the antibodies used for the studies. Although $CaCl_2$ can enhance antibody-Protein A interaction by shielding electrostatic repulsion, as a chaotropic salt, it weakens non-polar interactions. It seems that the effect of weakening hydrophobic interactions overwhelmed that of masking electrostatic repulsion and therefore resulted in reduced retention volume (time).

In the case of Protein L chromatography, the presence of $CaCl_2$ in the mobile phase enhanced antibody binding, and

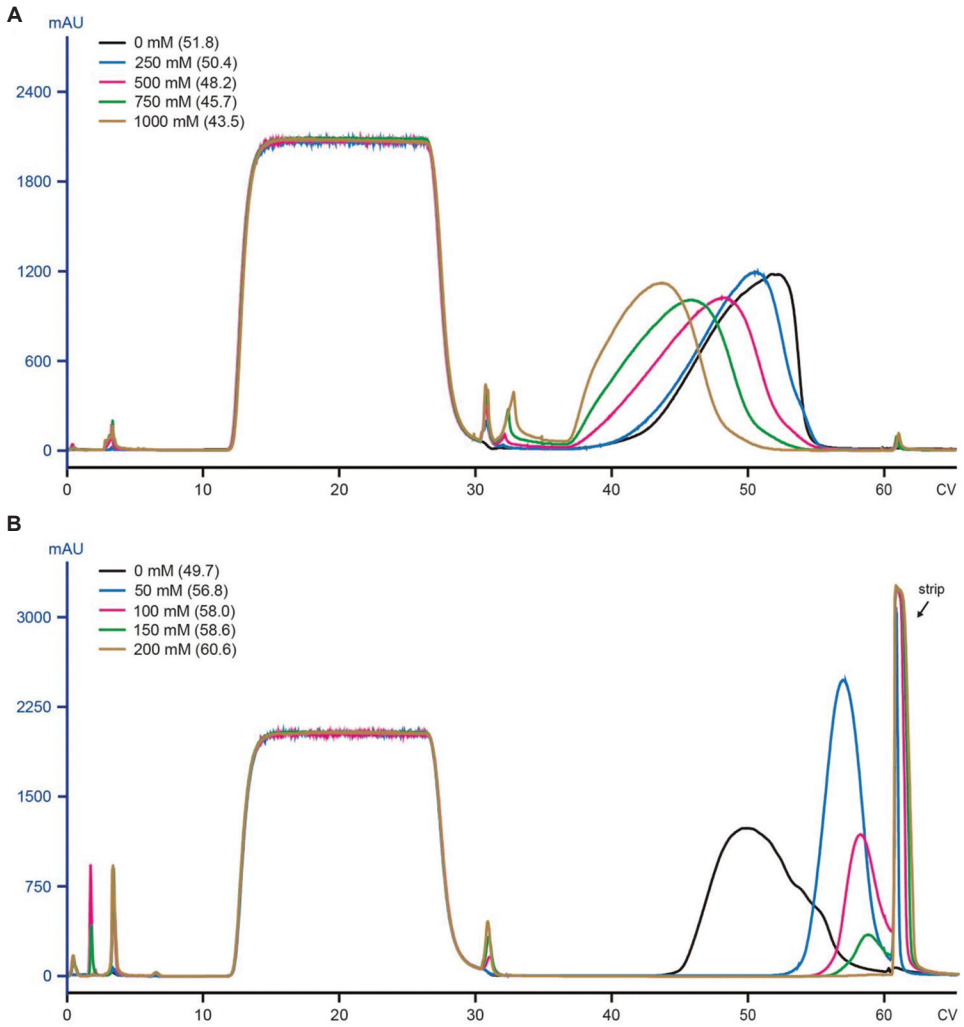

Figure 2. Overlay of (A) Protein A and (B) Protein L chromatograms. Runs were conducted under linear pH gradient elution with different amounts of $CaCl_2$ being added to the mobile phase. The numbers in parentheses are retention volumes in terms of column volume at the elution peak ultraviolet maximum.

hence increased antibody retention volume (time) (Figure 2B). Even a small increase in $CaCl_2$ concentration resulted in a big difference in antibody recovery from the eluate. At the highest $CaCl_2$ concentration tested (i.e., 200 mM), the bound antibody could not be eluted. At the presence of 150 mM and 100 mM of $CaCl_2$, approximately 10% and 40% of the loaded antibody was recovered from the eluate, respectively. Thus, the effect of $CaCl_2$ on protein elution in Protein L chromatography was dramatically opposite to that in Protein A chromatography. Like NaCl, $CaCl_2$ had no effect on hydrogen bonding and masked electrostatic repulsion between antibody and Protein L ligand under low pH. Therefore, $CaCl_2$ strengthened antibody-Protein L interaction. The difference in effects exerted by $CaCl_2$ on Protein A and Protein L lies in that $CaCl_2$ weakens hydrophobic interactions (the predominant force for antibody-Protein A interaction) whereas has no effect on hydrogen bonding (the predominant force driving antibody-Protein L interaction).

3.3. Effect of Arg·HCl on antibody elution in Protein A and Protein L chromatography

Previous studies demonstrated that arginine promoted antibody elution in Protein A [21-23]. Specifically, arginine effectively elutes mAbs at mildly acidic pH. Moreover, arginine was shown to promote protein elution in ion exchange [24], hydrophobic interaction [24,25], and mixed-mode [26-30] chromatography. Arginine's effect on antibody elution in Protein A chromatography was confirmed in the current study. As shown in Figure 3A, antibody retention in Protein A was reduced with increasing concentration of Arg·HCl in the mobile phase. Although Arg·HCl could shield electrostatic repulsion at low pH, it diminished hydrophobic interactions, which predominated antibody-Protein A interaction. The overall effect of Arg·HCl resulted in a significantly weakened interaction between the antibody and Protein A ligand.

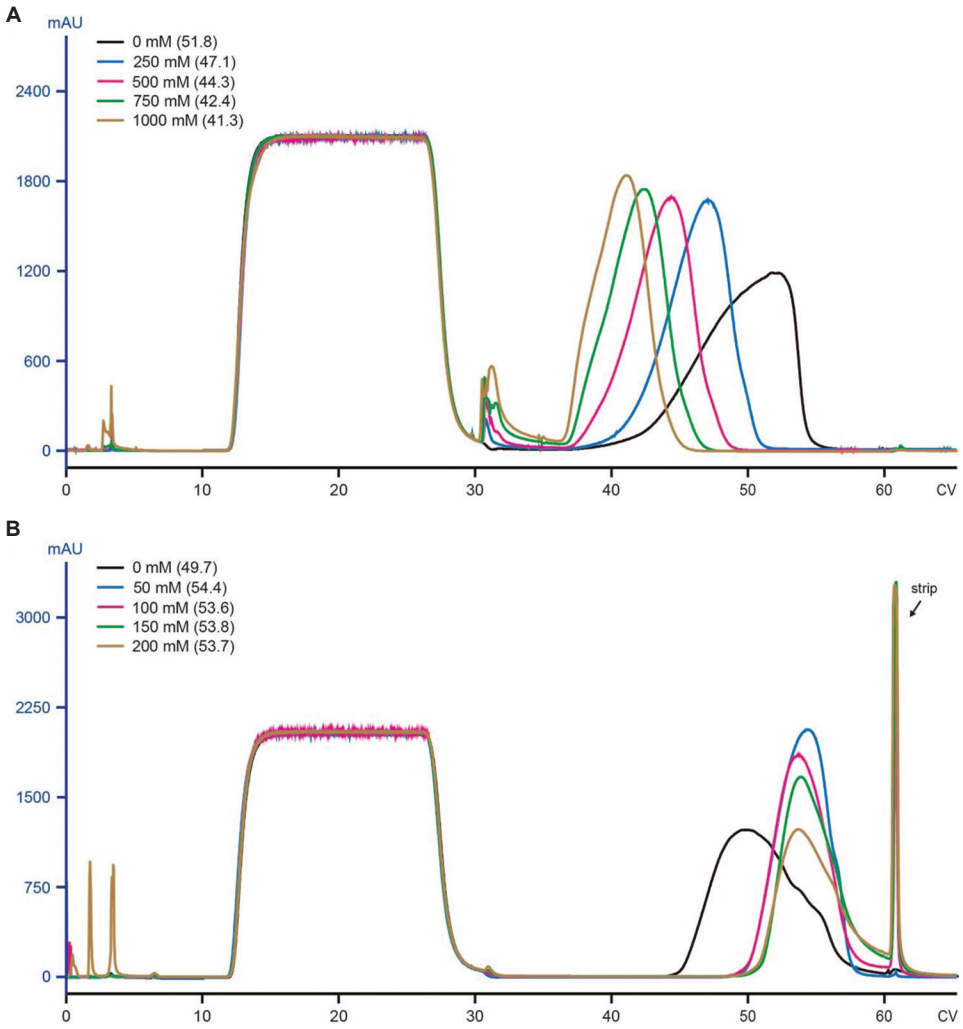

Figure 3. Overlay of (A) Protein A and (B) Protein L chromatograms. Runs were conducted under linear pH gradient elution with different amounts of Arg·HCl being added to the mobile phase. The numbers in parentheses are retention volumes in terms of column volume at the elution peak ultraviolet maximum.

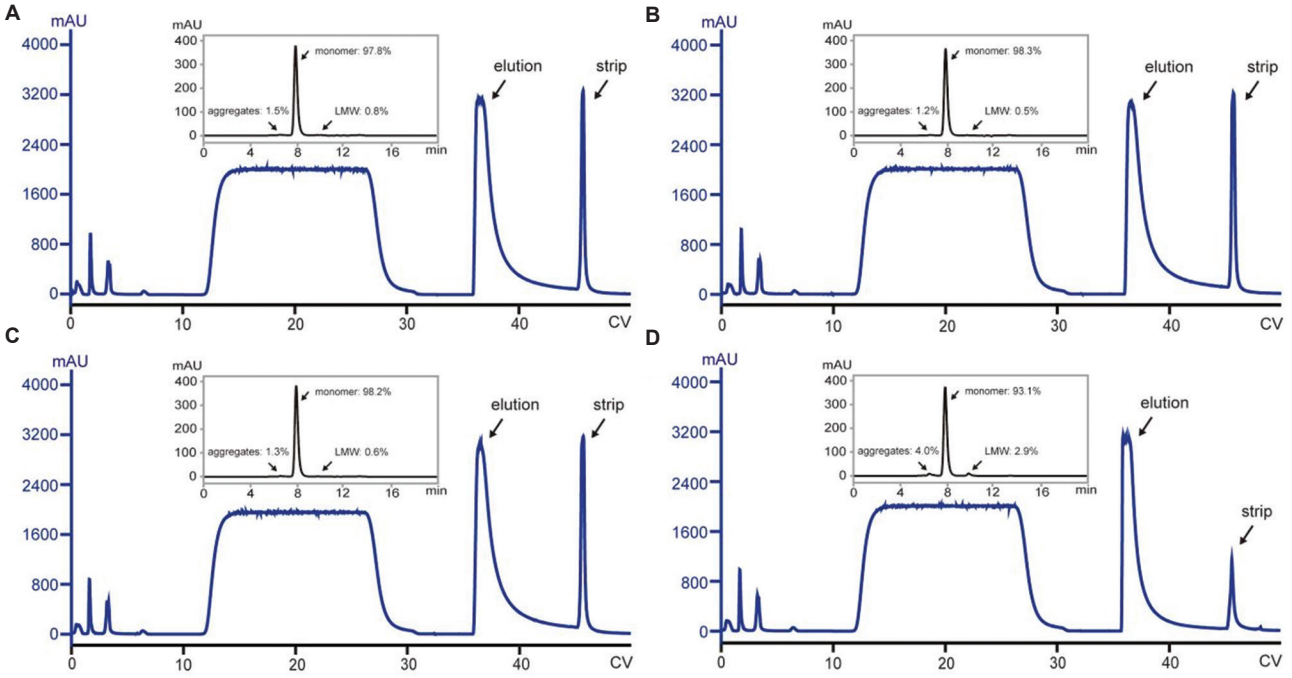

Figure 4. Protein L chromatograms of four runs with (A) 13 mM NaCl, (B) 10 mM $CaCl_2$, (C) 15 mM Arg·HCl, and (D) no salt addition to the pH 4.0 elution buffer. Inset, size-exclusion chromatography-high performance liquid chromatography profile of the elution pool.

As shown in Figure 3B, adding Arg·HCl to the Protein L mobile phase increased the retention volume (time), suggesting that Arg·HCl strengthened antibody-Protein L interaction. At the highest Arg·HCl concentration (i.e., 200 mM), only ~70% of the loaded antibody was recovered from the eluate. When Arg·HCl concentration was increased to 500 mM and above, antibody elution was completely inhibited under the pH gradient (data not shown). Thus, the effect exerted by Arg·HCl on antibody elution in Protein L chromatography was opposite to that in Protein A chromatography. Although Arg·HCl can shield electrostatic repulsion, it disrupts hydrogen bonding (arginine's guanidinium group and carboxyl group can form hydrogen bonding with Protein L ligand and, therefore, arginine can compete with the antibody for binding), the predominant force for antibody-Protein L interaction. Considering the overall effect of Arg·HCl, a combination of masking of electrostatic repulsions and weakening of hydrophobic interactions, resulted in significantly weakened binding in Protein A chromatography, the overall effect of Arg·HCl on antibody binding in Protein L chromatography was unexpected.

3.4. Improved aggregate removal by Protein L chromatography under conductivity gradient using different salts

Previous studies suggested that Protein L chromatography could provide good aggregate separation under linear conductivity gradient at a defined pH [13]. As the three salts under study (NaCl, $CaCl_2$, and Arg·HCl) had the same suppression effect on antibody elution in Protein L chromatography, we compared their effects on aggregate removal when they were separately used as conductivity gradient generators. For this study, we used the same loading material, which contained approximately 8.1% of aggregates, as that used for the study on antibody elution. For each salt, we initially tested a linear conductivity gradient (100-0 mM) at a defined pH (i.e., 4.0) (data not shown). Eventually, a stepwise conductivity gradient was developed based on the linear gradient results. As shown in Figure 4, controlled conductivity using each salt at the defined pH (i.e., 4.0) allowed for the effective separation of monomers from aggregates. For runs whose mobile phase conductivity was adjusted with NaCl, $CaCl_2$, and Arg·HCl, the content of aggregates in the eluate was reduced to 1.5%, 1.2%, and 1.3%, respectively (Figure 4A-C). For the run without using any salt additive, the content of aggregates in the eluate was 4.0% (Figure 4D). The data suggested that Protein L had good aggregate-removing capability under regular conditions and the effect could be further improved by adding the above salts to the mobile phase.

4. CONCLUSION

In the current study, we compared the effects of three salt additives (i.e., NaCl, $CaCl_2$, and Arg·HCl) on antibody elution in Protein A and Protein L chromatography. The data suggested that NaCl suppressed antibody elution in both Protein A and Protein L chromatography whereas $CaCl_2$ and Arg·HCl promoted antibody elution in Protein A but

suppressed antibody elution in Protein L chromatography. The different effects exhibited by $CaCl_2$ toward Protein A and Protein L chromatography is easy to understand as $CaCl_2$ weakens hydrophobic interactions but has no effect on hydrogen bonding (hydrophobic interactions and hydrogen bonding are the predominant forces driving antibody-Protein A and antibody-Protein L interactions, respectively). However, a straightforward explanation for the different effects exhibited by Arg·HCl toward Protein A and Protein L chromatography is not readily available. Arg·HCl is known to weaken both hydrophobic interactions and hydrogen bonding. In addition to Protein A chromatography, arginine was shown to promote protein elution in ion exchange [24], hydrophobic interaction [24,25], and mixed-mode [26-30] chromatography. Thus, it seems that the effect exhibited by arginine in Protein L chromatography is unique as it is opposite to that observed in all other types of chromatography.

Affinity chromatography can be considered as mixed-mode since normally more than one type of interaction is involved. A difference between Protein L and those mixed-mode media lies in that hydrogen bonding is the predominant and secondary force in bindings mediated by the former and the latter, respectively. Arginine can form hydrogen bonds with both Protein L ligands and antibodies. In addition, arginine is known to form clusters [31]. It is possible that while arginine can compete with antibodies for binding sites in the Protein L ligand, the arginine cluster, at the same time, can bridge the interaction between the Protein L ligand and the antibody, resulting in strengthened interactions. The exact mechanism through which Arg·HCl strengthens antibody-Protein L interaction warrants further investigation.

In consistency with the similar effect exhibited by all three salts (i.e., NaCl, $CaCl_2$, and Arg·HCl) on antibody elution in Protein L chromatography, they provided equally good separation between monomer and aggregates when used as conductivity gradient generators. The information provided by the current study should be conducive to the selection of conditions/additives for improving by-product removal by Protein A and Protein L chromatography.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: Yifeng Li
Data curation: All authors
Formal analysis: All authors
Methodology: Yifeng Li
Investigation: Yifeng Li
Writing – original draft: Yifeng Li
Writing – review & editing: All authors

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Informed consent was obtained from all participants

CONSENT FOR PUBLICATION

The participants gave consent to publish their data in this study.

AVAILABILITY OF DATA

The data and supportive information are all included in the article.

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