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Tailoring polishing steps for effective removal of polysorbate-degrading host cell proteins in antibody purification

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Abstract

Ensuring the quality and safety of biopharmaceutical products requires the effective separation of monoclonal antibodies (mAbs) from host cell proteins (HCPs). A major challenge in this field is the enzymatic hydrolysis of polysorbates (PS) in drug products. This study addresses this issue by investigating the removal of polysorbate-degrading HCPs during the polishing steps of downstream purification, an area where knowledge about individual HCP behavior is still limited. We investigated the separation of different mAb formats from four individual polysorbate degrading hydrolases (CES1F, CES2C, LPLA2, and PAF-AH) using cation exchange (CEX) and mixed-mode chromatography (MMC) polishing steps. Our research identified a key challenge: The similar elution behavior of mAbs and HCPs during chromatographic separation. To investigate this phenomenon, we performed high-throughput binding screenings for recombinant polysorbate degrading hydrolases and representative mAb candidates on CEX and MMC chromatography resins. We then employed a three-step strategy that also served as a scale-up process, optimizing separation conditions and leading to the successful removal of specific HCPs while maintaining high mAb recovery rates (>96%). This strategy involved the use of surface response models and miniature columns for screening, followed by validation on larger columns using a chromatography system. Our results highlight the critical role of the inherent properties of mAbs for successful separation from HCPs. These results underscore the need to tailor the purification process to leverage the slight differences in binding behavior and elution profiles between mAbs and specific HCPs. This approach lays the foundation for developing more effective strategies for overcoming the challenge of enzymatic polysorbate degradation, paving the way for improved quality and safety in biopharmaceutical products.

KEYWORDS

downstream development, host cell protein, hydrolase, monoclonal antibody, polysorbate

1 | INTRODUCTION

Monoclonal antibodies (mAbs) are the leading molecule class among biopharmaceuticals with significant therapeutic potential (Johnson, 2018). To enhance protein stability and extend shelf-life, surfactants are applied in mAb formulations to protect against interfacial stresses. Among the surfactants commonly used in biopharmaceutical drug formulations, polysorbates, specifically polysorbate 20 (PS20) and polysorbate 80 (PS80), are prone to degradation and hydrolysis under conditions commonly used in the industry (Hecht et al., 2022). This degradation can compromise the stability and therefore the efficacy of the mAb formulations, leading to potential safety concerns and reduced therapeutic efficacy (Kishore et al., 2011).

The purification of mAbs from host cell proteins (HCPs), especially the removal of polysorbate-degrading HCPs, remains a critical challenge. HCPs are process-related impurities introduced during mAb generation by the mammalian production cell lines and must be reduced to appropriate levels to ensure product stability and safety.

Most mAb purification trains involve an initial affinity chromatography step followed by two polishing steps. The HCPs found in the Protein A eluate are primarily the result of interactions with either the chromatography resin and ligands or the mAbs (Levy et al., 2014). Despite a significant reduction in total HCP concentrations in the Protein A elution pools, typically several hundred HCPs are still detected after this unit operation (Oh et al., 2023). Since most of the HCPs are more acidic than mAbs, a large fraction of the HCPs can be successfully separated in the subsequent polishing steps (Jin et al., 2010).

Monitoring HCP levels in the downstream process is crucial, and enzyme-linked immunosorbent assays (ELISAs) are commonly used because of their efficiency and speed. However, they have limitations, such as specificity, sensitivity, and lack of information on individual HCPs. Some studies even suggest that certain polysorbate degrading hydrolases may remain undetected by commercial HCP ELISAs (Gupta et al., 2023). As the risks posed by individual HCP species vary, it is essential to implement methods that can provide information on individual HCPs as an orthogonal strategy for risk reduction (Bracewell et al., 2015).

While there is existing knowledge of overall HCP content, little is known about the behavior of individual HCPs. The incomplete knowledge of their identities and their extremely low abundance, often less than 1 ppm, has made this task particularly difficult (Li et al., 2022). To address these challenges, significant research efforts have been devoted to the identification, detection, and quantification of HCPs responsible for polysorbate degradation (Jones et al., 2021; Li et al., 2021; Yang et al., 2022). These approaches have identified several HCPs involved in polysorbate degradation, including carboxylesterases (CES2C and CES1F) (Hu, Molden, Qiu, et al., 2022; Kovner et al., 2023; Zhang et al., 2020), lipoprotein-associated phospholipase A2 (LPLA2, also known as Pla2g15) (Hall et al., 2016), platelet-activating factor acetylhydrolase (PAF-AH, also known as Pla2g7) (Dehghani et al., 2023; Li et al., 2021), palmitoyl¯protein thioesterase 1 (PPT1) (Graf et al., 2021; Kovner et al., 2023), lipoprotein lipase (LPL) (Chiu et al., 2017), and lysosomal acid lipase (LIPA) (Dehghani et al., 2023; Zhang et al., 2022).

Recent studies suggested that ultra-low affinity HCP-mAb interactions may allow polysorbate degrading hydrolases to evade purification processes (Kerwin, 2008; Khan et al., 2015). However, there is still a need for a more comprehensive understanding of the behavior of individual polysorbate-degrading HCPs during downstream purification steps, as well as the development of targeted approaches to minimize their levels in the final product.

In this study, we aim to fill this knowledge gap by systematically characterizing the behavior of four polysorbate-degrading HCPs during downstream polishing chromatography steps. To achieve this, we first generated these enzymes by recombinant expression in a CHO host cell line, followed by purification via orthogonal steps. Our study employed a three-step strategy involving high-throughput screenings on slurry plates, separation optimization on miniature columns, and validation of optimized separation conditions on packed bed columns. This comprehensive approach allowed for the systematic investigation of the binding behavior of individual polysorbate degrading hydrolases and mAbs under various conditions, as well as the identification of optimal separation conditions. By understanding the factors that influence the separation efficiency between mAbs and polysorbate-degrading HCPs, we can optimize the purification process to get rid of these culprit enzymes. Using this three-step strategy, the study successfully identified and validated optimal purification conditions for the effective separation of specific polysorbate-degrading HCPs from various mAb formats.

2 | MATERIALS AND METHODS

2.1 | Model antibodies

In this study, several model antibodies were selected based on their differences in molecular format, molecular weight (MW), and isoelectric point (pI) (Table 1). These criteria were chosen to cover a wide range of behavior during the polishing steps of the purification process. All model antibodies were produced in-house (Boehringer Ingelheim Pharma GmbH & Co. KG).

TABLE 1 Characteristics of the five monoclonal antibodies (mAbs) investigated in this study.

Molecule	Classification	pI	MW (kDa)
mAb – 1	IgG1	9.6	145
mAb – 2	IgG4	7.8	146
mAb – 3	IgG4	7.4	144
mAb – 4	Bispecific mAb	9.3	151
mAb – 5	IgG-scFv	8.7	199

Abbreviations: MW, molecular weight; pI, isoelectric point.

TABLE 2 Characteristics of the expressed and purified polysorbate hydrolases with abbreviations used and their Uniprot accession numbers.

Polysorbate hydrolase	Abbreviation	Gene name	pI	MW (kDa)	Uniprot accession
Carboxylesterase 1f	CES1F	*Ces1f*	7.9	65	A0A061I7X9
Carboxylic ester hydrolase	CES2C	*Ces2c*	5.8	65	G3IIG1
Group XV phospholipase A2	LPLA2	*Pla2g15*	6.3	50	G3HKV9
1-alkyl⁻2-acetylglycerophospho-choline esterase	PAF-AH	*Pla2g7*	8.6	52	G3HTI7

Note: The theoretical pI was calculated based on the primary sequence.
Abbreviations: MW, molecular weight; pI, isoelectric point.

2.2 | Expression and purification of recombinant host cell proteins

Hydrolase expression was achieved using a transposase system and a glutamine selection system, with hydrolase sequences extracted from the CHO K1 transcriptome and cloned into a transposon harboring expression plasmid. Posttransfection, cells were subjected to selection pressure, expanded, and then transferred to a shaking flask for cultivation. Stable hydrolase-expressing cell pools were cultivated and harvested. For the purification of recombinant hydrolases, a two-step capture process was followed by size exclusion chromatography. The process involved the use of immobilized metal affinity chromatography (IMAC) and a Strep-Tactin column, with the final purified protein aliquoted and stored at −70°C. Protein concentrations were determined by absorbance at 280 nm using NanoDrop protein quantification (Thermo Fisher Scientific). Hydrolases used for this study are listed in Table 2.

2.3 | High-throughput batch binding screenings

We performed K_p (partition coefficient) screenings with hydrolases and mAbs to identify optimal separation conditions by calculating separation factors. This approach was inspired by the high-throughput screening method described by Kelley, Switzer, et al. (2008), Kelley, Tobler, et al. (2008), and McDonald et al. (2016).

To determine the K_p for purified hydrolases and mAbs, automated high-throughput batch binding screenings were performed using MultiScreen® 96-well 0.45 µm filter plates, prefilled with 25 µL of target resin (POROS™ XS or Capto adhere).

A Tecan Fluent automated liquid handling system was used for resin preparation and liquid transfer. The K_p-Screening included two equilibration steps, one loading step, and two stripping phases, each with a 200 µL liquid phase volume. Equilibration and loading were conducted on 48 wells of the resin plate, with specific pH and counter ion concentration combinations, enabling 48 unique load conditions per experiment. For CEX and MMC K_p-Screening, a multicomponent buffer system was used according to Kröner and Hubbuch (2013) The mAb and HCP stock solutions were exchanged into the corner buffers using a 53 mL HiPrep 26/10 Desalting Column.

The K_p value is the quotient of the protein concentration bound (q_p) to the resin and the protein concentration in the supernatant (c_p) as described by the following formula:

$$K_p = \frac{q_p}{c_p}. \quad (1)$$

Using the K_p value of an HCP (impurity) and the mAb (target) the separation factor α was calculated for a given counterion concentration and pH, according to the following:

$$\alpha = \frac{K_{p\text{impurity}}}{K_{p\text{target}}}. \quad (2)$$

The holdup volume, which is the volume of the solution occupying the voids in the resin matrix, was taken into account in the determination of the K_p.

2.4 | High-throughput miniature column experiments

To determine the optimal point of antibody separation from HCP impurities, design of experiments (DoE) was performed using 600 µL miniature columns packed with either POROS™ XS (Thermo Fisher Scienfic) or Capto adhere (Cytiva). A surface response model with a quadratic I-optimal design and 16 runs was employed to optimize the separation conditions for miniature columns. The two factors investigated were pH and counter ion concentration, while the three responses were log(K_p) HCP, log(K_p) mAb, and log(α). The center point for each experiment was selected based on the areas with the lowest separation factor α for CEX and the highest for MMC observed in batch-binding screenings, reflecting the application of CEX in bind/elute mode and MMC in flow-through mode. To simulate lab-scale column chromatography conditions, miniature columns were equilibrated with 5 CV of equilibration buffer at a flow rate of 200 µL/min. They were then loaded with 5 CV of buffer-exchanged sample at 120 µL/min, washed with 1 CV of equilibration buffer at 200 µL/min, and eluted with 3 CV of strip buffer at 300 µL/min. Before the experiment, mAb or HCP samples were buffer exchanged into the

respective equilibration buffer using a 53 mL HiPrep 26/10 desalting column and further diluted to achieve a load challenge of 12.5 g/L resin for mAbs and <4 g/L resin for HCPs. Concentrations of all fractions and loads were determined by measuring absorbance at 280 nm in triplicates. Statistical analysis was performed using Design Expert (ver. 13.05.0). Analysis of variance (ANOVA) was initially performed for model construction by including all significant parameters having a $p \leq 0.05$. Model quality was assessed using the model F value, lack of fit value, and adjusted R^2 statistics.

2.5 | Lab-scale verification experiments

To verify the transferability of the optimal separation conditions identified in the miniature column DoE experiments for mAb and HCP, validation experiments were performed on the Äkta Avant 25 system using both HiTrap Capto adhere (Cytiva) and POROS™ GoPure™ XS 1 mL columns (Thermo Fisher Scientific). Samples were buffer exchanged and diluted to achieve a loading of 37.5 g/L resin for mAbs and 2.5 g/L resin for HCPs. Peak fractions were pooled, and the specific concentrations of HCP and mAbs were quantified individually. Two different biolayer interferometry (BLI) assays were used for these measurements: an in-house developed assay targeting recombinant hydrolases for HCP quantification, and an antibody quantification assay using Protein A sensors.

2.6 | Biolayer interferometry for quantification of IgGs and specific HCPs

IgGs and HCPs were specifically quantified using BLI on an Octet Red384 instrument (ForteBio). Protein A sensors were used for IgG quantification according to the manufacturer's instructions. The procedure included five steps: (1) prehydration (10 min), (2) baseline (60 s), (3) sample loading (30 s), (4) regeneration (30 s) with 10 mM glycine buffer (pH 1.5), and (5) neutralization (30 s). Specific HCPs were quantified using an in-house developed assay against recombinant proteins. This assay measures in a linear quantification range of 10–300 ng/mL. Anti-His biosensors were prehydrated, and the BLI method involved eight steps, including loading, washing, and antibody binding. Quadruplicate measurements were performed for lab-scale validation experiments. Data acquisition and analysis were performed using the Octet Data Acquisition Software 9.0 and Data Analysis Software 11.1, respectively.

3 | RESULTS

3.1 | Binding behavior of critical host cell proteins in the polishing steps

High-throughput binding screenings were performed to investigate the binding behavior of the polysorbate-degrading HCPs CES1F, CES2C, LPLA2, and PAF-AH. These recombinantly expressed HCPs were first purified using a three-step process consisting of two capture steps (Ni-NTA and StrepTactin), followed by size exclusion chromatography. The primary output of the binding screenings, the partition coefficient (K_p), served as a key indicator of HCP binding or flow-through under specific pH and counter ion concentration conditions. Each screening generated 48 unique data points, providing a comprehensive insight into specific HCP behavior under various conditions.

The binding behavior of CES2C, LPLA2, PAF-AH, and CES1F was investigated on the CEX resin Poros XS, using a combination of eight sodium counter ion concentrations (38–500 mM) and six pH levels (4–8). Figure 1 shows contour plots to illustrate the logarithmic K_p for each polysorbate degrading hydrolase, represented across a range of pH levels and Na^+-counterion concentrations (mM). All HCPs examined displayed a higher sensitivity to pH than Na^+-counter ion concentration, as indicated by the vertically oriented black contour lines defining the HCP partitioning regions on the resin. The impact of Na^+-counter ions on resin binding was more significant for PAF-AH (Figure 1c) than for CES2C (Figure 1a), as indicated by the more horizontal shifts of the strong binding contour line ($\log(K_p) = 1$) for LPLA2 and PAF-AH in the contour plots. The contour plots also show that HCPs exhibiting higher pI values, such as PAF-AH (pI = 8.6), demonstrated a shift in the strong binding region towards higher pH ranges compared to CES2C (pI = 5.8). CES2C is the only polysorbate degrading hydrolase with a parallel transition of partitioning regions, where a shift from strong binding to flow-through occurs within a pH range of 0.5. LPLA2 showed two distinct flow-through regions at pH > 7 (Figure 1b), while CES1F (Figure 1d) shows a small flow-through area at pH higher than 7.75 and Na^+-counter ion concentrations below 100 mM. Notably, PAF-AH has no flow-through region in the tested ranges (Figure 1c).

The binding properties of CES2C, LPLA2, PAF-AH, and CES1F on the MMC resin Capto adhere were examined using eight distinct chloride counter ion concentrations (60–1000 mM) and six pH levels (4–10). The logarithmic K_p for each HCP, as a function of pH and Cl^--counter ion concentration, showed unique binding patterns on mixed mode resin as shown in contour plots (Figure 2). CES2C binds strongly to the resin over the entire range tested, except for a narrow pH range of 4–5.2 and below 500 mM Cl^--counter ions (Figure 2a). With increasing salt concentrations, hydrophobic interactions may intensify, leading to stronger binding in this area. Conversely, LPLA2 and CES1F exhibit greater sensitivity to pH than to Cl^--counter ion levels with respect to binding transitions (Figure 2b,d). PAF-AH shows salt sensitivity evidenced by its tilted oval-shaped strong binding contour line (Figure 2c). Here, electrostatic repulsion seemingly outweighs hydrophobic interaction, resulting in weakened resin-protein interactions at salt concentrations above 500 mM.

These results indicate that the binding behavior of polysorbate degrading hydrolases to Capto adhere resin varies and lacks common trends as observed with CEX resin POROS™ XS.

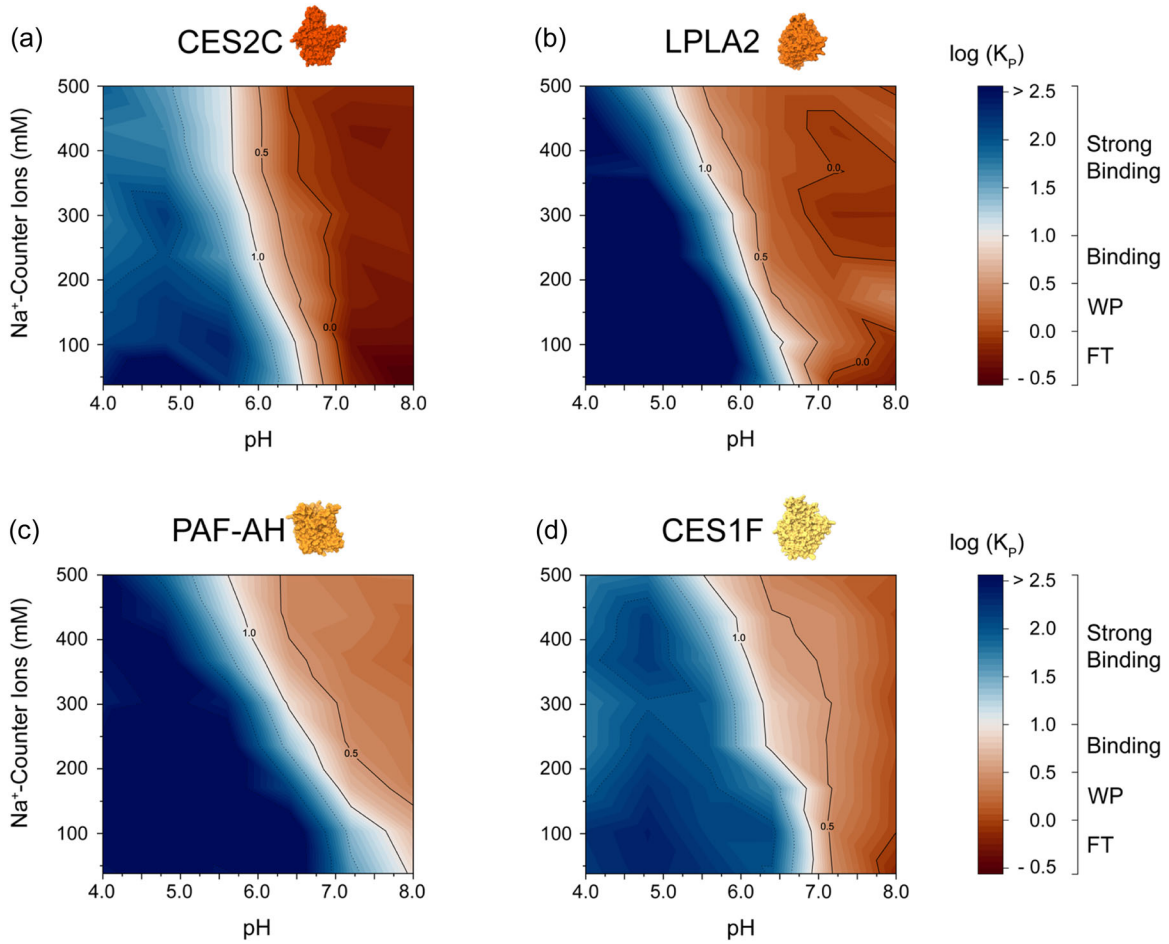

FIGURE 1 High-throughput polysorbate hydrolase binding screening on Poros XS resin. The contour plots illustrate the binding behavior of polysorbate hydrolases during bind-and-elute cation exchange chromatography, with log (K_p) values represented across varying pH levels and concentrations of Na+-counter ions (mM) for (a) CES2C, (b) LPLA2, (c) PAF-AH, and (d) CES1F. Binding affinity to the resin is represented by log (K_p) values, categorized as <0 (flow-through, FT), 0 ≤ 0.5 (weak partitioning, WP), 0.5 ≤ 1.0 (binding), and >1.0 (strong binding).

3.2 | Behavior of mAbs in the polishing steps

To tackle the challenge of separating the HCP impurities from the final mAb product, it is vital to compare the binding profiles of mAbs and specific HCPs under identical screening conditions, thereby pinpointing differences in their binding behaviors. The high-throughput screenings were performed for various antibody formats under the same conditions as for the polysorbate-degrading hydrolases. Both cation exchange and mixed-mode screenings were performed. The cation exchange resin contour plots, illustrating the logarithmic K_p as a function of pH and Na^+ concentration, revealed a decreasing requirement of Na^+ ions for mAb binding with increasing pH (Figure 3). In particular, mAb-5 showed robust binding over the entire pH screening range when Na^+ counterion concentrations were maintained below 250 mM (Figure 3d). These results indicate that the Na^+ counterion concentration has a greater influence on mAb-resin interactions than pH during CEX chromatography. Antibody binding was also examined on the mixed mode (MMC) resin (Figure 4). The IgG1 (mAb-1), IgG-scFv (mAb-5), and bispecific-mAb (mAb-4) displayed a partially oval-shaped transition from flow-through to strong binding, indicating that the binding behavior is almost equally influenced by pH and Cl^--counter ion concentration, but the size of each partitioning area depended on the molecule (Figure 4a,d,e). In contrast, the binding behavior of the tested IgG4-subtypes was more sensitive to pH levels than the concentration of Cl^--counter ions (Figure 4b,c). In particular, the two tested IgG4 mAbs showed a narrow flow-through area within the examined pH range near pH 4 and a Cl^--counter ion concentration below 200 mM. These results highlight that, similar to HCPs, the binding behavior of mAbs to MMC resin varies from molecule to molecule, with no global trends as observed for CEX resin.

3.3 | Separation of specific host cell proteins from mAbs

To identify optimal separation conditions for specific mAbs and polysorbate degrading hydrolases, the separation factor α was

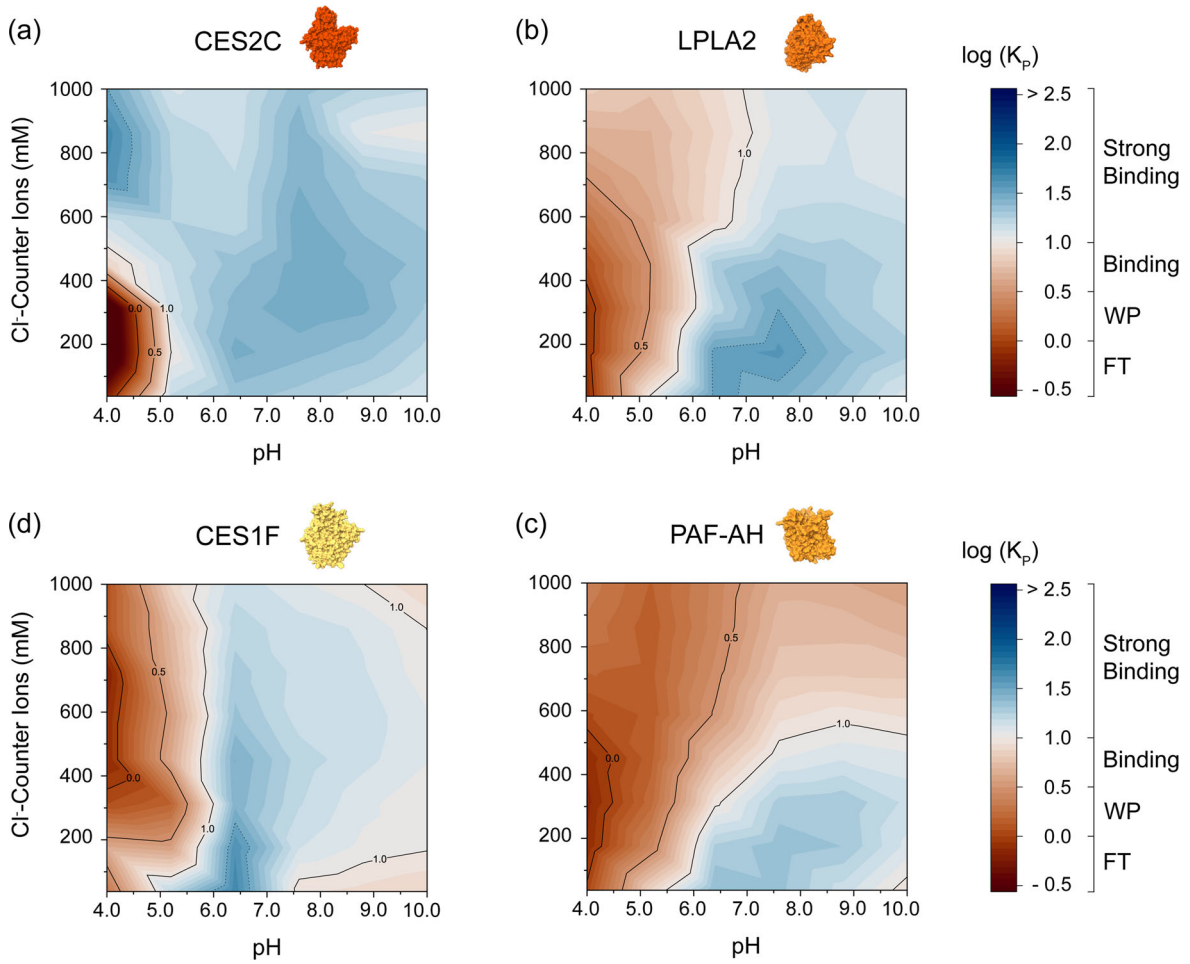

FIGURE 2 High-throughput polysorbate hydrolase binding screening on Capto adhere resin. Contour plots illustrate the binding behavior of polysorbate hydrolases during flow-through mixed-mode chromatography, with log (K_p) values represented across varying pH levels and concentrations of Cl^--counter ions (mM) for (a) CES2C, (b) LPLA2, (c) CES1F, and (d) PAF-AH. Binding affinity to the resin is indicated by log (K_p) values, with ranges: <0 (flow-through, FT), 0 ≤ 0.5 (weak partitioning, WP), 0.5 ≤ 1.0 (binding), and >1.0 (strong binding).

calculated for each combination of pH and counterion concentration. The binding behavior of four HCPs and five mAbs was investigated on two chromatography resins (POROS™ XS and Capto adhere). This comprehensive analysis resulted in a total of 40 separation factor α contour plots, all of which are presented in Supporting Information S1: Figures 1 and 2. In this section, we present a selection of these plots that best illustrate our key findings. The POROS™ XS CEX resin operates in bind-elute mode, requiring mAb binding to the resin and HCP flow-through. Separation conditions are indicated by log(α) values, with lower values indicating better separation. In this mode, mAb binding to the resin (log(K_p) > 0.5) and HCP flow-through (log(K_p) < 0) are required. Log(α) values below zero indicate improved separation, while values above zero indicate no separation between mAb and HCP. The selected log(α) contour plots for mAb-5 and four HCPs (CES2C, LPLA2, PAF-AH, and CES1F) are shown in Figure 5. A broad separation range for CES2C, LPLA2, and CES1F is observed at pH values above 6 and Na^+-counter ion concentrations below 250 mM, with a log(α) range of −1.3 to −2.5 (Figure 5a,b,d). For PAF-AH, the log(α) range of −1.3 to −1.9 is limited to pH values above 7 and Na^+-counter ion concentrations below 225 mM (Figure 5c). This highlights the sensitivity of the interaction forces between the mAb and the resin to Na^+ counter ions, and the influence of pH on the complex MMC interactions of the HCP, thereby creating a separation window. However, this separation window is not present for mAb-3 due to the lack of strong binding at pH 6.26 (Figure 3). These results suggest that suitable separation conditions cannot be determined here for all HCPs tested, especially CES2C and LPLA2 (Figure 6).

The Capto adhere MMC (mixed mode) resin operates in flow-through mode and requires mAb flow-through (log(K_p) < 0) and HCP binding (log(K_p) > 0.5). MAb and HCP separation is indicated by log(α) values above zero, with higher values indicating better separation. Log(α) values below zero indicate no separation. Figure 7 shows log(α) contour plots for mAb-1 (IgG1) and the four HCPs as a representative example. PAF-AH shows weak separation (log(α) < 0.75) (Figure 7c). The other three polysorbate degrading hydrolases showed a narrow separation range (log

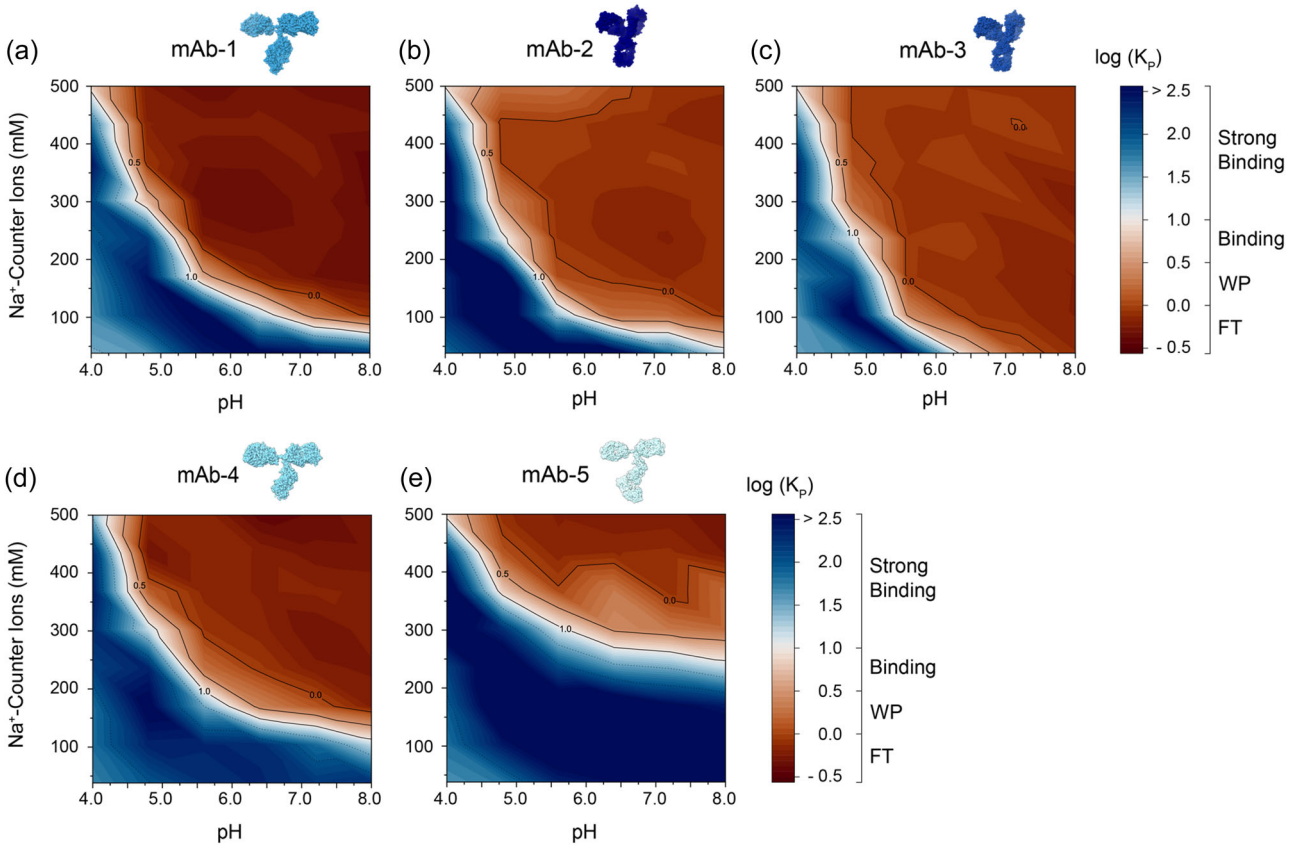

FIGURE 3 High-throughput mAb binding screening on Poros XS resin. Contour plots illustrate the binding behavior of mAbs during bind-and-elute cation exchange chromatography, with log (K_p) values represented across varying pH levels and concentrations of Na^+-counter ions (mM) for (a) mAb-1 (IgG1), (b) mAb-2 (IgG4), (c) mAb-3 (IgG4), (d) mAb-5 (IgG-scFv), and (e) mAb-4 (bispecific-mAb). The binding affinity to the resin is represented by log (K_p) values, categorized as <0 (flow-through, FT), 0 ≤ 0.5 (weak partitioning, WP), 0.5 ≤ 1.0 (binding), and >1.0 (strong binding). mAbs, monoclonal antibodies.

(α) > 1.1) around pH 6 and 100 mM Cl^- concentration (Figure 7a–c). The Capto adhere resin exhibited a narrower separation window (log(α) > 1) compared to the CEX resin, which corresponds to the proximate partitioning areas for HCPs and mAbs. These observations highlight that, within MMC, there is a broad spectrum where log(α) > 0, but achieving optimal separation requires log(α) values greater than 1.0. This is limited by the overlapping partitioning regions of HCP and mAb. An example of this overlap can be observed between mAb-2 and CES2C (Supporting Information S1: Figure 2).

3.4 | Separation of specific host cell proteins from mAbs on miniature columns

To transfer the identified separation regions to a packed bed column format and to improve the separation conditions, a surface response model with a quadratic I-optimal design was applied to 600 µL miniature columns. This was investigated for two distinct mAb-HCP combinations: mAb-5 (IgG-scFv) with CES2C on CEX resin, and mAb-1 (IgG1) with LPLA2 on MMC resin.

In the first scenario, involving mAb-5 (IgG-scFv) and CES2C, a 16-run design was utilized, with a screening range of 50–250 mM Na^+-counter ions and pH values between 5.75 and 7.75. The most favorable separation condition was identified at pH 7.28 and 151.4 mM Na^+-counter ions, where the antibody showed strong binding to the resin while the polysorbate degrading hydrolase was found in the flow-through. This optimal separation condition, as shown in Figure 8, met the criteria for the lowest log (K_p) CES2C, lowest log (α), and a high log (K_p) for mAb-5, indicated by the red circle.

For the second scenario, focusing on mAb-1 (IgG1) and LPLA2, the same DoE model was implemented, using a screening range of 75–275 mM Cl^--counter ions and pH values ranging from 4.75 to 6.75, derived from previous high-throughput binding screening. The MMC resin Capto adhere operates in flow-through mode, requiring mAb flow-through (K_p < 0) and strong HCP binding to the resin (K_p > 1) for packed bed columns. LPLA2 and mAb-1 displayed higher sensitivity to pH than to Cl^--counter ions within this screening range, as evidenced by the vertically oriented contour lines (Figure 9a,b). The design identified an optimal separation region where log (α) is greater than 2.0, specifically for pH values ranging from 5.9 to 6.2 and Cl^--counter ions less than 100 mM (Figure 9c). The optimal

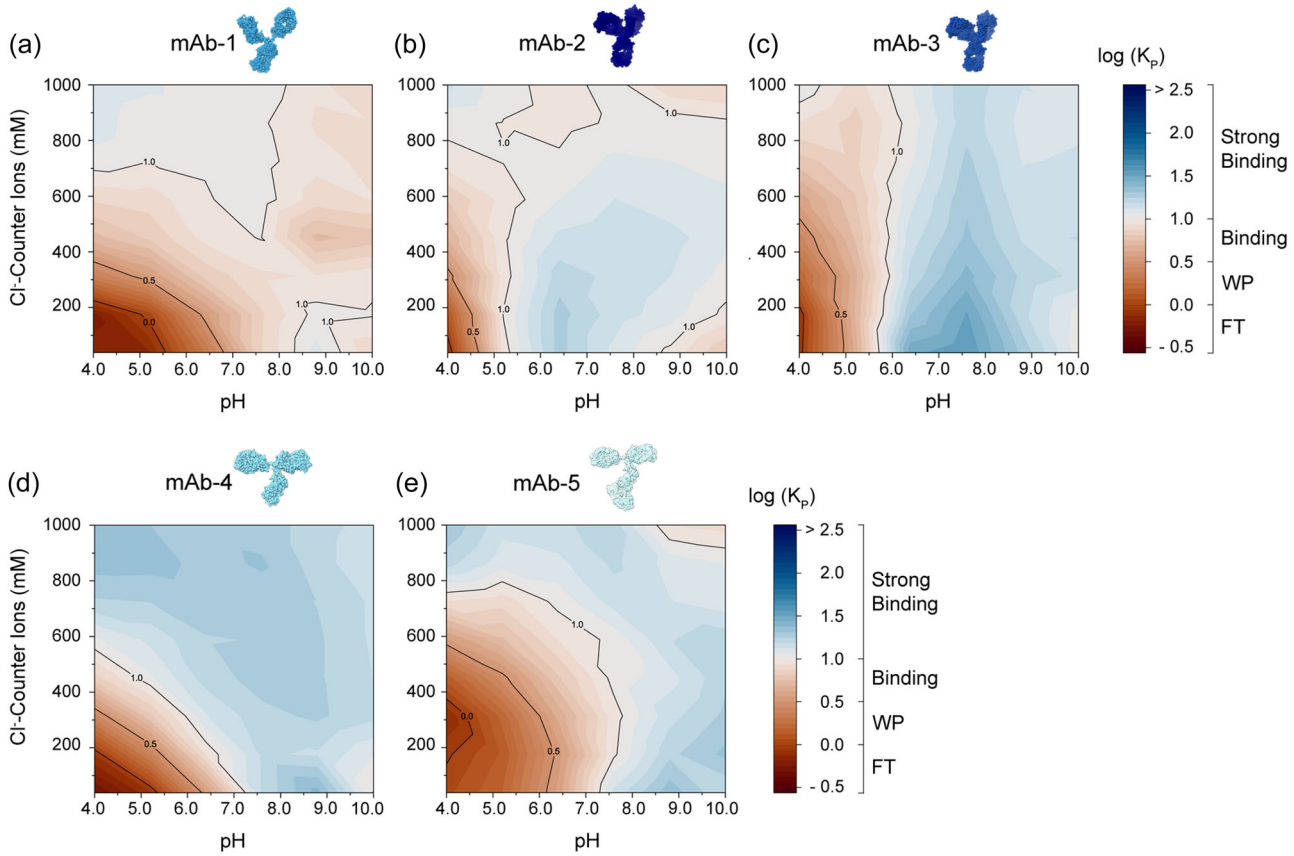

FIGURE 4 High-throughput mAb binding screening on Capto adhere resin. Contour plots illustrate the binding behavior of mAbs during flow-through mixed-mode chromatography, with log (K_p) values represented across varying pH levels and concentrations of Cl^--counter ions (mM) for (a) mAb-1 (IgG1), (b) mAb-2 (IgG4), (c) mAb-3 (IgG4), (d) mAb-5 (IgG-scFv), and (e) mAb-4 (bispecific-mAb). The binding affinity to the resin is represented by log (K_p) values, categorized as <0 (flow-through, FT), 0 ≤ 0.5 (weak partitioning, WP), 0.5 ≤ 1.0 (binding), and >1.0 (strong binding). mAbs, monoclonal antibodies.

separation condition was determined to be at pH 5.87 and 107 mM Cl^--counter ions, satisfying the requirements of a log (K_p) below 0 for mAb-1, a maximized log (K_p) for LPLA2, and a maximized log (α).

3.5 | Verification of host cell protein and mAb separation conditions on lab-scale columns

The final step of our study was to verify mAb and polysorbate degrading hydrolase separation conditions using packed bed columns on a chromatography system. To assess the potential impact of the presence of mAb on the binding and elution behavior of polysorbate degrading hydrolases due to nonspecific binding, both components were evaluated simultaneously and individually for comparison. This verification was performed specifically for mAb-5 and CES2C using Poros XS cation exchange and for mAb-1 and LPLA2 using Capto adhere mixed mode.

The optimal separation conditions for mAb-5 and CES2C on a POROS™ XS column were verified based on results from the miniature experiments at pH 7.28 and 151.4 mM Na^+. Under these separation conditions, 98.7% of CES2C was separated from mAb-5 (Figure 10). The presence of mAb-5 slightly increased the survival rate of CES2C. However, no significant differences were observed between the combination of mAb-5 and CES2C in the control runs. The majority of mAb-5 was eluted at satisfactory levels in the salt gradient for mAb-5 and CES2C in combination (96.5%) and the mAb alone (98.5%).

The verification for mAb-1 and LPLA2 was performed on a MMC column (Capto adhere) at pH 5.87 and 107 mM Cl^- counter ions. The generated chromatograms included the combined mAb-1 and LPLA2, as well as each molecule individually (Figure 11). Remarkably, 99.3% of LPLA2 was separated from the antibody, with no significant changes observed in the flow-through fractions for the HCP LPLA2, whether in combination with the antibody or alone. An antibody recovery rate of 99.3% was achieved. Interestingly, the majority of LPLA2, which exhibited strong binding to the resin, was not eluted during the salt gradient process but was instead found during the column cleaning steps. These results suggest that the separation conditions identified in the miniature column experiments can be successfully transferred to the packed bed columns. Furthermore, the data suggest that the presence of the mAb-1 has no significant impact on LPLA2 levels in the flow-through fraction.

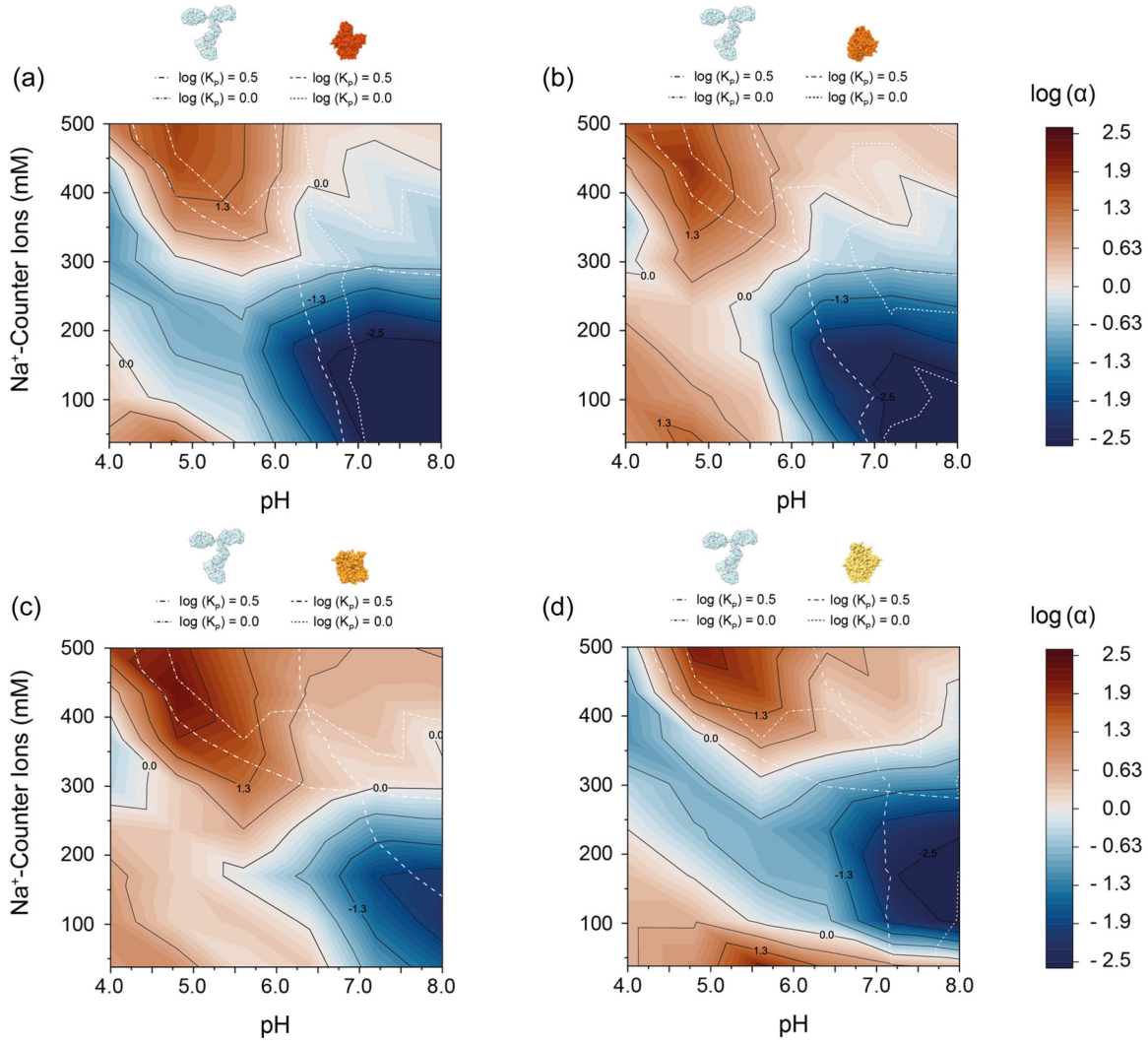

FIGURE 5 Identification of cation exchange separation conditions between mAb-5 and four polysorbate hydrolases. Contour plots show the logarithmic separation factor α (log (α)) as a function of pH and Na^+-counter ions (mM) for the target protein mAb-5 (IgG-scFv) and the HCP impurities (a) CES2C, (b) LPLA2, (c) PAF-AH, and (d) CES1F. In case of bind-and-elute cation exchange chromatography mAb and HCP separation is indicated by decreasing log (α) values < 0 (white to dark blue). The white lines indicate the weak partitioning region of each protein with log (K_p) values of 0 and 0.5. log (K_p) = 0: transition line between flow-through and weak-partitioning (mAb-5: short dashed-dotted; HCP: short dashed), log (K_p) = 0.5: transition from weak partitioning to binding (mAb-5: dashed-dotted; HCP: dashed). HCP, host cell protein; mAbs, monoclonal antibodies.

4 | DISCUSSION

The objective of this study was to address two major gaps in the current understanding of polysorbate degrading HCP behavior during downstream processing, focusing specifically on cation exchange and mixed-mode chromatography as polishing steps. The first gap concerns the limited knowledge of the binding behavior of the specific HCPs CES2C, LPLA2, PAF-AH, and CES1F, to the different resin types. The second gap concerns the identification of separation conditions for effective removal of critical HCPs from the antibody. Bridging these gaps is critical as polysorbate degrading HCPs continue to be identified in antibody formulations, highlighting the need for improved understanding and optimization of the purification process (Hu, Molden, Hu, et al., 2022; Kovner et al., 2023). With respect to the binding behavior of polysorbate degrading HCPs and mAbs during the polishing steps of a downstream purification process, our results illustrated different pattern under the investigated conditions. For CEX, we observed that pH has a more significant effect on the interactions between polysorbate degrading hydrolases and the resin compared to the concentration of counter ions. Interestingly, these results are consistent with McDonald et al., who found strong binding over the pH range of 5 to 6.5 and Na^+-counter ion concentrations of 50–225 mM for general HCP content (McDonald et al., 2016). This suggest that our findings are consistent with the overall HCP behavior. Binding screenings of mAbs using CEX revealed a pattern that as pH increases, a reduced number of Na^+-counter ions are required for the mAb to shift from strong binding to moderate binding (Figure 3). This observation is consistent with several studies that have highlighted

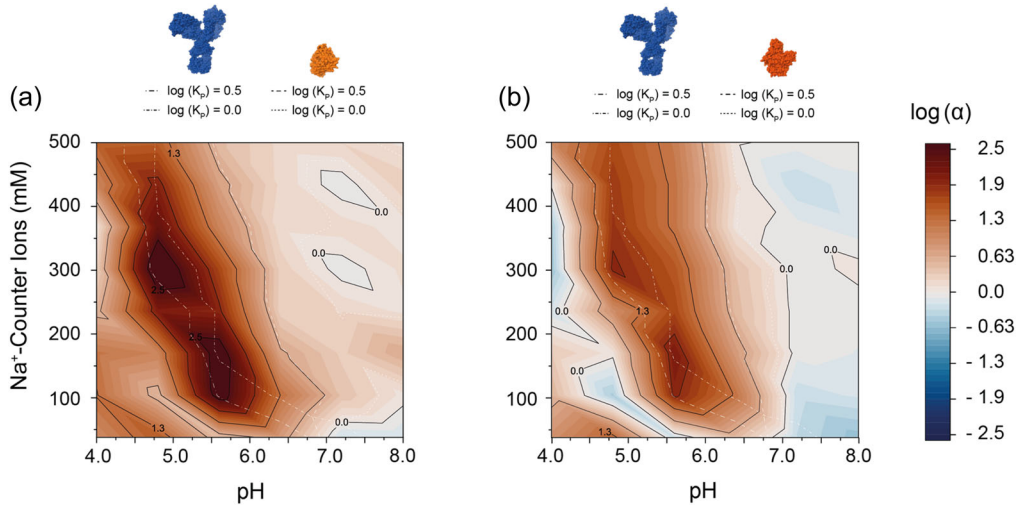

FIGURE 6 Identification of cation exchange separation conditions between mAb-3 and two polysorbate hydrolases. Contour plots show the logarithmic separation factor α (log (α)) as a function of pH and Na^+-counter ions (mM) for the mAb-3 and the polysorbate hydrolases (a) LPLA2 and (b) CES2C. mAb, monoclonal antibody.

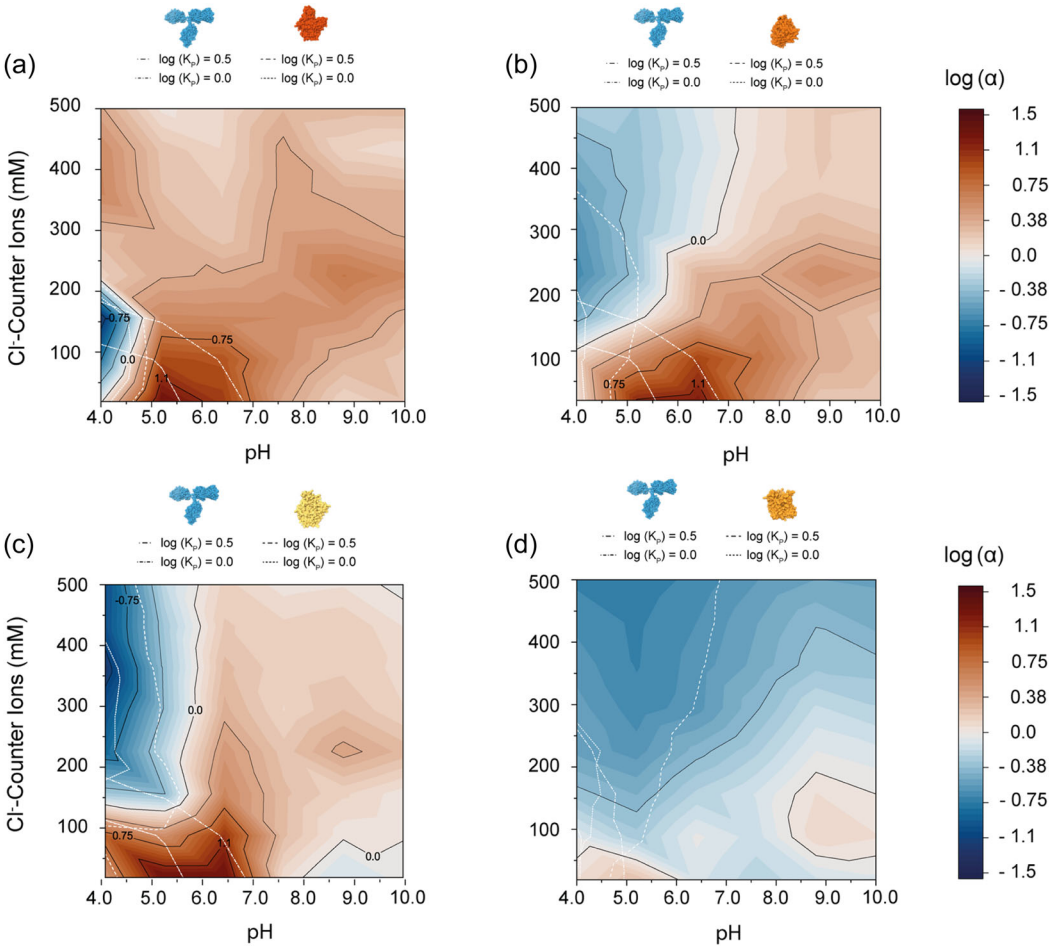

FIGURE 7 Identification of mixed mode separation conditions between mAb-1 and polysorbate hydrolases. Contour plots display the logarithmic separation factor α (log (α)) as a function of pH and Cl^--counter ions (mM) for mAb-1 and the polysorbate hydrolases (a) CES2C, (b) LPLA2, (c) PAF-AH, and (d) CES1F. In case of mixed-mode chromatography mAb and HCP separation is indicated by increasing log (α) values > 0 (white to dark red). The white lines indicate the weak partitioning region of each protein with log (K_p) values of 0 and 0.5. log (K_p) = 0: transition line between flow-through and weak-partitioning (mAb-1: dashed-dotted; polysorbate hydrolase: dashed), log (K_p) = 0.5: transition from weak partitioning to binding (mAb-1: short dashed-dotted; HCP: short dashed). HCP, host cell protein; mAbs, monoclonal antibodies.

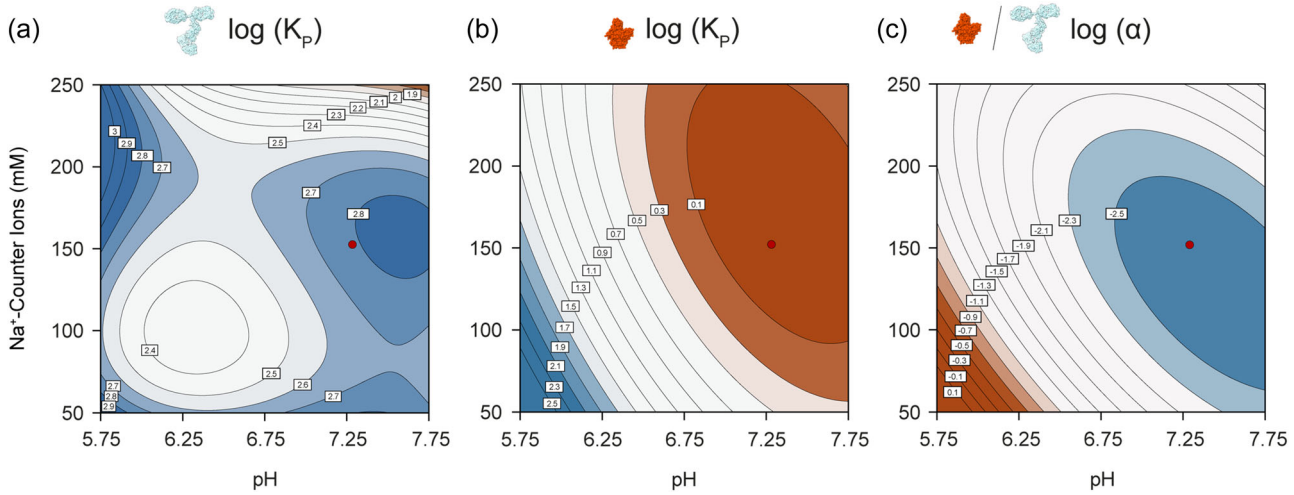

FIGURE 8 Determination of the optimal separation conditions for mAb-5 and CES2C on Poros XS miniature columns. Contour plots illustrate the influence of pH and Na^+-counter ions (mM) on the log (K_p) of the target protein mAb-5 (a), the log (K_p) of impurity CES2C (b), and the logarithmic separation factor log (α) (c), based on a quadratic I-optimal design. The red dot represents the optimal separation condition for mAb-5 and CES2C. mAb, monoclonal antibody.

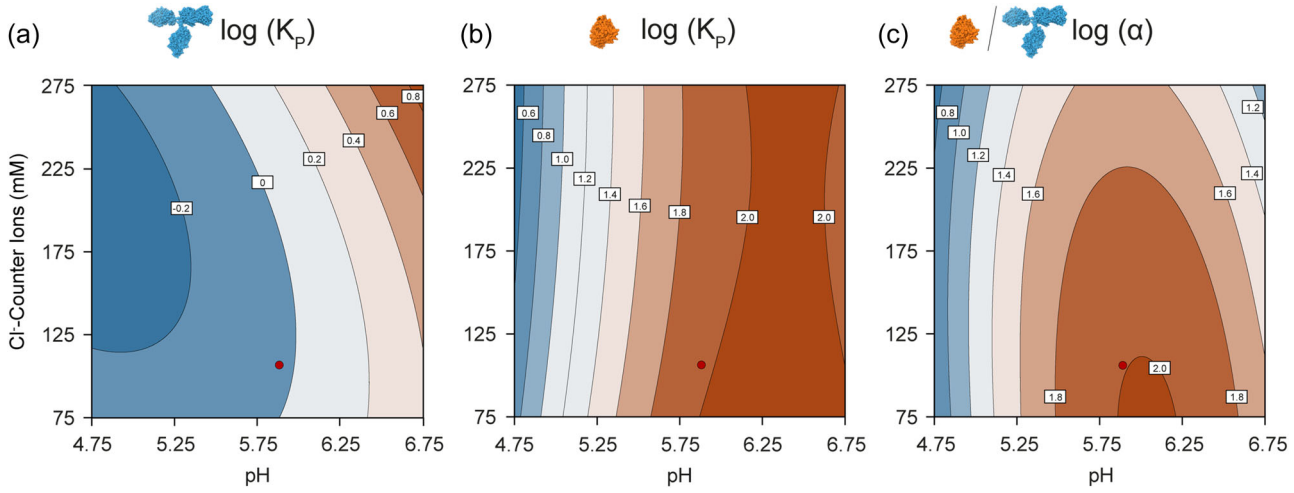

FIGURE 9 Determination of the optimal separation conditions for mAb-1 and LPLA2 on Capto adhere miniature columns. Contour plots illustrate the influence of pH and Cl^--counter ions (mM) on the log (K_p) of the target protein mAb-1 (a), the log (K_p) of the impurity LPLA2 (b), and the logarithmic separation factor log (α) (c), using a quadratic I-optimal design. The red dot represents the optimal separation condition for mAb-1 and LPLA2. mAb, monoclonal antibody.

the more significant effect of Na^+-counter ion concentration on mAb-resin interactions relative to pH (Kelley, Switzer, et al., 2008; Kelley, Tobler, et al., 2008; Petroff et al., 2015; Welsh et al., 2014). The study by Welsh and coworkers further supports the approach of this research, showing that different loading challenges (5–120 g/L Resin) had minimal effect on the overall observed binding behavior of mAbs to Poros XS (Welsh et al., 2014). Notably, this resin specificity is not limited to Poros XS, as similar behavior was observed by McDonald et al. for the CEX resins Poros50HS and SPSFF (McDonald et al., 2016). These findings suggest that the results of this study may also be applicable to other CEX resins. Another notable observation supported by literature, is that the pI of a mAb cannot reliably predict the exact rank order in which antibodies shift from binding to nonbinding conditions (Lorek et al., 2023). For example, although the two IgG4 molecules have pI values within a similar range, mAb-2 has a broader range in which it strongly binds the resin than mAb-3 (Figure 3). However, a general trend was observed that antibodies with higher pIs exhibited a broader range of strong binding, facilitating their separation from similarly charged polysorbate degrading hydrolases. On the other hand, antibodies with lower pIs may present challenges during purification from similarly charged polysorbate degrading hydrolases. This was evident in the case of mAb-3, where no separation window was identified in this process step. For the removal of polysorbate degrading HCPs by CEX, higher pIs are generally more favorable.

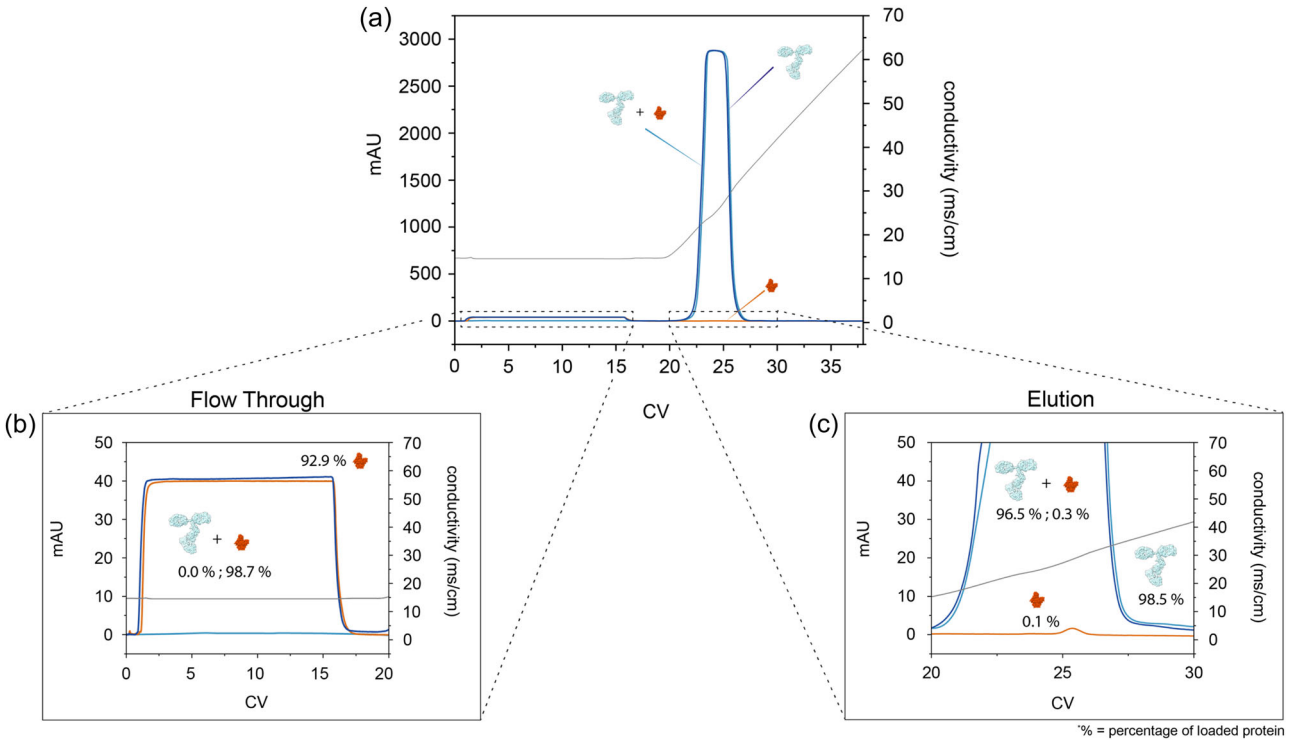

FIGURE 10 Verification of separation conditions for mAb-5 and CES2C using a 1 mL POROS XS column. Chromatogram (a) shows cation exchange chromatography runs in bind-and-elute mode for mAb-5 and CES2C combined (cyan), mAb-5 alone (blue), and CES2C alone (orange). The flow-through (b) and elution (c) fractions show the percentage of loaded protein. Conductivity (mS/cm) is shown in gray. Flow-through: 0–18 column volumes (CV), wash: 18–20 CV, and gradient elution: 20–38 CV. mAb, monoclonal antibody.

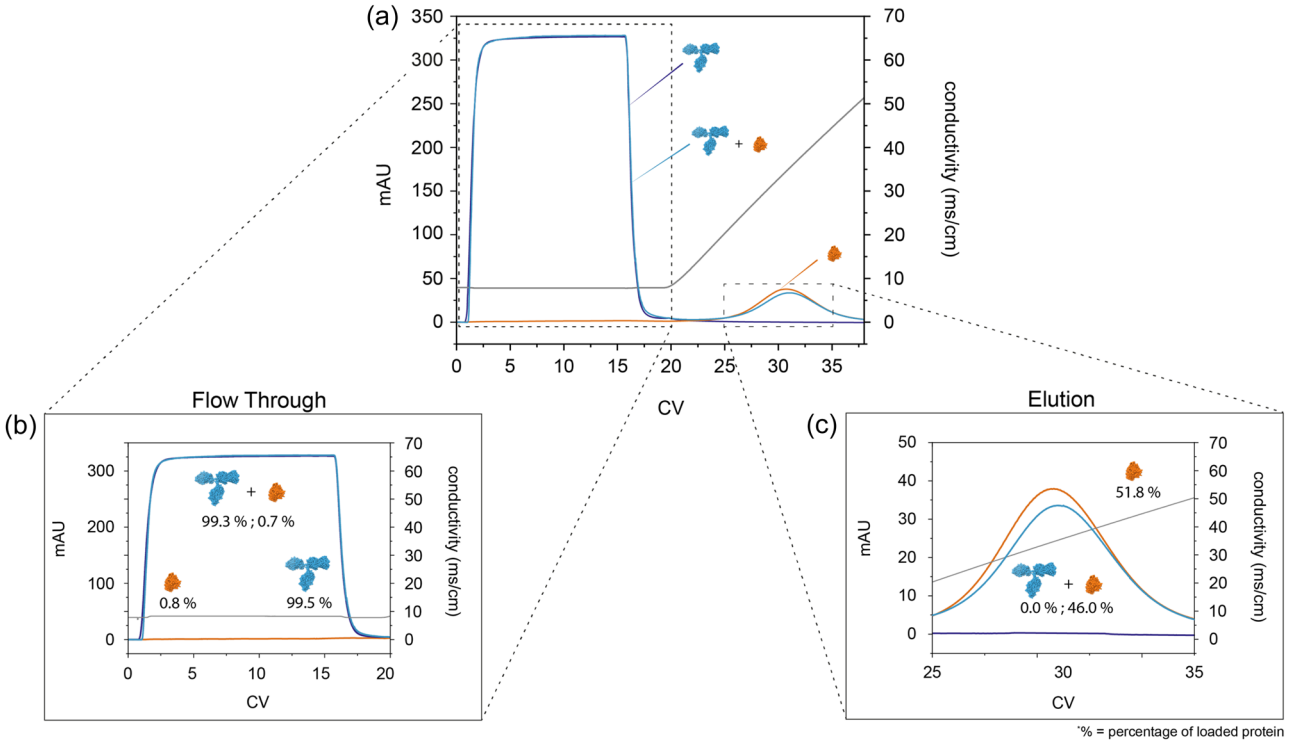

FIGURE 11 Validation of separation conditions for mAb-1 and LPLA2 using a 1 mL Capto adhere column. Chromatograms (a) show flow-through mixed mode chromatography runs for mAb-1 and LPLA2 combined (cyan), mAb-1 alone (blue), and LPLA2 alone (orange). Flow-through (b) and elution (C) fractions show the percentage of loaded protein. Conductivity (mS/cm) is shown in gray. Flow-through: 0–18 CV column volumes (CV), wash: 18–20 CV, gradient elution: 20–38 CV. mAb, monoclonal antibody.

When mixed-mode chromatography resins were used, the binding behavior of polysorbate degrading hydrolases showed significant variation among individual molecules, with no discernible general trends.

These observations highlight the importance of understanding the unique binding characteristics of individual HCPs to optimize downstream processing steps, especially when targeting the separation of a specific polysorbate degrading hydrolase.

The results of this study suggested that polysorbate degrading hydrolases may persist through polishing steps without specific tailoring of column conditions due to similar binding behavior. In both CEX and MMC for mAbs and the polysorbate degrading hydrolases analyzed, a consistent trend shows that optimal separation occurs at low counter-ion concentrations (60 to 150 mM), which is consistent with the findings by Kelley and colleagues for overall HCP removal (Kelley, Tobler, et al., 2008). Regarding pH, the ideal range for CEX was determined to be typically above 6.5, while for MMC, it is between 5.5 and 6.5 (Figures 1 and 2).

This study also provides insight into how a three-step strategy can identify downstream separation opportunities demonstrated for two different mAb-HCP combinations. The identified separation conditions were successfully validated on packed bed columns, achieving satisfactory reduction of CES2C in CEX and LPLA2 in MMC. In this context, it is important to note that the quantification of the polysorbate hydrolases was achieved using specific tags. This approach was necessitated by the lack of commercially available antibodies specific to native CHO proteins. Furthermore, accurately measuring individual HCPs in the presence of a mAb at extremely low levels (below 10 ppm) via mass spectrometry presents significant challenges. Given the relatively small size of the His- and Strep-tag compared to the HCPs, their influence on partitioning behavior is considered negligible. These tags, composed of short amino acid sequences, are unlikely to substantially alter the hydrophobicity or charge distribution of the proteins, thereby not affecting their binding characteristics in cation exchange and mixed mode chromatography. Although the presence of the tags could induce slight shifts in the weak partitioning region, the determined separation conditions—with log(Kp) values less than 0 for CEX and greater than 1 for MMC—are sufficiently distinct from this range. Thus, even if minor shifts in the weak partitioning region occur, effective separation is still achieved.

Interestingly, the data suggest that coelution, rather than hitchhiking, seems to be the main reason for the copurification of certain polysorbate degrading hydrolases (and maybe also other HCPs) in the downstream process. Hitchhiking involves nonspecific binding of HCPs to mAbs or other target molecules during purification, whereas coelution occurs when mAbs and HCPs have similar properties, causing them to elute together. The study by Hecht et al. found that LPLA2 has low affinity for IgG1 and IgG4 antibodies, which could contribute to polysorbate hydrolase survival through the downstream processing (Hecht et al., 2022). Our results for LPLA2 and mAb-1 (IgG1) on MMC suggest that low affinity does not cause additional LPLA2 to elute (Figure 11). A minimal effect of hitchhiking on the screened polysorbate degrading hydrolases is supported by CEX chromatography column runs with CES2C and mAb-5. Another study by Zhao et al. investigated the separation of different HCPs through size exclusion chromatography. The polysorbate degrading hydrolase CES1F could be separated from the mAb under native conditions, indicating no effective binding between the mAb and this hydrolase (Zhao et al., 2022).

Determining the optimal separation factor for all mAbs, especially for mAb-3 (IgG4) in this study, can be challenging. The binding behavior of mAb-3 in MMC and CEX is largely consistent with that of the four polysorbate degrading hydrolases studied (Supporting Information S1: Figures 1 and 2). Since separation depends on the different binding behavior of the HCPs and the target mAb to the resin, similar binding patterns result in coelution. The efficiency of separation of specific HCPs from mAbs during the MMC and CEX steps is strongly influenced by the molecular properties of the mAbs. The results of this study indicate that complete removal of polysorbate degrading hydrolases might not be possible for all mAb-HCP combinations. Hence, alternative approaches are needed to achieve further removal of polysorbate degrading hydrolases. This could include the optimization of the capture step. The observed coelution during the polishing steps does not explain the persistence of HCPs during the capture steps. Some studies have suggested that HCPs may also associate with aggregates (Hu, Molden, Hu, et al., 2022; Zhao et al., 2022) or survive the capture step due to mAb cluster formation (Luo et al., 2022; Oh et al., 2022). However, these possibilities are beyond the scope of this publication. Optimizing the capture step involves optimization in binding and wash conditions, reducing the burden on subsequent polishing steps. However, it must be ensured that the antibody remains structurally intact, and the yield is maintained under these conditions. Another potential approach to remove particularly critical polysorbate degrading hydrolases from certain mAbs could be to modify the antibody backbone to alter the physicochemical properties of the mAb. This modification might potentially prevent coelution, thereby improving the separation and purification process. However, this approach requires careful consideration of the potential impact on the antibody's efficacy and stability. Structural modifications might affect the antibody's binding affinity, half-life, and overall therapeutic performance, necessitating extensive validation. In addition, the use of cell line engineering to knock out polysorbate degrading hydrolases is a promising strategy, but several challenges must be addressed. Knocking out specific hydrolases could lead to compensatory upregulation of other hydrolases, potentially affecting cell viability, growth rates or protein production. Some hydrolases may play critical roles in cellular functions, making their knockout problematic. Extensive validation is required to ensure the modified cells perform equivalent to their non modified counterparts. To date, this approach has so far only been successfully demonstrated for the removal of lipoprotein lipase (LPL) in CHO cells, (Chiu et al., 2017; Dovgan et al., 2021) indicating the need for further research to extend this approach to other hydrolases involved in polysorbate degradation.

5 | CONCLUSION

Our study is the first in the field to take a detailed look at hydrolase-antibody coelution, focusing specifically on cation exchange (CEX) and mixed-mode chromatography (MMC). Our high-throughput binding screenings revealed unique binding behaviors of various HCPs and mAbs, highlighting the importance of understanding these characteristics to optimize downstream processing. Lower counter ion concentrations were found to improve HCP clearance in both CEX and MMC. We employed a three-step strategy, consisting of high-throughput binding screenings, miniature column experiments, and packed bed column experiments, to evaluate the binding behavior of HCPs and mAbs under different conditions and identify differences in resin interaction. This approach allowed us to identify optimal separation conditions for both CEX and MMC that resulted in HCP reduction while maintaining high mAb recovery rates. However, the separation of some HCPs from certain mAbs remains challenging, indicating the need for alternative approaches. It is important to note that considering other resins and chemistries may further optimize the purification process. The variability in binding behavior, especially observed in the mixed mode resin, highlights the potential for further enhancements in the purification process through careful resin selection. Furthermore, to gain a more comprehensive understanding of the overall purification process, it is necessary to extend research efforts to other steps in the bioprocess. This will provide a more holistic view of the process and may reveal additional opportunities for optimization.

AUTHOR CONTRIBUTIONS

Melanie Maier: Conceptualization; data collection; data analysis and interpretation; writing—original draft; writing—review and editing. **Stefan Schneider:** Data Collection; data analysis and interpretation; writing—review and editing. **Linus Weiss:** Data collection; writing—review and editing. **Daniel Lakatos:** Writing—review and editing. **Simon Fischer:** Supervision; writing—review and editing. **Joey Studts**: Supervision; resources; writing—review and editing. **Matthias Franzreb:** Supervision; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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