Research Article

# CH1-specific affinity resins possess the potential of separating heterodimer from homodimers in asymmetric bispecific antibody purification

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## Abstract

CaptureSelect CH1-XL and Praesto 70 CH1 are two affinity media that specifically bind to the CH1 domain of an antibody. In the current work, we first demonstrated that these two CH1-specific affinity media bound to different monoclonal antibodies (mAbs) with varied strengths under identical conditions. We previously had observed the same on a Protein L-conjugated resin and showed that such a property could facilitate homodimer removal in asymmetric bispecific antibody (bsAb) purification. Next, using Praesto 70 CH1, we showed that a small difference in binding between two mAbs could be significantly exaggerated by adding sodium chloride to the mobile phase, further demonstrating this resin can potentially play a role in bsAb purification. Finally, with a concrete bsAb case study, we showed that, like Protein L, Praesto 70 CH1 could separate the target heterodimer from the homodimer by-product. Homodimers are common product-related impurities associated with the recombinant production of asymmetric bsAbs, which can be difficult to remove. Their removal, even a partial one, at the capture stage is a big advantage as it can alleviate the purification burden on subsequent polishing steps and render the overall process more robust. Therefore, Praesto 70 CH1's unique property is highly desirable, and this affinity resin can be a better alternative than Protein A for product capture in asymmetric bsAb purification.

**Keywords:** Bispecific antibody, CaptureSelect CH1-XL, Heterodimer, Homodimer, Praesto 70 CH1

## 1. INTRODUCTION

Protein A affinity chromatography has been extensively used in antibody purification [1,2]. Besides Protein A, other antibody-binding affinity media that bind to different regions of an antibody have also been developed [3-6]. For example, Protein L-conjugated resins bind to the variable region of kappa light chain [3,4]; KappaSelect and LambdaFabSelect media bind to the constant region of kappa and lambda light chains, respectively [5]; CaptureSelect FcXP binds to the CH3 domain of heavy chain [6]. Although most of these non-Protein A affinity media are originally developed and primarily used for capturing antibody fragments lacking the Fc region [7], they can be used to capture full-length antibodies as well. Furthermore, when used to capture full-length antibodies, some of these non-Protein A affinity media exhibit certain advantages over Protein A media. For instance, both Protein L and CaptureSelect FcXP resins have shown much stronger aggregate-removing capabilities than Protein A [8,9]. For regular monoclonal antibody (mAb) purification, the major task of the affinity step is product capture and removal of process-related impurities (e.g., host cell proteins and DNA), whereas removal of product-related impurities is often left to the polishing steps. However, for complex molecules such as bispecific antibodies (bsAbs), product-related impurities are often present at increased types and levels [10,11], and totally rely on the polishing steps for by-product removal in these cases will result in a less effective and non-robust process. Thus, affinity media possessing strong by-product-removing capabilities are highly desirable for the purification of challenging molecules, in which they can play a more important role than product capture [12].

CaptureSelect CH1-XL and Praesto 70 CH1 are two affinity media from Thermo Fisher and Purolite, respectively, which specifically bind to the CH1 domain on

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the heavy chain of human immunoglobulin G (IgG) [13,14]. CaptureSelect CH1-XL's ligand is based on a 13-kDa llama heavy chain antibody fragment, which recognizes the CH1 domain of human IgG (all four subclasses), and its matrix is epoxide-activated agarose [13]. For Praesto 70 CH1, the detailed information of the ligand is not disclosed by the vendor and its matrix is highly cross-linked agarose [14]. In the current study, we demonstrated that these two CH1-specific resins bound different antibodies with varied strengths under identical conditions (as indicated by different retention volumes of individual mAbs). Such a property was previously seen in a Protein L-conjugated resin [15]. More importantly, we demonstrated that this property could facilitate separation of heterodimer from homodimer by-products in asymmetric bsAb purification [15]. The separation is based on the mechanism that when the resin binds the two parental mAbs from which the asymmetric bsAb is derived with different strengths, it binds the heterodimer with an intermediate strength, which is different from that toward either of the two homodimers/parental mAbs (Figure 1). Using Praesto 70 CH1, we further showed that a small difference in binding between two mAbs could be significantly magnified by adding sodium chloride to the mobile phase, allowing for complete separation of the two mAbs. This suggested that separation between heterodimer and homodimers can be substantially improved by following the same strategy, further demonstrating the practical value of CH1-specific resins in asymmetric bsAb purification. Finally, with a real bsAb case, we exhibited that Praesto 70 CH1 could effectively separate the heterodimer from homodimer by-product as expected. For bsAb purification, removing product-related impurities, even partially, at the capture stage, is highly desirable, as it can significantly alleviate the purification burden on polishing steps and improve the robustness of the overall downstream process. Thus, the unique property of Praesto 70 CH1 makes this resin an attractive alternative to Protein A for product capture in asymmetric bsAb purification.

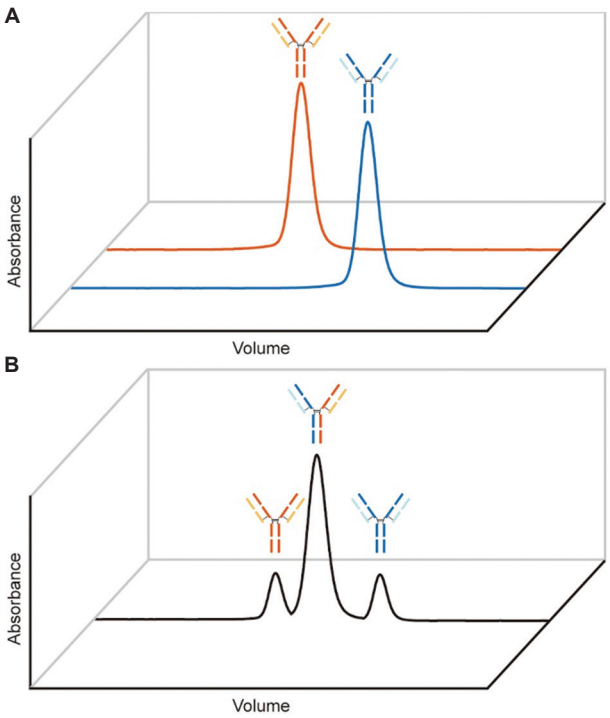


**Figure 1.** Schematic representation of rationale behind homodimer removal by affinity resins that exhibit varied binding strengths towards different monoclonal antibodies (mAbs). (A) Certain affinity resin may bind two mAbs with varied strengths under identical conditions as suggested by the different retention volumes. (B) When an asymmetric bispecific antibody is constructed based on the above two mAbs, the resin binds to the heterodimer with an intermediate strength, enabling separation of the heterodimer from the two potential homodimers.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Citric acid monohydrate, sodium chloride, sodium hydroxide, tri-sodium citrate dihydrate, and Tris(hydroxymethyl)aminomethane were purchased from Merck (Darmstadt, Germany). Acetic acid was procured from J.T. Baker (Phillipsburg, NJ, USA). MabSelect SuRe LX and Tricorn 5/150 column (0.5 cm I.D.) were bought from Cytiva (Uppsala, Sweden). CaptureSelect CH1-XL, 20X MES running buffer and 4X LDS sample buffer were from Thermo Fisher Scientific (Waltham, MA, USA). Praesto 70 CH1 came from Purolite (King of Prussia, PA, USA). Precast SurePAGE 4-12% gradient Bis-Tris gels were bought from GenScript (Nanjing, Jiangsu, China). Precision Plus Protein Unstained Standards was from Bio-Rad Laboratories (Hercules, CA, USA). Home-made protein markers were also used. All seven mAbs (human IgG1) and the bsAb (which was derived from human IgG4 with kappa light chain) used in the current study were expressed in stably transfected CHO-K1 cells grown in HyClone ActiPro culture medium supplemented with Cell Boost 7a and 7b (HyClone feeding supplements). In each case, the cell culture was allowed to grow for 14 days before harvest.

### 2.2. Equipment

An ÄKTA pure 150 system installed with Unicorn software version 7.8 (Cytiva, Uppsala, Sweden) was used for column chromatography. pH and conductivity were measured using SevenExcelence S470 pH/Conductivity Meter (Mettler-Toledo, Columbus, OH, USA). Protein concentration was measured on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The bioreactor system from Applikon Biotechnology (Delft, Netherlands) was utilized for cell cultivation.

### 2.3. MabSelect SuRe LX Protein A and CaptureSelect CH1-XL chromatography

MabSelect SuRe LX and CaptureSelect CH1-XL resins were individually packed in a 0.5-cm diameter column with a 15.0-cm and a 15.2-cm bed height, respectively. The column volumes (CVs) were approximately 2.9 and 3.0 mL, respectively. For both types of columns, they were loaded with clarified culture harvest of each of the seven mAbs (without dilution or further adjustment) at 5 mg of protein per mL of resin. After loading, the column was washed consecutively with 50 mM Tris, 42.3 mM HAc, 150 mM NaCl, pH 7.4 and 20 mM citrate, pH 6.0 (buffer A), each for 5 CV. The column was eluted with a linear pH gradient (buffer B: 20 mM citrate, pH 3.0; 0-100% B over 20 CV). The system was run at a flow rate of 180 cm/h (residence time: 5 min).

### 2.4. Praesto 70 CH1 chromatography

Praesto 70 CH1 resin was packed in a 0.5-cm diameter column with a 15.2-cm bed height. The CV was approximately 3.0 mL. For studies aimed at assessing the binding strength toward the seven different mAbs, for each antibody, the column was loaded with clarified culture harvest (without dilution or further adjustment) at 5 mg of protein per mL of resin. All runs were conducted by following the same protocol as previously described for MabSelect SuRe LX and CaptureSelect CH1-XL chromatography. For separation confirmation and optimization studies, artificial mixtures containing mAb 1 and mAb 5 or mAb 3 and mAb 5 were employed as load materials. For confirmation runs with the first and the second artificial mixtures (clarified culture harvests of mAb 1 and mAb 5 or mAb 3 and mAb 5, respectively, were combined at a 1:1 mass ratio), they were conducted according to the same protocol as that used for individual mAbs without addition of any salt to the two gradient generation buffers. For separation optimization runs with the second artificial mixture, different amounts of sodium chloride (50, 100 and 200 mM) were added to the two gradient generation buffers (buffer A: 20 mM citrate, pH 6.0; buffer B: 20 mM citrate, pH 3.0). For runs aimed at confirming Praesto 70 CH1's homodimer-removing capability in a real bsAb case, regular linear pH gradient elution was first applied. Next, 500 mM sodium chloride was added to both buffer A and buffer B to improve resolution. Finally, based on the result of the optimized linear gradient elution, stepwise gradient elution (40% and 90% buffer B) was performed. All runs were conducted at a flow rate of 180 cm/h (residence time: 5 min).

### 2.5. Non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Non-reducing SDS-PAGE was performed using precast SurePAGE 4-12% gradient Bis-Tris gels from GenScript as previously described [9]. Sample loading buffer (4X LDS) and gel running buffer (20X MES) from Thermo Fisher Scientific were used. All samples were heated at 75°C for 5 min. After cooling down, they were loaded at equal protein amount (~0.5 μg per well). Electrophoresis was carried out at 120 V for 100 min (or 100 V for 180 min in the bsAb case). Gels were stained and destained using eStain LG protein staining system from GenScript.

## 3. RESULTS AND DISCUSSION

### 3.1. CH1-specific affinity resins bind different mAbs with varied strengths under identical conditions

CaptureSelect CH1-XL is a relatively new affinity medium that specifically binds to the CH1 region of an antibody. While using this resin to capture different mAbs, we noticed that under identical conditions individual mAbs showed different retention volumes, suggesting that they bound to this resin with different strengths. We previously observed the same on a Protein L-conjugated resin and demonstrated that such a property of the resin could facilitate homodimer removal in asymmetric bsAb purification [15]. Therefore, we were led to find out whether CH1-specific affinity resins really share the same property with Protein L resins. To better characterize CH1-specific affinity resins, we designed a comparison study, which included MabSelect SuRe LX Protein A resin, CaptureSelect CH1-XL and Praesto 70 CH1 (another CH1-specific resin). Specifically, we separately processed the culture harvests of seven mAbs (all of them were human IgG1) by the three affinity resins. For each resin, culture harvests of the seven mAbs were loaded at the same density (i.e., 5 mg/mL of resin) and processed by following the same protocol (relevant information can be found in the Method section). For MabSelect SuRe LX Protein A resin, all seven mAbs showed close or identical retention volumes (Figure 2A), suggesting that they bound to the resin with similar strength. The picture/pattern was different for CaptureSelect CH1-XL and Praesto 70 CH1 affinity resins, whose chromatograms indicated that the seven mAbs exhibited appreciably different retention volumes (Figure 2B and 2C, respectively), although they were processed under identical conditions. This finding suggested that, under the same condition, CH1-specific affinity resins bind different mAbs with varied strengths. As aforementioned, the same phenomenon was previously observed with a Protein L-conjugated resin [15]. In addition, we figured that such a property could potentially facilitate homodimer removal in asymmetric bsAb purification [15]. As shown in Figure 1, the rationale is that when the affinity resin binds the two parental mAbs from which the asymmetric bsAb is derived with different strengths, it will bind the bispecific heterodimer with an intermediate strength, hence allowing for separation of the three species (i.e., heterodimer and two homodimers/

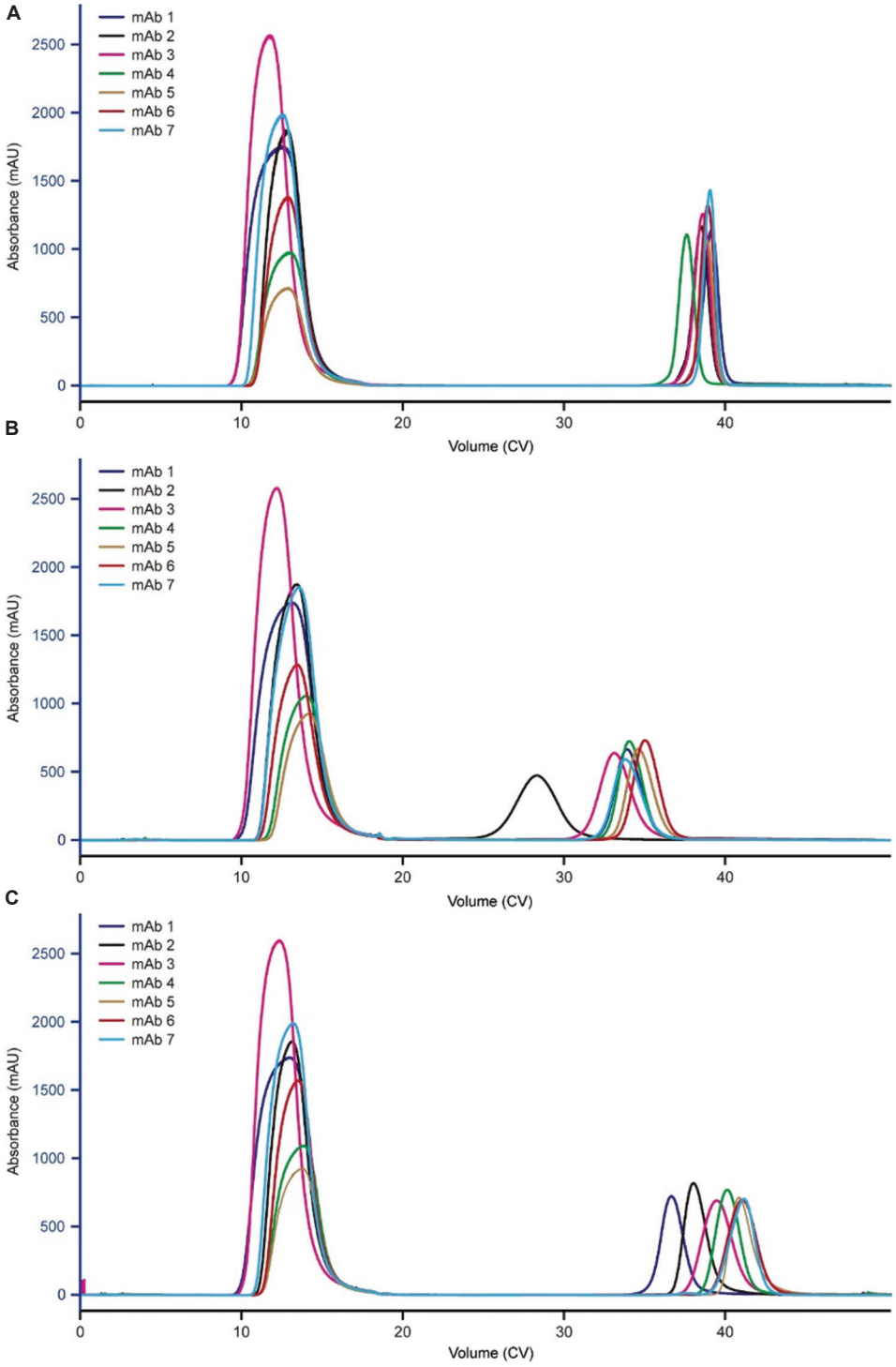


**Figure 2.** Overlay of (A) MabSelect SuRe LX Protein A, (B) CaptureSelect CH1-XL and (C) Praesto 70 CH1 chromatograms of seven monoclonal antibodies (mAbs). For each affinity resin, the column was loaded with culture harvests of seven mAbs at the same density (i.e., 5 mg/mL) and all runs were conducted following the same protocol.

parental mAbs). Intermediate binding of the heterodimer and effective separation of the heterodimer from homodimers mediated by a Protein L resin were confirmed in a real bsAb purification case [15]. Between the two CH1-specific resins under evaluation, Praesto 70 CH1 exhibited greater difference in binding towards different mAbs. In addition, Praesto 70 CH1 has a higher binding capacity and better alkali stability than CaptureSelect CH1-XL according to the vendors. Therefore, Praesto 70 CH1 was selected as a representative of CH1-specific resin for the subsequent studies.

### 3.2. The variable binding of Praesto 70 CH1 to different mAbs was confirmed with an artificial mixture containing two mAbs

To confirm the variable binding of Praesto 70 CH1 to different mAbs, an artificial mixture was made by combining the culture harvests of mAb 1 and mAb 5 (as suggested by the chromatograms shown in Figure 2C, these two mAbs showed the biggest difference in their retention volumes) at a 1:1 mass ratio. This artificial mixture was processed by Praesto 70 CH1 following the same protocol as that used for individual mAbs. The chromatogram of this run is shown in Figure 3. In consistency with the data shown in Figure 2C, the two components (mAb 1 and mAb 5) in the mixture were well separated, confirming that Praesto 70 CH1 binds these two mAbs with different strengths under the same condition.

### 3.3. A small difference in Praesto 70 CH1 binding between two mAbs can lead to complete separation upon optimization

As mentioned in the previous section, the unique binding property of Praesto 70 CH1 could potentially facilitate

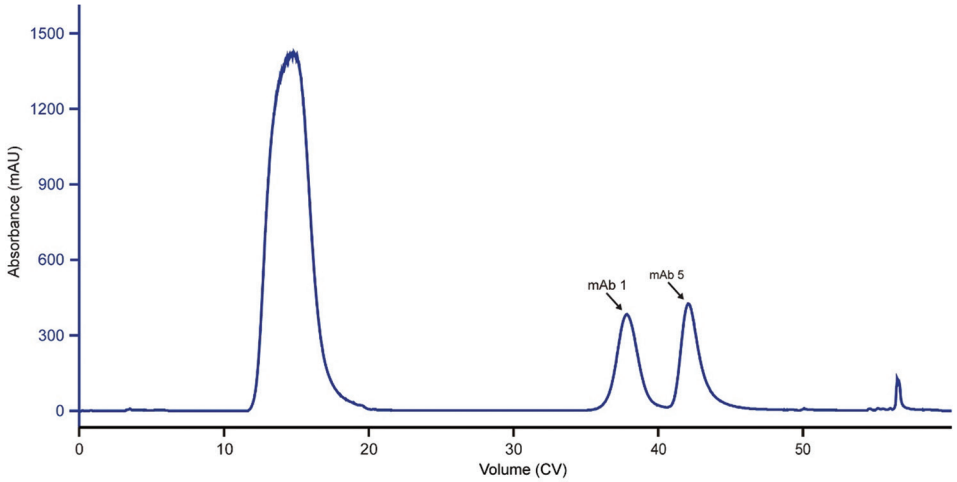


**Figure 3.** Praesto 70 CH1 chromatogram of a run loaded with an artificial mixture. The artificial mixture was generated by combining culture harvests of monoclonal antibody (mAb) 1 and mAb 5 at a 1:1 mass ratio.

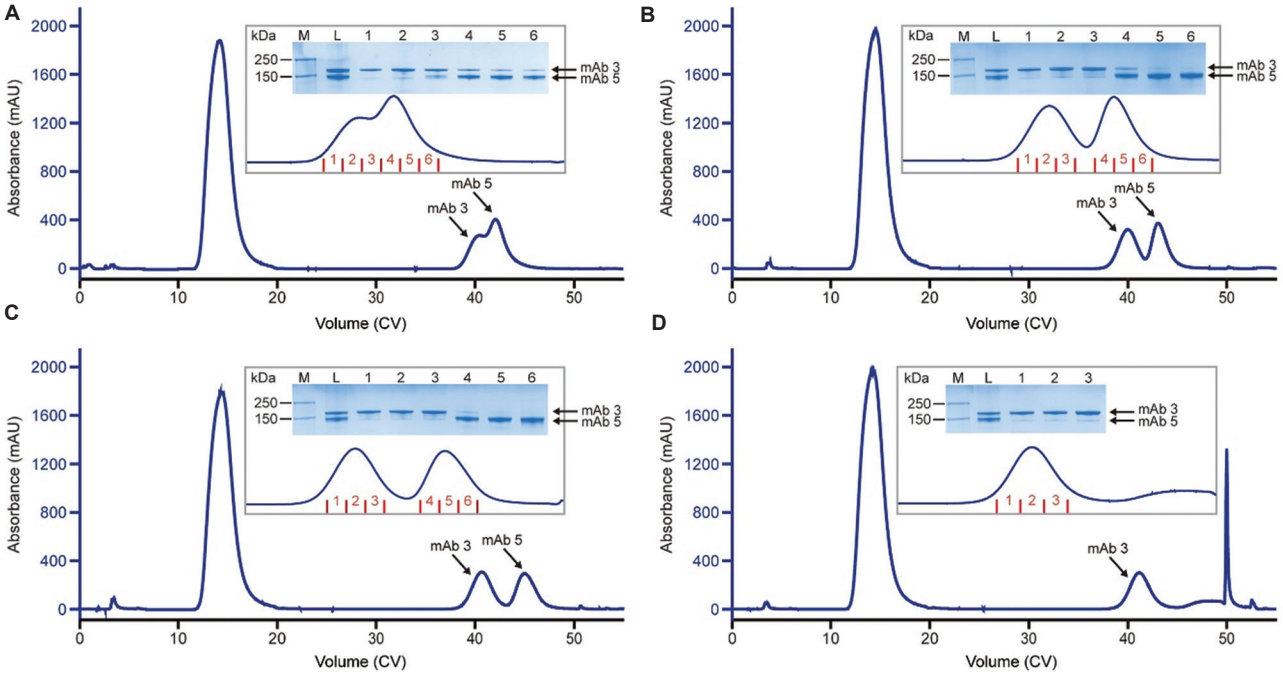


**Figure 4.** Praesto 70 CH1 chromatograms of runs conducted under (A) regular linear pH gradient and (B-D) optimized gradient (linear pH gradient with salt additive) elution. The load material used in the four runs was an artificial mixture generated by combining culture harvests of monoclonal antibody (mAb) 3 and mAb 5 at a 1:1 mass ratio. For the optimized gradient, (B) 50 mM, (C) 100 mM and (D) 200 mM NaCl were added to the mobile phase. Inset, a zoom-in of the elution peaks and SDS-PAGE analysis of relevant fractions. M: Protein marker; L: Load; Lanes 1-3/6: Relevant elution fractions.

separation of heterodimer from homodimers in asymmetric bsAb purification. Apparently, separation relies on the degree of binding difference between the two parental mAbs. The greater difference in binding the two parental mAbs exhibited that a better separation between heterodimer and homodimers can be expected. For practical applicaion of Praesto 70 CH1

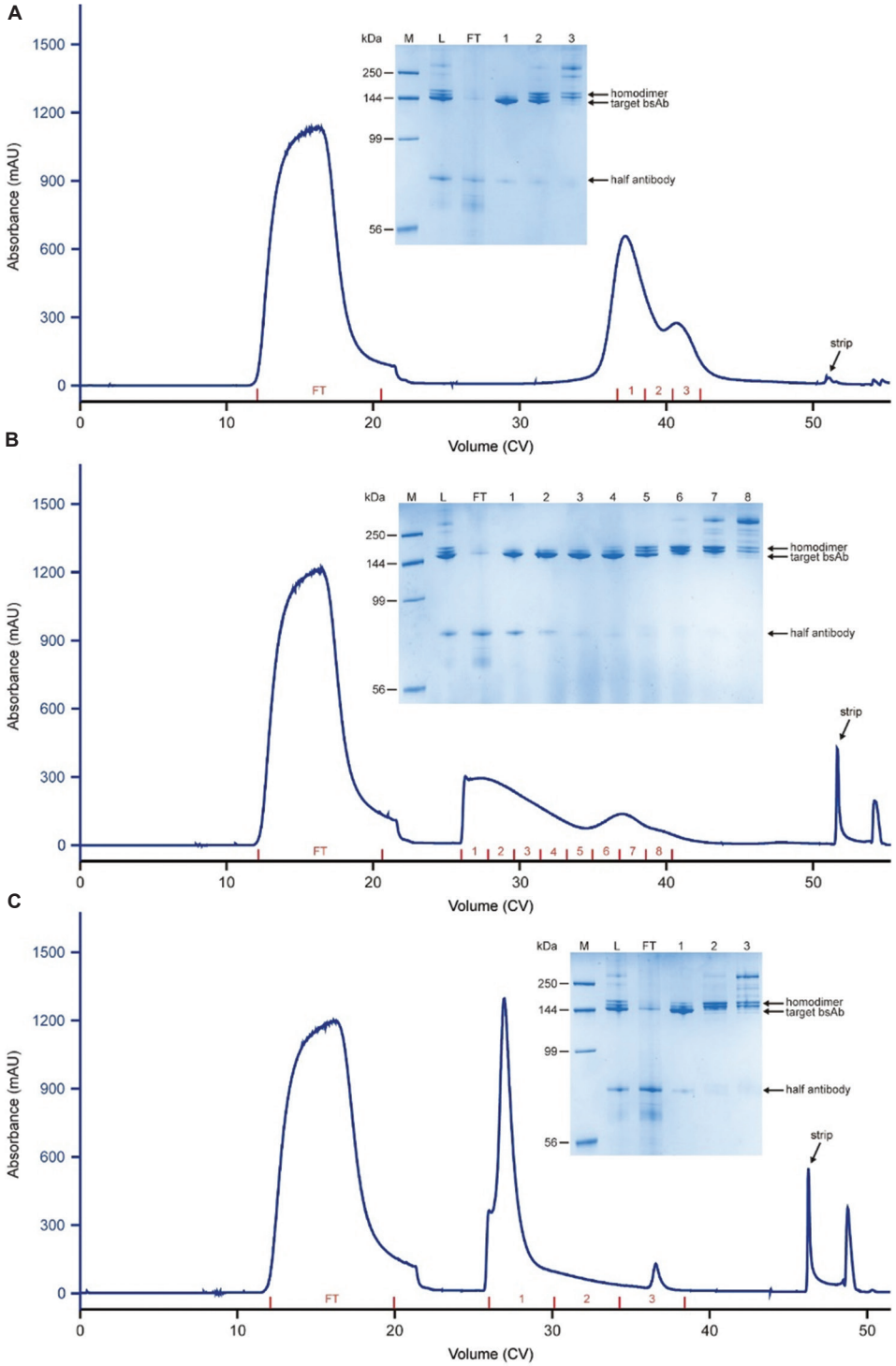


**Figure 5.** Homodimer removal by Praesto 70 CH1 chromatography. (A) Chromatogram of a run conducted under regular linear pH gradient elution. (B) Chromatogram of a run conducted under optimized linear gradient (pH gradient with salt additive) elution. (C) Chromatogram of a run conducted under stepwise pH gradient elution developed based on the optimized linear gradient elution. Inset, SDS-PAGE analysis of relevant fractions. M: Protein marker; L: Load; FT: Flow-through; Lanes 1-3/8: Relevant elution fractions. Position of bands corresponding to the target bispecific antibody, half antibody and homodimer are labelled. The homodimer migrated slightly slower than the heterodimer on gel and itself showed as two close bands due to the existence of conformational isoforms [15,21].

affinity resin in bsAb purification, it is critical to translate a small difference in binding into a complete separation on optimization. To explore such a possibility, another artificial mixture was made by intermingling the culture harvests of mAb 3 and mAb 5, which showed a small difference in their retention volumes when processed separately (Figure 2C). Consistently, when this artificial mixture was processed by Praesto 70 CH1 by following the original protocol, the corresponding elution peaks of the two mAbs largely overlapped (Figure 4A).

For Protein A and Protein L affinity chromatography, several previous studies showed that adding salt to the mobile phase could significantly improve resolution [16-19]. Thus, we applied this strategy to Praesto 70 CH1. As shown in Figures 4B-D, addition of salt to the mobile phase significantly improved resolution between the two mAbs. The previous two overlapping peaks were substantially separated when 50 mM NaCl was added to the two pH gradient generation buffers (Figure 4B). Separation between the two mAbs was further improved when the salt concentration was increased to 100 mM (Figure 4C). When the salt concentration was raised to 200 mM, only mAb 3 was eluted during the pH gradient (mAb 5 binding was too strong to be eluted under this condition), leading to further improved separation (Figure 4D). The data indicated that a small difference in binding exhibited by Praesto 70 CH1 toward two mAbs can be greatly magnified through simple optimization, allowing for complete separation of the two mAbs. This information suggests that desired separation between heterodimer and homodimers can be accomplished by following the same strategy even if the difference in binding between the two parental mAbs is relatively small.

### 3.4. Homodimer removal by Praesto 70 CH1 chromatography in a real bsAb case

We previously showed that a Protein L resin was capable of separating heterodimer from homodimer by-product in an asymmetric bsAb case [15]. As explained in the above sections, Praesto 70 CH1 shares the same binding property with Protein L, and is supposedly to be able to separate homodimer as well. We tested this hypothesis with a real bsAb case that we had previously worked on (this case was different from that used in Protein L related studies). When we developed the purification process for this project, the homodimer by-product had largely remained post Protein A capture and it was eventually removed using a mixed-mode resin [20]. With Praesto 70 CH1, we first processed the clarified culture harvest of this bsAb under regular linear pH gradient elution. As shown in Figure 5A, partial separation between the heterodimer and the homodimer was achieved. Use of the optimization strategy we previously worked out (adding salt to the two gradient generation buffers) attained more complete separation (Figure 5B). Eventually, a stepwise elution was developed based on the optimized linear gradient elution and equally good separation was achieved (Figure 5C). Data from this case study confirmed that Praesto 70 CH1 possesses the homodimer-removing capability as expected.

## 4. CONCLUSION

In the present study, we found that two CH1-specific resins (CaptureSelect CH1-XL and Praesto 70 CH1) bound different mAbs with varied strengths under identical conditions. This is a special property not possessed by Protein A affinity resins but shared by a Protein L-conjugated resin [15]. It is not clear at this point that why Protein L and CH1-specific resins but not Protein A resins have such a property. It is possible that the sequences in the antibody domains recognized by the first two resins (VL and CH1, respectively) are less conserved among different mAbs as compared to that in the Fc region (domain recognized by Protein A). Further studies are warranted to discover the mechanism underlying the variable binding to different mAbs exhibited by Protein L and CH1-specifc resins. Although the exact mechanism of variable binding is currently unknown, we found that this property is of practical value in asymmetric bsAb purification. Specifically, we previously demonstrated that the variable binding property of a Protein L resin enabled separation of heterodimer from homodimers (please see Figure 1 for the rationale) [15]. For Praesto 70 CH1, after showing that a small difference in binding it mediated can be significantly magnified, we further demonstrated that this resin, like Protein L, could effectively separate the heterodimer from the homodimer by-product in a real bsAb case. This result was coincident with the theoretical expectation for CH1-specific resins, which exhibit variable binding towards different mAbs. It is worth noting that as both CaptureSelect CH1-XL and Praesto 70 CH1 are designed to bind human IgGs, they cannot be used for purification of non-human antibodies.

Homodimers are common product-related impurities associated with the recombinant production of asymmetric bsAbs and their removal can be considerably challenging as they normally share close physicochemical properties with the target heterodimer. Therefore, the property of Praesto 70 CH1 that we discovered in this study is valuable. When used for capture of asymmetric bsAb, Praesto 70 CH1 can significantly reduce homodimer by-product and therefore alleviates the purification burden on polishing steps. As we previously showed, several antibody-binding affinity resins possess the capability of removing certain product-related impurities associated with bsAb production [12]. Information on the by-product removing potential of commonly used commercial antibody-binding affinity resins

is useful. When purifying complex molecules such as bsAbs, such information can help choose an appropriate affinity resin with desired by-product removing capability. If the potential of the selected resin can be fully exploited, the purification burden can be spread, allowing for a more robust downstream process. The current study provided updated information on Praesto 70 CH1's by-product removing potential and suggested that this resin could be a better alternative than Protein A for product capture in asymmetric bsAb purification, especially when the homodimer content is high.

For a given asymmetric bsAb case, Praesto 70 CH1 may outperform Protein L resin (which possesses similar properties) in homodimer separation, or vice versa, depending on the difference in binding between the two parental mAbs towards Praesto 70 CH1 and Protein L. The resin which shows larger difference in binding to the two parental mAbs will allow for better separation. With different bsAbs, the situation can vary. For example, mAb A and mAb B may exhibit greater difference in binding towards Protein L than towards Praesto 70 CH1. On the other hand, mAb C and mAb D may exhibit larger difference in binding towards Praesto 70 CH1 than toward Protein L. Therefore, Protein L is a better choice than Praesto 70 CH1 for the bsAb derived from mAb A and mAb B, but Praesto 70 CH1 is more promising than Protein L for the bsAb derived from mAb C and mAb D. Thus, these two resins can work as complementary tools for homodimer separation. For a given case, if one fails to provide adequate separation, the other provides additional options and possibilities.

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## CONFLICT OF INTEREST

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

## AUTHOR CONTRIBUTIONS

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

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## CONSENT FOR PUBLICATION

Not applicable.

## AVAILABILITY OF DATA

The data and supportive information are available within the article.

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