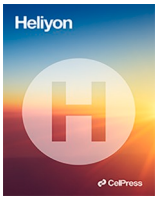
Heliyon 10 (2024) e35124

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

DOE-based process optimization for development of efficient methods for purification of recombinant hepatitis B surface antigen from *Pichia pastoris* feedstock using Capto adhere resin

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ARTICLE INFO

Keywords:
Process optimization
Design of experiments (DOE)
rHBsAg purification
Capto adhere
Downstream processing

ABSTRACT

Background: The multimodal chromatography resins, such as Capto adhere, are considered good candidates to be utilized in downstream processing due to their high capacity and selectivity; however, their multimodal interactions lead to an intricacy in the adsorption-desorption patterns and systematic characterization of conditions for process steps is necessary.
Methods: Capto adhere, a strong ion exchanger with multimodal functionality, was used in this study for the final aim of recombinant hepatitis B surface antigen (rHBsAg) purification from *Pichia pastoris* (*P. pastoris*) industrial feedstock. Optimization of various parameters was done using the design of experiments (DOE) approach to determine the best binding and non-binding conditions.
Results: Maximum rHBsAg binding on Capto adhere occurred in 20 mM sodium acetate, pH 4.5, and a binding capacity of about 0.75 mg/ml was achieved, which was much higher than rHBsAg binding capacity of other resins reported so far. In elution optimization investigations, it was revealed that 1 M arginine (buffered in 50 mM sodium phosphate, pH 6.5) was the most efficient eluting agent. The binding and elution optimal conditions were utilized for further purification of rHBsAg from *P. pastoris* industrial feedstock in bind-elute mode, and the recovery and purity of the obtained rHBsAg were about 60% and 100%, respectively. Following optimization in the flow-through purification mode, the target protein recovery was significantly increased (up to 97%) and the target protein purity of more than 95% was achievable. SEC-HPLC analysis showed that the obtained retention times for the purified rHBsAg were similar to those reported previously.
Conclusions: These results suggest that Capto adhere under such optimized conditions can be considered as a good candidate for efficient purification of rHBsAg from *P. pastoris* industrial feedstock in downstream processing.

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https://doi.org/10.1016/j.heliyon.2024.e35124
Received 20 August 2023; Received in revised form 22 July 2024; Accepted 23 July 2024
Available online 24 July 2024

1. Introduction

Commercial manufacturing of hepatitis B surface antigen (HBsAg) using recombinant yeasts has been established as an alternative to the antigen purification from the human plasma [1]. Based on the host yeast expressing rHBsAg, various methods have been utilized in downstream processes [2,3]. *Pichia pastoris* (*P. pastoris)* has been one of the most important workhorse cell factories for the production of rHBsAg because of advantages over other yeasts (such as efficient and low-cost production of recombinant proteins with suitable glycosylation) [4–7]. In rHBsAg vaccine production using *P. pastoris*, the conventional downstream processing is complex and costly due to using several chromatographic steps including immunoaffinity chromatography [3]; therefore, developing cheaper and more efficient downstream processing methods is of interest.

Using non-affinity chromatography methods imperils the selectivity and efficacy of the purification process due to the low orthogonality of single mode resins in a single-step [8]. Compared to single-mode resins, multimodal or mixed-mode chromatography (MMC) media provide higher adsorption capacity and unique selectivity due to their sophisticated ligand chemistry [9–12]. Orthogonality in multimodal resins, achieved through multiple interactions (hydrogen bonding, hydrophobic, electrostatic, and Wander Waals interactions), leads to discrimination between different proteins based on subtle changes in their surface properties and eventually increases selectivity and efficacy of a purification step [8–10,13–16].

Capto adhere, which is a salt-tolerant multimodal strong anion exchanger with N-benzyl-N-methyl ethanolamine ligand, can interact with proteins via ionic interaction, hydrogen bonding and hydrophobic interaction. Capto adhere is in the category of the commercial multimodal resins which contain ligands with particular incorporation of hydrogen bonding moieties in close proximity to ionic moieties leading to their salt-tolerance and consequently their ability to capture proteins directly from feedstocks with high conductivity. These features remove the necessity for feed dilution to reduce its conductivity, normally required for ion exchange (IEX) single-mode resins [17,18]. Capto adhere have been used in bind/elute and flow-through chromatography modes for purification of monoclonal antibodies and other proteins from feedstock with high conductivity (e.g., 15–30 mS/cm) [19–25].

However, protein adsorption–desorption in mixed-mode resins becomes complicated due to the involvement of several interactions with the ligand's functional groups [26,27]. Therefore, a meticulous optimization process for protein purification using multimodal resins is required. Using the one-factor-at-a-time (OFAT) approach for process optimization requires numerous experiments and is time-consuming and laborious. Also, it fails to calculate the global optimal conditions since the experiments result in partial knowledge about the design space, and the effect of the variables combination is disregarded. These limitations can be avoided using the design of experiment (DOE), since variables are simultaneously varied and this helps generate adequate information using the minimum number of experiments [28,29]. In the DOE approach, multidimensional modeling of the main and higher order effects (i.e., interaction and quadratic effects), which may occur during protein purification, assures robustness of the obtained optimal conditions [29–35]. Hence, the main and interaction effects of the most significant process parameters (i.e., factors) on the process responses are defined in a space using designed experiments [30–32]. Response surface methodology (RSM) is suggested as a DOE method for inclusive examining design space in optimization studies [36,37].

Based on evidence in the literature, there has been no report on rHBsAg purification using multimodal Capto adhere resin so far. In this study, Capto adhere was chosen as mixed-mode anion-exchange resin for purification of rHBsAg from *Pichia pastoris* feedstock in bind-elute and flow-through modes. The main objective of this study was rHBsAg purification with high purity from the *P. pastoris* industrial feedstock by multimodal Capto adhere resin in different purification modes. For this purpose, the important factors and responses were firstly defined using OFAT non-binding experiments. Then the target protein binding and non-binding conditions were defined using the DOE method. The optimal conditions were finally utilized to purify rHBsAg from *P. pastoris* industrial feedstock in two purification modes (i.e., bind-elute and flow-through) and the purified rHBsAg was assessed for purity and SEC-HPLC profile. Finally, the protein purity, purification yield, and capacity of Capto adhere were discussed to evaluate its efficacy and selectivity for rHBsAg purification in a single-step.

2. Methods

2.1. Defining design space elements for DOE

The main factors, including salt type/concentration (NaCl and $(NH_4)_2SO_4$) and pH, were selected to be evaluated in the binding step optimization using DOE. In the elution step, the chemicals (determined by the preliminary screening experiments) and pH were selected for DOE optimization. The factors including temperature and protein concentration were kept constant in each series of experiments. The step yield was considered as the main response in all experiments.

2.2. Defining factors ranges for DOE

To define the pH range, OFAT non-binding experiments were conducted using rHBsAg-API (obtained from Pasteur Institute of Iran) and Capto adhere resin. Briefly, rHBsAg-API was dialyzed into 25–50 mM buffers with various pH values (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5). The resin was distributed in eight Eppendorf microtubes in equivalent volumes (200 μl) and equilibrated (three times) with the pertinent binding buffer. The protein solutions (200 μl), prepared at different conditions by dialysis, were next added to the appropriate equilibrated resin, and the microtubes were placed at room temperature for 1 h (with gentle mixing at 5 min breaks). Protein solutions were separated from the resin beads by centrifugation at 300 *g* for 2 min for SDS-PAGE and densitometry (using Image lab software). The concentration ranges for salts and additives were defined by the literature review.

2.3. DOE-based optimization for rHBsAg binding on Capto adhere resin

Binding experiments were conducted based on a Central Composite Face-centered (CCF) design using version 11 (StatEase). The experimental layouts were created using two factors (i.e., pH and salt concentration) for NaCl and $(NH_4)_2SO_4$ (Tables 1a and 1d). The percentage of adsorbed rHBsAg was calculated as the DOE response. Based on the designed outlines, protein solutions were made in the relevant conditions for each of the 22 experiments. Capto adhere resin was aliquoted in equal volumes (100 μl) in 22 Eppendorf microtubes, equilibrated with different binding buffers, and subsequently exposed to the relevant protein samples (100 μl). The microtubes were placed at room temperature for 1 h with continuous mixing and eventually centrifuged at 300 g for 5 min to separate protein solutions for SDS-PAGE.

After densitometry, the difference between initial and final rHBsAg concentration was considered as the amount of adsorbed protein. The resulting data were transferred to the Design-Expert software and the most fitted model for the data was produced. Consequently, the optimal point was achieved and experimentally confirmed.

Table 1
The experimental outlines and obtained results for rHBsAg binding on Capto adhere resin in association with NaCl (a-c) and $(NH_4)_2SO_4$ (d-f). (a) and (d) are experimental layouts, (b) and (e) are ANOVA tables, and (c) and (f) are Fit Statistics tables.
(a).

Run	Space Type	A: pH	B: NaCl concentration (mM)	Adsorbed rHBsAg (%)
1	Factorial	4.5	0	80
2	Factorial	8	0	22
3	Factorial	4.5	600	70
4	Axial	6.25	0	22
5	Center	6.25	300	18
6	Center	6.25	300	18
7	Axial	4.5	300	36
8	Axial	6.25	600	15
9	Axial	8	300	17
10	Factorial	8	600	16
11	Center	6.25	300	20

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	0.0012	3	0.0004	25.87	0.0004	significant
A-pH	0.0008	1	0.0008	49.90	0.0002	
B–NaCl concentration	0.0001	1	0.0001	7.49	0.0290	
A^2	0.0003	1	0.0003	20.21	0.0028	
Residual	0.0001	7	0.0000			
Lack of Fit	0.0001	5	0.0000	4.42	0.1947	not significant
Pure Error	8.756E-06	2	4.378E-06			
Cor Total	0.0013	10				

Std. Dev.	Mean	C.V. %	R^2	Adjusted R^2	Predicted R^2	Adeq Precision
0.0039	0.0240	16.20	0.9173	0.8818	0.7493	13.2711

Run	Space Type	A: pH	B: $(NH_4)_2SO_4$ concentration (mM)	Adsorbed rHBsAg (%)
1	Factorial	4.5	0	80
2	Factorial	8	0	22
3	Factorial	4.5	600	74
4	Axial	6.25	0	22
5	Center	6.25	300	28
6	Center	6.25	300	22
7	Axial	4.5	300	70
8	Axial	6.25	600	46
9	Axial	8	300	40
10	Factorial	8	600	50
11	Center	6.25	300	24

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	5299.06	4	1324.76	32.50	0.0003	significant
A-pH	2308.40	1	2308.40	56.63	0.0003	
B- $(NH_4)_2SO_4$ concentration	385.28	1	385.28	9.45	0.0218	
AB	317.43	1	317.43	7.79	0.0315	
A^2	2287.95	1	2287.95	56.13	0.0003	
Residual	244.56	6	40.76			
Lack of Fit	224.25	4	56.06	5.52	0.1592	not significant
Pure Error	20.31	2	10.15			

Std. Dev.	Mean	C.V. %	R^2	Adjusted R^2	Predicted R^2	Adeq Precision
6.38	45.18	14.13	0.9559	0.9265	0.8135	13.3557

2.4. Screening of effective additives for rHBsAg elution from Capto adhere

For this purpose, various concentrations of chemicals including NaCl, potassium thiocyanate (KSCN), ethylene glycol, glycerol, arginine, urea, and ethanol (all made in 20 mM Tris buffer, pH 6.5), sodium acetate, and Tris buffer at different pH values were used in twenty-six OFAT experiments. To perform these experiments, a rHBsAg-API sample (2.8 ml) was prepared in the optimal binding state (achieved from the rHBsAg binding optimization investigations) and added to 2.8 ml equilibrated Capto adhere resin. The mix was placed at room temperature for 1 h, with periodic mixing. The supernatant was then separated from the resin beads, the resin was rinsed with the equilibration buffer and distributed in 26 Eppendorf microtubes in equivalent volumes of 100 μl. According to the OFAT experimental design outline, elution of rHBsAg from Capto adhere resin was made using 300 μl of each treatment (Table 3) in each microtube for 15 min. The supernatants were separated from the resin beads by centrifugation at 300 *g* for 5 min and analyzed by SDS-PAGE. Following densitometry, the percentage of rHBsAg recovery was considered as the response in each elution experiment.

2.5. DOE-based optimization for elution of rHBsAg from Capto adhere

In the matter of elution experiments, two efficient eluting agents obtained from OFAT elution experiments (section 2.4) and pH were used for CCF design in 3 levels, which generated 18 experimental runs. The experiments were conducted and the response (percentage of rHBsAg recovery) was analyzed as explained in section 2.4.

2.6. rHBsAg purification using Capto adhere resin from P. pastoris feedstock

2.6.1. rHBsAg purification in bind-elute purification mode

The crude extract containing rHBsAg originated from mechanically disrupted *P. pastoris* cells was acquired from the Pasteur Institute of Iran. The crude extract pH was decreased to 4.5 by adding 1 M HCl. The precipitated proteins (i.e., protein impurities that precipitated at their isoelectric points through decreasing pH to 4.5) were then separated by centrifugation at 10,000 g for 15 min. The supernatant was used for the preparation of a protein feedstock (400 μl) in the optimal binding state (i.e., condition achieved from the optimization investigation, section 2.3) using dialysis and mixed with 400 μl equilibrated Capto adhere resin. The mixture was placed at room temperature for 1 h, with periodic mixing. The supernatant was next separated from the resin using centrifugation (300 g, 5 min). The resin was aliquoted in equal volumes (200 μl) in two Eppendorf microtubes. For rHBsAg elution according to the elution optimized point resulting from the CCF outline, 300 μl of the elution agent was added to the resin in each microtube for a period of 15 min. The supernatant was finally separated using centrifugation (300 g, 5 min) and analyzed by SDS-PAGE. In these experiments, the elution yield and rHBsAg purity figures were considered as responses.

2.6.2. rHBsAg purification in flow-through purification mode

The feedstock having rHBsAg was dialyzed in sodium acetate buffer (pH 5.5) containing NaCl concentrations of 0, 200, 400, and 800 mM and in sodium phosphate buffer (pH 6.5) containing NaCl concentrations of 0, 400, and 800 mM. Equal volumes of the protein samples were mixed with the equilibrated resins for 1 h. After separation using centrifugation, the supernatant was evaluated by SDS-PAGE and densitometry.

2.7. Analytical methods

2.7.1. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein samples were diluted 1:1 with the sample buffer, placed in boiled water for 3 min, and used for SDS-PAGE (12% acrylamide). Molecular weights were estimated using a prestained protein ladder (SL7012, Sinaclon, Tehran, Iran) or active pharmaceutical ingredient (API) of rHBsAg.

2.7.2. Silver staining method

After electrophoresis, the gels were fixed in 50% (v/v) ethanol for 1 h, and 10% (v/v) acetic acid for at least 30 min. The gels were rinsed in water three times for 10 min in each rinse. Then they were impregnated for 15–30 min in silver-ammonia solution with 2% (v/v) of 25% ammonia and rinsed three times (5 min for each rinse) in water. The protein bands were developed in an acidic developer containing 1% (v/v) of citric acid. After appearing bands with desired intensity, the developer solution was discarded and the development process was stopped using the stop solution (12% (v/v) acetic acid). The densitometry of the gels was performed using Image Lab densitometry software.

To check the sensitivity and linearity of the silver staining method at various protein concentrations, different amounts of rHBsAg-API (0.25, 0.5, 1.0, 2.0, 4.0, and 15 μg) were run on the SDS-PAGE gel. After the silver staining and densitometry, the density of the bands was plotted against the protein concentration.

2.7.3. Size-exclusion high-performance liquid chromatography (SEC-HPLC)

The particle profile was analyzed using SEC-HPLC in a TSKgel 5000 PW column with a dimension of 7.5 mm ID × 60 cm (Tosoh Bioscience GmbH, Griesheim, Germany). A mobile phase of phosphate-buffer saline (1X, pH 7.4) was run at a flow rate of 0.5 ml/min at 25 °C.

3. Results

3.1. Linearity of the silver staining at various protein concentrations

The relationship between the calculated densitometry volume and various concentrations of rHBsAg on the SDS-PAGE gel showed a good correlation, with R2 = 0.98 (Fig. 1a and b and Fig. S1). As it is evident from these figures, rHBsAg at a concentration as low as 0.25–0.5 μg could be detected by this silver staining method. Linearity of the silver staining at various protein concentrations demonstrated the consistency of measurements over the entire range of densitometry measurements.

3.2. Defining of design space elements and ranges

The determination of design space elements (including factors and response) was done through literature review. Throughout binding/elution optimization experiments, step yield was considered as the response.

The results of non-binding investigations demonstrated that the maximal rHBsAg adsorption on Capto adhere resin detected at pH values between 4.0 and 5.0. Lower adsorption occurred with the steady rise in pH up to 7.5 (Fig. 2a and b and Fig. S2).

Based on these non-binding experiment results, the pH values ranging between 4.5 and 8.0 were selected for further binding optimization studies using DOE. NaCl and $(NH_4)_2SO_4$ at concentrations up to 600 mM were chosen for such binding optimization.

3.3. DOE-based optimization for rHBsAg binding on Capto adhere resin

The CCF design was selected to elucidate all effect types (i.e., main, interaction, and curvature effects). The experimental results were collected through conducting 22 CCF runs (Tables 1a and 1d) and subsequently analyzed.

Transformation of the raw data obtained from the binding experiments in the company of NaCl was according to BOX-COX plot recommendation (Power transformation with Lambda: -1.21, Constant: 0) and a reduced quadratic model (F-value = 25.87) was created (Fig. 3a and Table 1b).

The model expressions with P-values <0.1000 were remained (i.e., A, B, A^2). As shown in Table 1b, the model is well-fitted to the data (i.e., Lack of Fit F-value is not significant). As demonstrated in Table 1c, the reasonable agreement between Predicted R^2 and Adjusted R^2 (i.e., a difference less than 0.2) and an adequate signal to noise ratio (Adeq Precision >4) indicate the suitability of this model to navigate the design space.

A reduced quadratic model (F-value = 32.50) was created using Power transformation (Lambda: 1.01, Constant: 0) of the raw data obtained from the binding experiments in the company of $(NH_4)_2SO_4$ (Fig. 3c). The non-significant B^2 term was removed from the

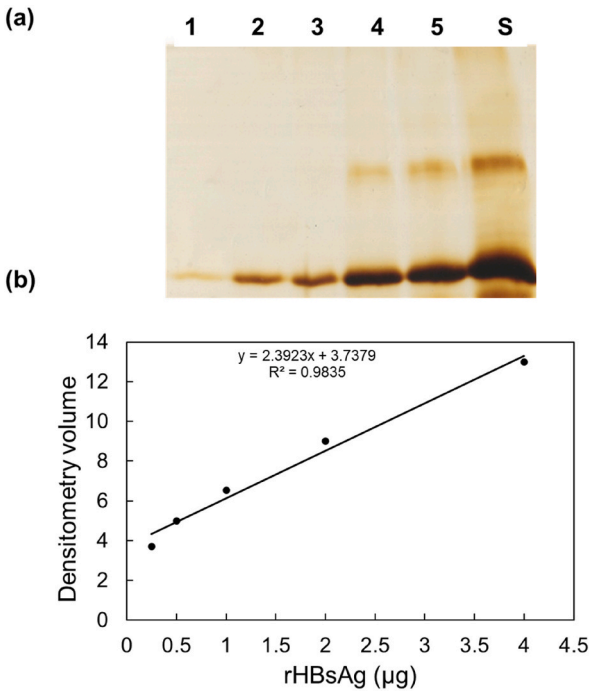

Fig. 1. SDS-PAGE and densitometry analysis of rHBsAg-API at various concentrations. a) SDS-PAGE gel staining by silver nitrate method. Lane: S – 15 μg API used as the reference volume in the densitometry software; 1 to 5–0.25, 0.5, 1.0, 2.0, and 4.0 μg API, respectively. b) Densitometry calculated volume versus various rHBsAg-API quantities.

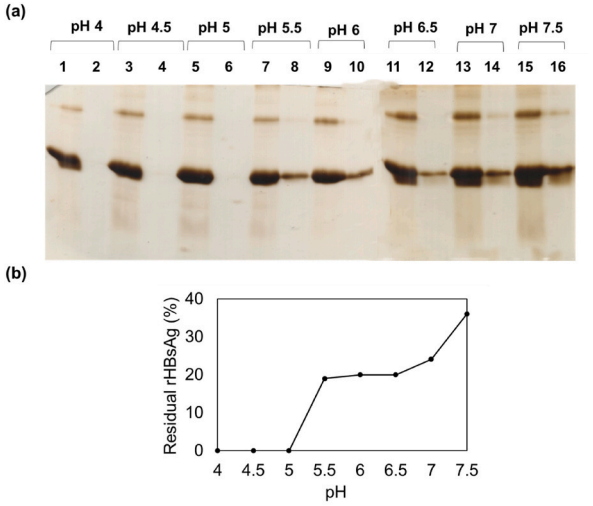

Fig. 2. The non-binding experiments for rHBsAg and Capto adhere resin. a) SDS-PAGE analysis of samples collected from non-binding experiments. Lanes 1, 3, 5, 7, 9, 11, 13 and 15 belong to rHBsAg-API before exposure to Capto adhere. Lanes 2, 4, 6, 8, 10, 12, 14, and 16 are relevant to supernatants collected after exposure to Capto adhere resin at various pH values, respectively. b) The percentage of residual rHBsAg after exposure to Capto adhere at different pH values.

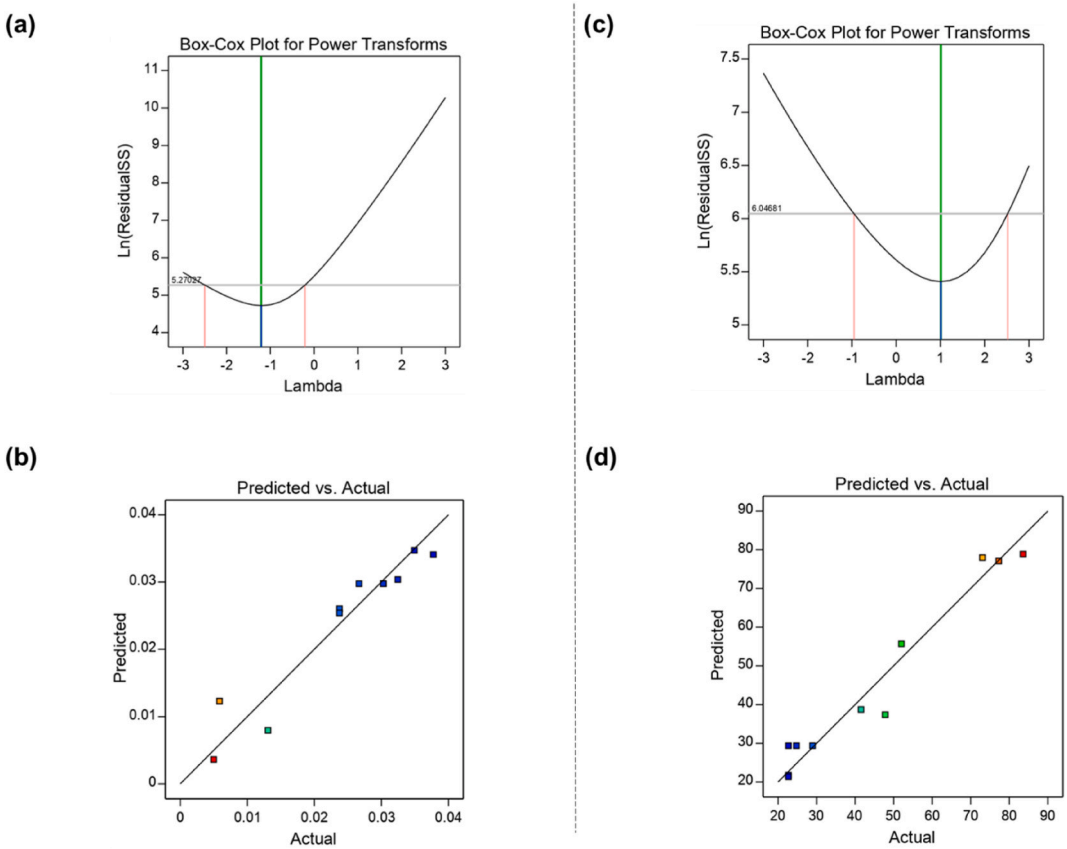

Fig. 3. The results obtained from rHBsAg binding optimization study on Capto adhere resin in connection with NaCl and $(NH_4)_2SO_4$. (a and c) are the BOX-COX plot and (b and d) are the predicted vs. actual plots for NaCl and $(NH_4)_2SO_4$, respectively.

model. The non-significant Lack of Fit confirmed that the model was correctly fitted to the data (Table 1e). The closed Predicted R^2 and Adjusted R^2 values and adequate signal to noise ratio (Table 1f) verified the model.

According to the predicted versus actual results, presented in Fig. 3b and d, the observations are well predicted by the model, due to the smooth scattering of the data points around the 45-degree line.

As revealed by the counter and 3D plots for NaCl (Fig. 4a and b), the maximum rHBsAg binding yields were detected at the pH values of 4.5 and 4.7 and salt concentrations lower than 300 mM. The maximal adsorption was attained at low pH values close to 4.5,

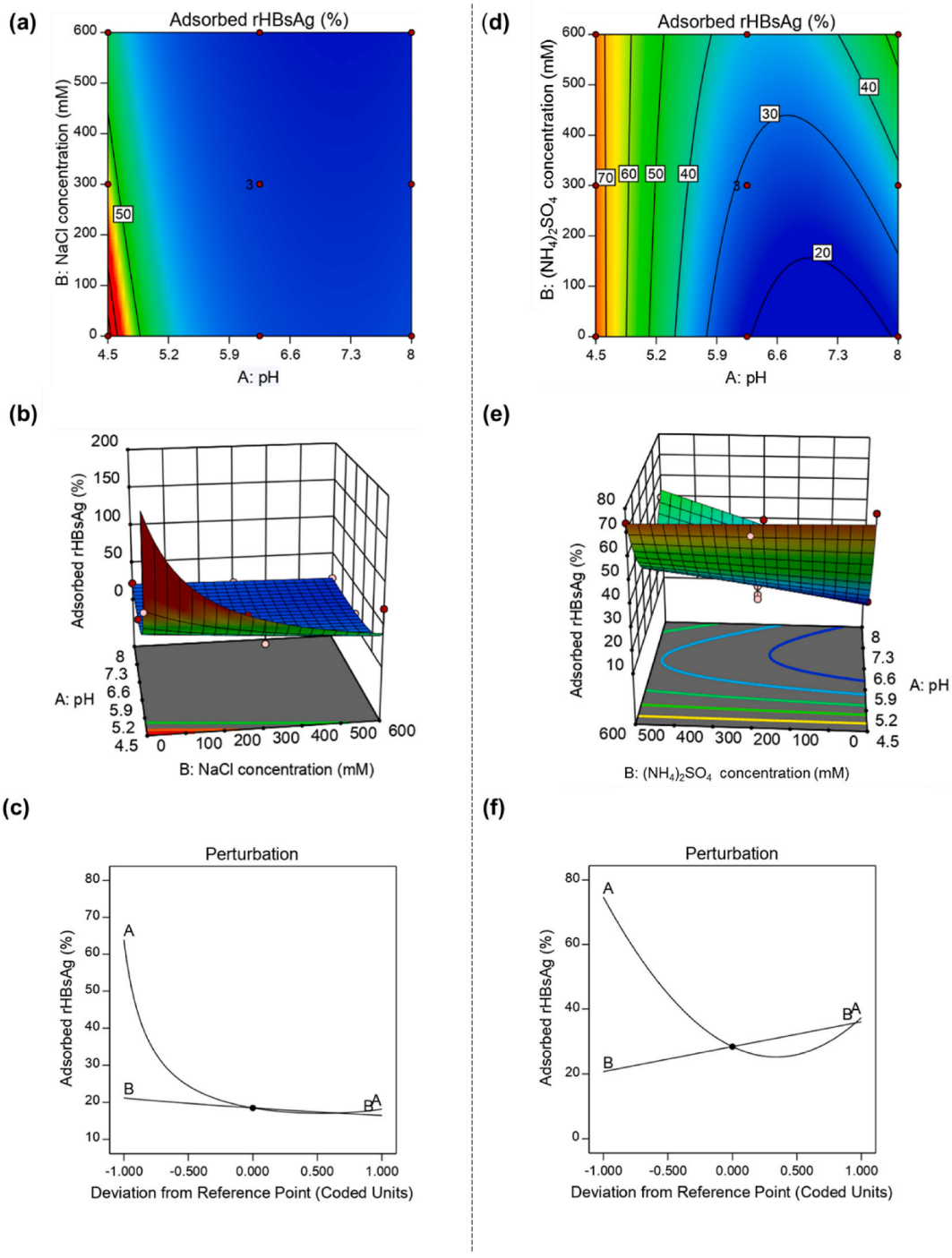

Fig. 4. The graphical presentation of data obtained from experiments designed for rHBsAg binding on Capto adhere resin in association with NaCl and $(NH_4)_2SO_4$. Counter plots (a,d), 3D plots (b,e), and perturbation plots (c,f).

in the absence of any salt. The perturbation plot showed that pH was a factor which had the uppermost effect on rHBsAg adsorption on Capto adhere resin, and the effect of NaCl was very mild (Fig. 4c).

As evident from the counter and 3D plots for $(NH_4)_2SO_4$ (Fig. 4d and e), the highest binding yield was attained at the pH values lower than 4.6 and the salt concentration had no effect on rHBsAg adsorption on the resin. At a constant salt concentration, the binding yield was decreased, with the continuing rise in pH value up to 8.0.

The perturbation plot indicated that pH was the factor with the highest effect on rHBsAg adsorption and $(NH_4)_2SO_4$ had more synergistic influence at upper pH values (Fig. 4f).

The optimal rHBsAg binding took place at the pH levels around 4.5 and salt concentrations close to zero (Tables 2a and 2b). The first three optimal points presented in Tables 2a and 2b were executed again for the model verification. During the optimization procedure for rHBsAg binding on Capto adhere resin, rHBsAg adsorption of 100% was achieved at a low pH and without salts.

3.4. Screening of effective additives for eluting rHBsAg from Capto adhere resin

When it comes to elution of proteins from multimodal resins, the interactions are very complicated, and often additives like amino acids, polyhydroxy alcohols are needed to achieve an efficient elution. As declared in previous studies [29], the effects of the additives are difficult to predict; therefore, the unsuitable pH value attained from the non-binding experiments and binding optimization investigations in this study (i.e., pH 6.5) was taken for planning twenty-six OFAT experiments in favor of determining the most efficient additives/salts for elution. According to the results presented in Table 3, 1 M arginine was the most effective eluting condition and resulted in around 60% rHBsAg recovery from the resin.

3.5. DOE-based optimization procedure for eluting rHBsAg from Capto adhere

DOE was used to elucidate the synergistic effects of the most efficient elution agents determined by OFAT experiments. A reduced 2FI model (F-value = 16.23) was generated using Power transformation (Lambda: 0.56, Constant: 0) of the raw data obtained from rHBsAg elution in the presence of 3 factors including arginine, NaCl, and pH (Fig. 5a, Table 4a). The non-significant terms (i.e., A, AB, and AC) were removed from the model (Table 4b). Due to a non-significant Lack of Fit, reasonable agreement between Predicted R^2 and Adjusted R^2 values, adequate signal to noise ratio (Table 4b and 4c), and evenly distribution of the data points in predicted versus actual results (Fig. 5b) were seen. This model was selected for further optimization studies.

Based on the counter and 3D plots (Fig. 5c and 5d), the highest rHBsAg recovery was attained in the absence of NaCl and in the presence of 1 M arginine. The perturbation plot (Fig. 5e) shows that 0.5 M NaCl could reduce rHBsAg recovery from 60% to 30%. Thus, the combination of these chemicals was unfavorable due to their antagonist effects. As shown in Table 4d, the best pH (in the range between 5.0 and 8.0) for elution was 6.5.

3.6. rHBsAg purification using Capto adhere resin from P. pastoris feedstock in bind-elute and flow-through methods

For purification of rHBsAg in bind-elute mode, the feedstock sample was loaded on Capto adhere resin under optimal binding

Table 2
The optimal conditions for rHBsAg binding on Capto adhere resin in connection with NaCl (a) and $(NH_4)_2SO_4$ (b).
(a).

Number	pH	NaCl concentration (mM)	Adsorbed rHBsAg	Desirability	
1	4.500	0.000	100	1.000	Selected
2	4.506	72.027	82.652	1.000	
3	4.542	38.262	80.376	1.000	
4	4.524	47.675	83.009	1.000	
5	4.500	30.000	94.559	1.000	
6	4.504	8.837	99.346	1.000	
7	4.553	25.573	80.026	1.000	
8	4.538	23.820	84.372	1.000	
9	4.531	2.186	91.371	1.000	
10	4.511	16.239	94.398	1.000	

Number	pH	$(NH_4)_2SO_4$ concentration (mM)	Adsorbed rHBsAg (%)	Desirability	
1	4.500	0.000	75.516	0.922	Selected
2	4.500	7.280	75.496	0.922	
3	4.500	172.408	75.029	0.914	
4	4.500	187.450	74.986	0.913	
5	4.513	0.027	74.891	0.911	
6	4.500	255.000	74.795	0.910	
7	4.500	269.129	74.755	0.909	
8	4.500	288.381	74.701	0.908	
9	4.500	306.036	74.651	0.907	
10	4.500	339.640	74.556	0.906	

Table 3
The rHBsAg recovery yields achieved during OFAT experiment.

Run	Treatment	Elution yield (%)
1	pH6.5	0
2	Tris pH6.5 + 1M Urea	0
3	Tris pH6.5+ 2M Urea	0
4	Tris pH6.5+ 3M Urea	0
5	Tris pH6.5 + 0.5 M Arginine	2.5
6	Tris pH6.5 + 1 M Arginine	60
7	Tris pH6.5 + 0.5 M NaCl	3
8	Tris pH6.5 + 1 M NaCl	6
9	Tris pH6.5 + 1.5 M NaCl	1.5
10	Tris pH6.5 + 2 M NaCl	6
11	Tris pH6.5 + 1M KSCN	1.5
12	Tris pH6.5+ 2M KSCN	6
13	Tris pH6.5+ 3M KSCN	5
14	Tris pH6.5 + 15 % ethanol	0
15	Tris pH6.5 + 20 % ethanol	0
16	Tris pH6.5 + 20 % ethylene glycol	0
17	Tris pH6.5 + 30 % ethylene glycol	0
18	Tris pH6.5 + 40 % ethylene glycol	0
19	Tris pH6.5 + 50 % ethylene glycol	0
20	Tris pH6.5 + 5 % glycerol	0
21	Tris pH6.5 + 10 % glycerol	0
22	Tris pH6.5 + 15 % glycerol	0
23	Tris pH6.5 + 20 % glycerol	1
24	Acetate pH3	0
25	Tris pH8	0
26	Tris pH11	0

conditions obtained from section 3.3, and the target protein elution was made by 1 M arginine at pH 6.5. The experiment was done in duplicate (Fig. 6 and S3). As shown in these figures, the recovery and purity of the eluted rHBsAg are around 60% and 100%, respectively.

In the flow-through mode rHBsAg purification experiments, higher rHBsAg purity and recovery numbers were achieved at pH 6.5. As shown in Fig. 7 and S4, at pH 5.5, rHBsAg along with a relatively high amount of protein impurities observed in the supernatant; however, a purity of around 60% and a recovery of greater than 81% can be achieved at this pH point. At pH 6.5, both recovery and purity values were improved as the adsorption of rHBsAg to Capto adhere resin decreased with increasing NaCl concentration. Impurities were more in the supernatant in the absence of NaCl salt, but they adsorbed to the resin with increasing concentration of NaCl salt (the purity increased from 77% to more than 97% with the rise in NaCl concentration). The rHBsAg purity and recovery figures in the flow-through mode purification under various pH values and salt concentrations have been summarized in Table 5.

3.7. SEC-HPLC profile of rHBsAg purified using Capto adhere resin

By using optimal binding and elution conditions, purification of rHBsAg from the feedstock resulted in around 100% target protein purity based on SDS-PAGE (Figs. 6 and 7). SEC-HPLC was utilized to evaluate the particle profile of the purified rHBsAg (Fig. 8). The retention times of 22 and 27 min detected in the SEC-HPLC analysis in this investigation were analogous to those disclosed in other investigations [38,39] and substantiated an equivalent particle profile of the purified rHBsAg.

4. Discussion

In the optimization work for rHBsAg adsorption on Capto adhere resin (Tables 1 and 2, Figs. 3 and 4), the pH value close to 4.5 was optimal for binding rHBsAg to Capto adhere resin, which agreed with the non-binding experiments. This was consistent with a study in which Capto adhere high capacity for human serum albumin was achieved without salt addition [20]. It can be construed that anion exchange and hydrophobic properties of Capto adhere resin concurrently led to rHBsAg adsorption which is diminished by increasing either pH or NaCl concentration. In comparison with studies on other resins, in which very low rHBsAg binding capacity has been reported [40], Capto adhere resin showed a greater binding capacity in relation to its multimodal interactions. However, rHBsAg as a VLP-based protein with large particle size encounters difficulties in diffusing through chromatographic resin pores [41–44].

The intricacy of different multimodal resins and their unforeseeable performance in interactions with rHBsAg was revealed from the results of this study and the ones previously performed on Capto MMC resin [43]. For instance, rHBsAg adsorption on Capto adhere resin decreased with an increase in NaCl concentration, while in the case of Capto MMC resin, an increase in rHBsAg adsorption was observed with a rise in NaCl concentration. Also, the effect of the salt $(NH_4)_2SO_4$ on rHBsAg adsorption was dissimilar for these two multimodal resins (Table 6).

During the screening of additives for eluting rHBsAg from Capto adhere resin (Table 3), arginine was the most efficient eluting agent at a concentration of 3 M. This observation was in the manner of other investigations carried out for releasing different proteins

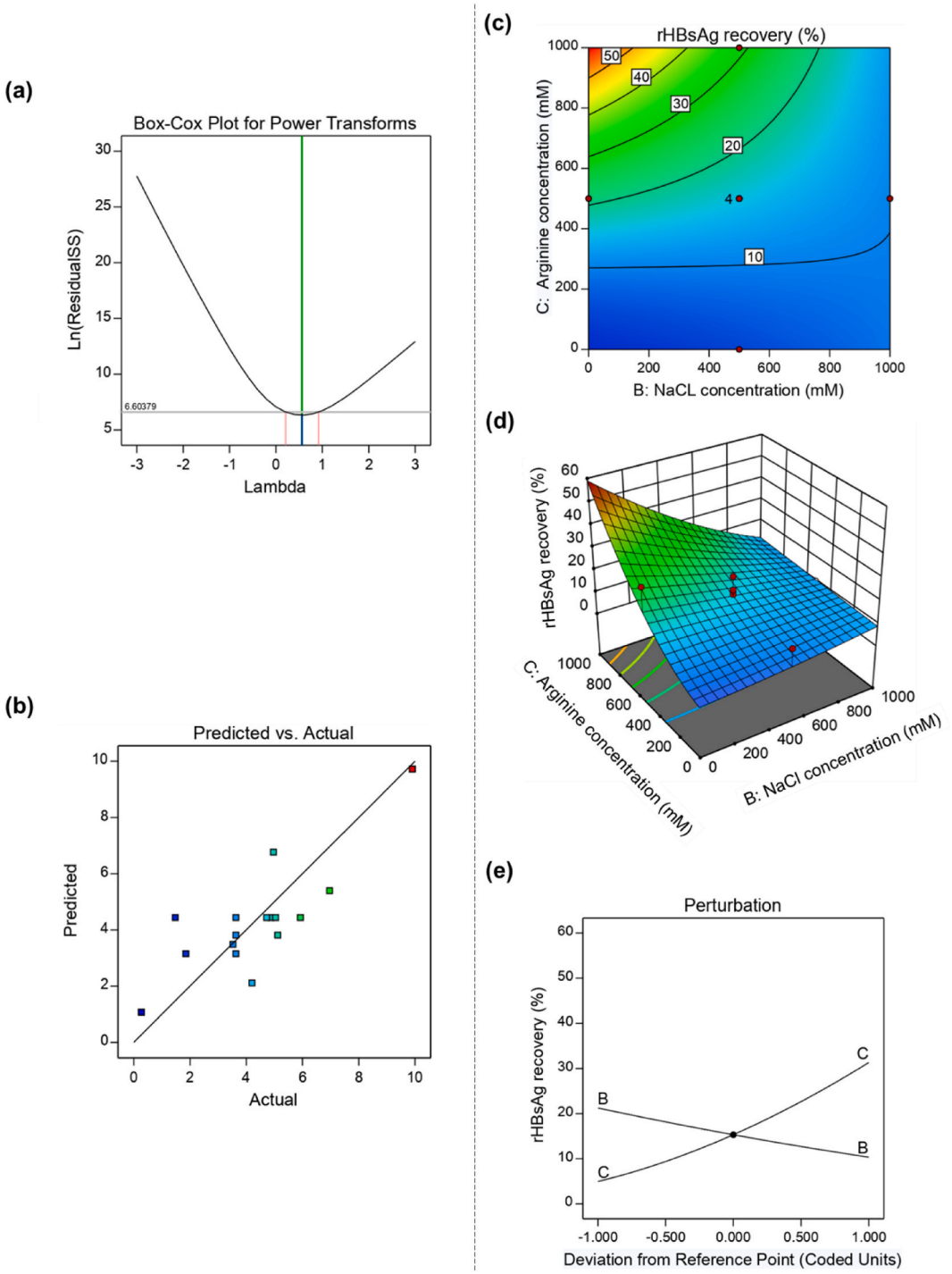

Fig. 5. The graphical presentation of data obtained from the experiments designed for eluting rHBsAg from Capto adhere resin using various concentrations of arginine and NaCl. (a) BOX-COX plot, (b) the predicted vs. actual plots, (c) counter plots, (d) 3D plot, and (e) perturbation plot.

from the resin [45]. As shown in Table 4, urea, glycerol, ethanol, and ethylene glycol could not effectively elute rHBsAg from Capto adhere. It has been previously reported in a study that increasing NaCl concentration from 200 mM to 800 mM could disrupt the multimodal interactions between Capto adhere and target protein and lead to protein recovery from the resin [20]; however, the rise in salt concentration in our study did not enhance rHBsAg recovery from Capto adhere resin. A rise in arginine concentration from 0.5 M to 1 M resulted in an improved rHBsAg recovery (from 2.5% to 60%). This finding was consistent with a study that declared arginine concentration of more than 0.25 M was required for the elution of the target protein [22]. As strong multimodal interactions (i.e.,

Table 4
The CCF design results for rHBsAg elution from Capto adhere resin using various chemicals. (a) Experimental layout, (b) ANOVA, (c) Fit- Statistics, and (d) Optimal points list.
(a).

Run	Space Type	A: pH	B: NaCl concentration (mM)	C: Arginine concentration (mM)	rHBsAg recovery (%)
1	Factorial	5	0	1000	60
2	Axial	6.5	1000	500	9.5
3	Factorial	8	0	0	0.1
4	Axial	8	500	500	10
5	Center	6.5	500	500	17
6	Axial	6.5	500	1000	17.5
7	Axial	5	500	500	2
8	Factorial	8	0	1000	60
9	Factorial	5	1000	0	10
10	Axial	6.5	0	500	32
11	Center	6.5	500	500	24
12	Axial	6.5	500	0	13
13	Factorial	5	0	0	0.1
14	Factorial	5	1000	1000	10
15	Factorial	8	1000	0	3
16	Factorial	8	1000	1000	18.5
17	Center	6.5	500	500	16
18	Center	6.5	500	500	18

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	95.30	3	31.77	16.23	<0.0001	significant
B-NaCL concentration (mM)	9.13	1	9.13	4.67	0.0486	
C- Arginine concentration (mM)	54.25	1	54.25	27.71	0.0001	
BC	31.92	1	31.92	16.31	0.0012	
Residual	27.41	14	1.96			
Lack of Fit	26.54	11	2.41	8.35	0.0534	not significant
Pure Error	0.8669	3	0.2890			
Cor Total	122.71	17				

Std. Dev.	Mean	C.V. %	R^2	Adjusted R^2	Predicted R^2	Adeq Precision
1.40	4.44	31.50	0.7767	0.7288	0.6660	13.1201

Number	pH	NaCL concentration (mM)	Arginine concentration (mM)	rHBsAg recovery (%)	Desirability	
1	6.500	0.000	1000.000	58.078	0.991	Selected
2	6.500	6.191	1000.000	57.688	0.987	
3	6.500	48.290	999.998	55.070	0.961	
4	6.500	0.001	920.211	50.920	0.919	

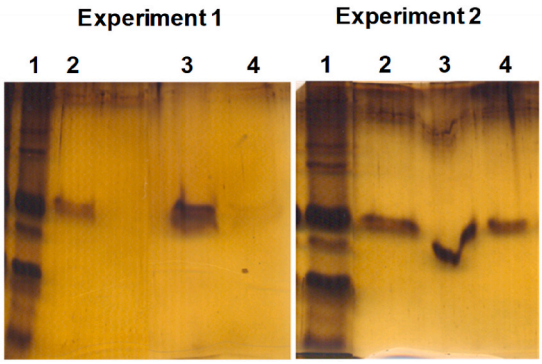

Fig. 6. SDS-PAGE analysis of different samples taken during rHBsAg purification with Capto adhere resin in bind-elute state. Lanes: 1– load; 2– wash with sodium acetate, pH 4.5; 3 and 4– elution by 1 M arginine.

hydrophobic and electrostatic) were included in protein adsorption on Capto adhere which led to low rHBsAg recovery yield, high concentrations of eluting agents were necessary for the elution step [22,24,25]. Hydrophobic, JI– JI interactions and hydrogen bonding between Capto adhere and arginine were accounted for the efficient protein elution from Capto adhere resin [46].

In the current study, it was demonstrated that the protein impurities that were present in *P. pastoris* feedstock samples interact stronger than rHBsAg with Capto adhere resin in both bind-elute and flow-through purification methods. As a result of such stronger connections of protein impurities with Capto adhere resin, a better rHBsAg recovery could be obtained under the defined conditions in

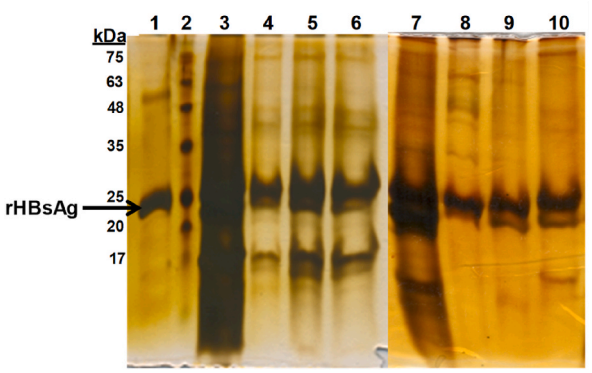

Fig. 7. SDS-PAGE analysis of samples taken during rHBsAg purification with Capto adhere resin in flow-through state. Lanes: 1– rHBsAg-API; 2– protein markers; 3, 7– load; 4, 5, 6– flow-through fractions at pH 5.5 and NaCl concentrations of 0, 400, and 800 mM, respectively; 8, 9, 10– flow-through fractions at pH 6.5 and NaCl concentrations of 0, 400, and 800 mM, respectively.

Table 5
Results achieved from the consecutive buffer exchange and various methods of rHBsAg purification from *P. pastoris* feedstock with Capto adhere resin.

Process	Recovery (%) obtained from Capto adhere[a]	rHBsAg purity (%)	Purification fold (PF) obtained from Capto adhere purification[b]	Purification mode
Sodium acetate, pH 4.5	60 ± 5	97 ± 2	2.75	Bind-elute
Sodium acetate, pH 5.5	38 ± 5	60 ± 5	1.80	Flow-through
Sodium acetate, pH 5.5, 400 mM NaCl	60 ± 5	60 ± 5	1.80	Flow-through
Sodium acetate, pH 5.5, 800 mM NaCl	78 ± 2	60 ± 5	1.80	Flow-through
Sodium phosphate, pH 6.5	55 ± 5	72 ± 3	2.08	Flow-through
Sodium phosphate, pH 6.5, 400 mM NaCl	80 ± 2	95 ± 2	2.7	Flow-through
Sodium phosphate, pH 6.5, 800 mM NaCl	97 ± 2	98 ± 2	2.75	Flow-through

[a] rHBsAg in the fraction/rHBsAg in the feedstock.
[b] rHBsAg purity after purification step/rHBsAg purity in the feedstock.

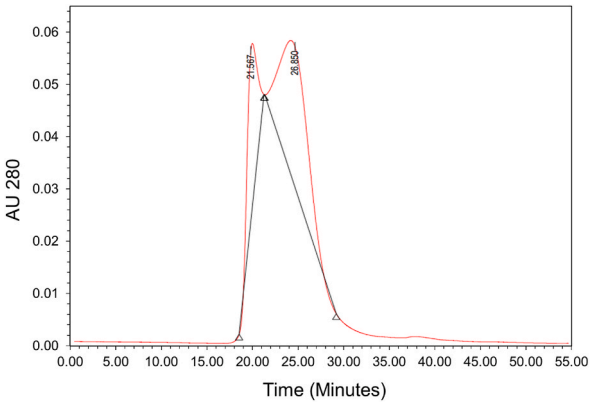

Fig. 8. Particle profile evaluation of the purified rHBsAg using SEC-HPLC analysis.

the flow-through purification mode. These findings were agreed with other studies which revealed that Capto adhere resin was more suitable for use in flow-through purification method and utilization of this resin in bind-elute purification mode led to higher host cell protein (HCP) removal but lower target protein recovery [27,47,48]. Also, by comparing the results of these studies with those reported previously [43], it was revealed that the optimal conditions achieved for efficient rHBsAg purification from the yeast feedstock using Capto adhere resin (in both bind-elute as well as flow-through manners) were different from the optimal conditions attained for rHBsAg purification using Capto MMC resin. These findings highlight the importance of implementing the DOE approach to overcome the complexity of multimodal resin due to their unpredictable behaviors. Moreover, achieving high purity in a single step demonstrates

Table 6
Comparison of results obtained from various resins utilized in the process of rHBsAg purification from *P. pastoris* feedstock.

Resin name	Capto adhere	Capto MMC [43]	Q Sepharose [38]	DEAE Sepharose FF [44]
Resin type	Multimodal	Multimodal	Single-mode	Single-mode
Investigation approach	DOE	DOE	OFAT	OFAT
Optimum binding conditions	pH 4.5, No salt	pH 4.5, 2M NaCl or 0.6 M $(NH_4)_2SO_4$	pH 5.0	pH 8.5, conductivity 3.2 mS/cm
Optimum elution conditions	pH 6.5, 1 M Arginine	Tris pH 8, 3 M KSCN	pH 5.0, 2 M NaCl	pH 8.5, 500 mM NaCl, conductivity ~ 50 mS/cm
Optimum FT conditions	pH 6.5, 800 mM NaCl	pH 5.5, 600 mM NaCl	NA	NA
Purification mode(s)	BE and FT	BE and FT	BE	BE
Highest percentage of purity/recovery achieved in BE	99/60	99/65	95/80	90/7
Highest percentage of purity/recovery achieved in FT	98/97	99/75	NA	NA
Binding capacity	0.75	0.14	ND	ND

BE= Bind-Elute.
FT= Flow-Through.
ND = Not Determined.

the high potential of a multimodal resin (i.e., Capto adhere) for developing a highly selective purification step. Although the capacity of Capto adhere for rHBsAg was higher than those values reported in previous studies and the industrial immunoaffinity, there is a strong need for developing resins with improved diffusion properties for VLP-based proteins in order to increase chromatographic process efficacy in a bind-elute mode.

5. Conclusions

The multimodal bioprocess resins for production-scale chromatography provide an opportunity for improvement of the established downstream processes. Capto adhere, a strong ion-exchanger with multimodal functionality, was used in this study for the final aim of rHBsAg purification from the industrial *P. pastoris* feedstock. Due to the number and complexity of protein-ligand interactions in multimodal resins, optimization works were conducted for binding and elution steps based on the DOE method.

In the binding optimization process using CCF design, conditions for high rHBsAg binding capacity were identified. The obtained higher Capto adhere binding capacity compared to single-mode resin reported in other studies [40], being attributed to the multimodal interactions of the resin. Although strong multimodal interactions were involved in the protein binding to Capto adhere, a reasonable recovery (ca. 60%) was achieved using 1 M arginine, pH 6.5, after elution optimization.

The obtained optimal conditions were finally used for purification of rHBsAg from *P. pastoris* industrial feedstock in both bind-elute and flow-through purification modes. The same rHBsAg binding capacity and elution recovery figures were achieved during rHBsAg purification from the industrial feedstock. In the flow-through purification mode, the target protein recovery was substantially increased up to 97%, without significant decrease in rHBsAg purity. These findings were in agreement with other studies which encourage the usage of this resin in the flow-through mode.

Comparison of this study with our previous study [43] highlights the complexity of various multimodal resins, their unpredictable behavior in interactions with rHBsAg and necessity of using the DOE approach to deconvolute complex protein-resin interactions.

The SEC-HPLC of the purified rHBsAg (with 100% purity) showed that the retention times of the elution step were similar to those reported elsewhere. The binding capacity, recovery and purity of rHBsAg obtained from these optimization studies show that Capto adhere can be considered as prospective resin in downstream processing of rHBsAg originated from recombinant *P. pastoris*.

Funding statement

This research was financed by the Research Project 1037 (contract No. 97/0201/2886) and the grant number BP-9585 for the PhD thesis of Maryam Moazami Goodarzi, together arranged by Pasteur Institute of Iran (1316943551, Tehran, Iran).

Data availability statement

The data relevant to this research work are publicly accessible in “figshare” at http://doi.org/10.6084/m9.figshare.25909204.

CRediT authorship contribution statement

Maryam Moazami Goodarzi: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Reza Jalalirad:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Delaram Doroud:** Writing – review & editing, Supervision,

Project administration, Methodology, Conceptualization. **Hamidreza Hozouri:** Writing – review & editing, Validation, Resources, Formal analysis. **Mohammad Reza Aghasadeghi:** Writing – original draft, Validation, Resources, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to express their gratitude to Elham Erami, Kourosh Mahboudi, Davoud Nouri Inanlou and Tahereh Sadeghcheh for their valuable assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35124.

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