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Application Note

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Capture of an Acid-Sensitive Protein From CHO Cell Culture Supernatant Using HyperCel STAR AX Salt Tolerant Anion Exchange Chromatography Resin

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1. Summary

- This study describes the use of HyperCel STAR AX "salt tolerant" anion exchange resin for the capture of an acid-sensitive model protein (α-amylase) spiked in Chinese Hamster Ovary (CHO) cell culture supernatant (CCS).
- HyperCel STAR AX resin and other commercial anion exchangers were evaluated to purify biological-active α-amylase, after optimization of wash and elution conditions.
- HyperCel STAR AX was the only resin in the study to allow an efficient capture of α-amylase directly from both crude (undiluted) or diluted CCS with good purity, yield and productivity.
- This model could be representative of typical target and contaminant concentrations in various recombinant protein expression systems.

2. Introduction

CHO cell culture is well established for the production of recombinant proteins. Anion exchangers (Q or DEAE resins) are commonly used as the first steps in the purification process.Conventional anion exchangers require CCS dilution to lower ionic strength or diafiltration to achieve sufficient capacity. These operations, however, increase buffer consumption and processing time, and limit throughput. Using a "salt tolerant" anion exchanger, such as the HyperCel STAR AX resin, would allow direct capture from undiluted feed and would result in significant process economics benefits at production scale.

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Table 1
Properties of HyperCel STAR AX resin

Average particle size	80 µm
Ion exchange ligand	Primary amine
Dynamic binding capacity[1] at conductivity 15 mS/cm	>100 mg BSA/mL within pH range 7.5 – 8.0
Recommended operating range of feedstock conductivity	2 – 15 mS/cm
Recommended cleaning conditions[2]	1 M NaOH

[1] Determined using 5 mg/mL BSA in 25 mM Tris-HCl, 0.14 M NaCl at 2 minute residence time.
[2] Injection of 5 column volumes (CV) of 0.5 – 1 M NaOH, 1 hour contact time.

3. Materials and Methods

3.1. CHO Feedstock Spiked With α-amylase

The model feedstock was prepared by spiking pure α-amylase from Aspergillus oryzae (Sigma) (MW 51 kDa, pI 3.5) in CHO CCS, pH 7.5, conductivity 12 mS/cm. Two different concentrations of α-amylase were used in the feedstock:

- a "high" concentration of 2 mg/mL (for preliminary screening studies, results in 4.1.1),
- and a "regular" (more challenging) concentration of 0.5 mg/mL for further comparisons (results shown in 4.1.2).

Before loading, CCS was filtered through a 0.2 µm membrane.

3.2. Analytical Methods

Total protein quantification	BCA assay kit (Pierce Thermo Scientific)
SDS-PAGE in non-reducing conditions	Nupage* 4-12% Bis-Tris precast gels, staining with Coomassie SimplyBlue* SafeStain (Life Technologies)
CHO host cell proteins (CHOP) assay	ELISA assay kit (Cygnus Technologies)
α-amylase activity	Ceralpha assay kit (Megazyme)

* Nupage and SimplyBlue are trademarks of Life Technologies Corporation.

Recovery and purity were calculated as follows:

- α-amylase recovery (% of load) = $\frac{\text{α-amylase in elution [mg]} \times 100}{\text{α-amylase in load [mg]}}$

- α-amylase purity [%] = $\frac{\text{α-amylase [mg]} \times 100}{\text{CHOP [mg]} + \text{α-amylase [mg]}}$

3.3. Chromatography Runs

3.3.1. Preliminary Screening: Dynamic Binding Capacity (DBC) On Three Different Resins

DBC was determined in 0.5 cm I.D. columns packed with 0.5 mL of HyperCel STAR AX resin and two other AEX resins: rigid Q agarose and Q polymeric resins. DBC at 10% breakthrough ($DBC_{10\%BT}$) was evaluated by the quantification of α-amylase in the flowthrough fractions.

Resins were equilibrated in 10 CV of 50 mM Tris-HCl, pH 7.5, 12 mS/cm, CCS containing 2 mg/mL of α-amylase was loaded at 0.5 mL/min (1 minute residence time).

3.3.2. Comparison with Two Selected Resins: DBC vs. CCS Dilution on HyperCel STAR AX Resin and Rigid Q Agarose Resin

After preliminary screening on three resins, the two best candidates were selected (HyperCel STAR AX resin and rigid Q agarose resin). To mimic a more challenging feedstock, the target protein concentration was decreased to 0.5 mg/mL and the CCS was diluted 2- and 4-fold in 50 mM Tris-HCl, pH 7.5.

Resins were equilibrated in 10 CV of this buffer adjusted at 12, 8 and 5 mS/cm with NaCl. Next $DBC_{10\%BT}$ was determined by loading undiluted and 2- and 4-fold diluted CCS (respectively 12, 8 and 5 mS/cm) as described in 3.3.1.

3.3.3. Optimization of Elution Conditions

The optimal conductivity for α-amylase elution was determined in 1 mL columns of HyperCel STAR AX resin and rigid Q agarose resin. CCS was spiked with α-amylase at 0.5 mg/mL.

Equilibration and load	Equilibration: 10 CV of 50 mM Tris-HCl, pH 7.5 adjusted at 12 and 8 mS/cm with NaCl Load: Amounts of feedstocks (undiluted or 2-fold diluted) corresponding to 60% of the $DBC_{10\%BT}$
Wash	15 CV wash in equilibration buffer
Elution	30 CV salt gradient from equilibration buffer to 50 mM Tris-HCl, pH 7.5, + 1 M NaCl
Strip elution	5 CV of same buffer+ 2 M NaCl
Clean-in-place	1 M NaOH, 30 minutes

3.3.4. Optimization of Wash Conditions

The optimal wash conditions were determined in 1 mL columns, as detailed in 3.3.3.

Two-step wash: 10 CV in 50 mM Tris-HCl, pH 7.5, followed by second 10 CV wash using either again equilibration buffer, 50 mM sodium acetate, pH 4.5; 50 mM Tris-HCl, pH 7.5, 15 mS/cm; or 50 mM sodium acetate, pH 4.5, 5 mS/cm.

Elution: 10 CV NaCl (up to 1 M) using 50 mM Tris-HCl, pH 7.5 at the optimized conductivity previously determined. Strip elution and CIP as detailed in 3.3.3.

4. Results and Discussion

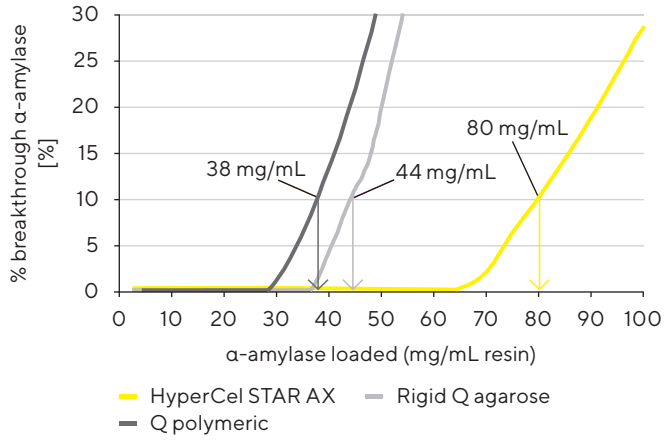
4.1. Dynamic Binding Capacity Comparisons in Different Conditions

4.1.1. Preliminary Screening: Comparison of DBC Using Crude Feedstock Loaded on Three Different Anion Exchangers

To compare the capacity performance of the three resins tested with undiluted feedstock (pH 7.5, 12 mS/cm), a first evaluation of DBC was performed in the "favorable" conditions designed to enhance the binding of α-amylase (i.e., high concentration of α-amylase in CCS: 2 mg/mL). Data shown in Figure 1 demonstrates a clear difference between HyperCel STAR AX resin and conventional anion exchange (AEX) resins. HyperCel STAR AX resin provided a 2-fold higher capacity: $DBC_{10\%BT}$ = 80 mg/mL, compared to the other resins: 44 mg/mL for rigid Q agarose resin, and 38 mg/mL for Q polymeric resin.

Figure 1

$DBC_{10\%BT}$ of α–amylase on HyperCel STAR AX Resin and AEX Resins Using Crude CCS



4.1.2. Selection of Two Resins and Influence of Feedstock Dilution on $DBC_{10\%BT}$

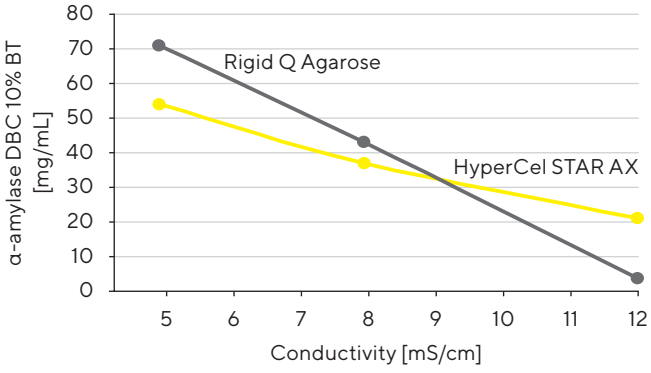
For the remainder of the study, only rigid Q agarose and HyperCel STAR AX resins were selected. In order to address the effect of feedstock dilution, a second evaluation of DBC was performed on HyperCel STAR AX resin and rigid Q agarose resin with CCS adjusted at different conductivities, while using a more challenging α-amylase concentration mimicking that of a real feedstock (0.5 mg/mL).

As expected, lower DBC was obtained compared to the previous experiment run in the more favorable conditions (see 4.1.1), confirming that a high ratio of contaminants towards target protein concentration facilitates competitive interactions of contaminants with the resins. However, HyperCel STAR AX resin provided the highest capacity (20 mg/mL) with crude feedstock (Figure 2), about 5-fold higher than that of rigid Q agarose resin. This confirms the "salt tolerance" of HyperCel STAR AX resin. With 2-fold diluted CCS (8 mS/cm), both resins had similar capacity (~40 mg/mL). The highest capacity for both resins was obtained with 4-fold dilution (5 mS/cm). However, this high dilution would not be a favorable choice for process scale-up and economics.

Capture using only crude and 2-fold diluted feedstock was further investigated.

Figure 2

$DBC_{10\%BT}$ of α-amylase on HyperCel STAR AX Resins and Rigid Q Agarose Resin Using Undiluted and Diluted CCS



CCS undiluted (12 mS/cm), diluted 2-fold (8 mS/cm) and 4-fold (5 mS/cm), spiked at 0.5 mg/mL. Each data point is the mean of 3 separate measurements.

4.2. Optimization of α-amylase Purification and CHOPs Removal

4.2.1. Optimization of Elution Conditions by NaCl Gradient

Crude and 2-fold diluted CCS were loaded on the rigid Q agarose and HyperCel STAR AX resins. Standard optimization of elution conditions using a NaCl gradient up to 1 M were performed and similar elution profiles were obtained with crude and diluted CCS for both resins (example with 2-fold diluted CCS load shown in Figure 3).

On Rigid Q Agarose Resin:

Two peaks were observed during the salt gradient on the rigid Q agarose resin (Figure 3A). The α-amylase activity was only detected in the first peak. The second elution peak likely corresponds to nucleic acids (high ratio A_{260nm} /A_{280nm}).

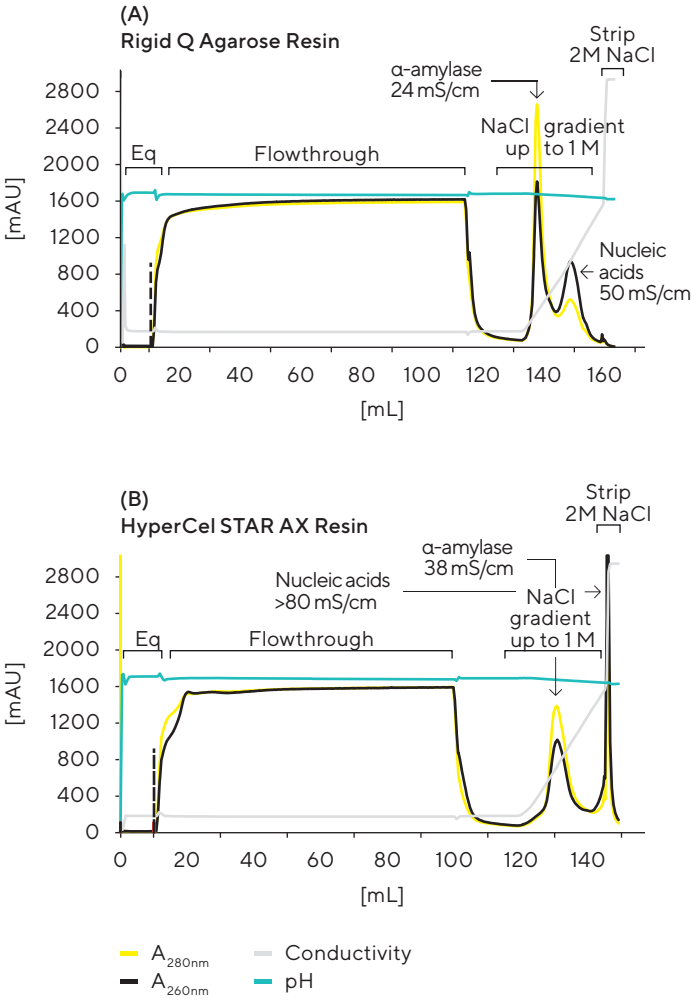
On HyperCel STAR AX Resin:

Only one peak containing α-amylase activity was visible during the salt gradient (Figure 3B). A major peak was also visible during the 2 M NaCl strip. The high A_{260nm} /A_{280nm} ratio indicated that the nucleic acids were well separated from the protein peak and were eluted only during the high salt strip. The strong affinity of HyperCel STAR AX resin for nucleic acids would therefore guarantee efficient removal of these contaminants.

Note: Elution conductivity for α-amylase was respectively 38 mS/cm on HyperCel STAR AX resin and 24 mS/cm on rigid Q agarose resin. As expected, higher elution conductivity was required for HyperCel STAR AX resin, due to the stronger interaction of its ligand with proteins in the presence of salt ("salt tolerance"). However, HyperCel STAR AX resin allowed complete elution of bound proteins during the elution gradient up to 1 M NaCl.

Figure 3

Optimization of Elution Conductivity on Rigid Q Agarose and HyperCel STAR AX Resins with 2-fold Diluted CCS as Loading Feed



Refer to Figure 4 for amounts of α-amylase loaded.

4.2.2. Optimization of Wash Conditions: Impact on Enzyme Activity, CHOPs Elimination and Yield

Four different wash conditions were tested to address their impact on α-amylase purity (as shown in Figure 4):

- Equilibration conditions (No wash)
- pH 4.5 – 2 mS/cm (pH wash)
- pH 7.5 – 15 mS/cm (High salt wash)
- pH 4.5 – 5 mS/cm (pH + moderate salt wash)

Note: Stability and activity of α-amylase at pH 4.5 were verified in previous experiments (data not shown)

Elution was performed according to optimization shown in 4.2.1. NaCl elution was applied with a 10 mS/cm safety margin to guarantee a complete elution of α-amylase (48 mS/cm for HyperCel STAR AX resin and 34 mS/cm for rigid Q agarose resin).

Initial purity of α-amylase evaluated as the ratio of α-amylase to total protein (α-amylase + CHOP) in CCS was 62%; as shown in Figure 4, purification on AEX resins using all the different wash conditions tested improved the final purity by 20 to 30%, with various impacts on yield and activity.

With 2-fold Diluted Feedstock (Figure 4, A and B)

- **pH wash:** Both resins achieved their best performances with "pH wash" conditions, leading to the improved elimination of CHOPs while maintaining high yield. Both resins provided similar purity at 93% but HyperCel STAR AX resin allowed 6% higher yield up to 94% compared to rigid Q agarose resin.
- **High salt wash:** pH 7.5, 15 mS/cm wash improved CHOPs elimination for both resins but significantly decreased the yield on rigid Q agarose resin, due to elution of α-amylase during the wash (65 to 70% yield of recovery at elution).
- **pH + moderate salt wash:** The wash at pH 4.5, 5 mS/cm significantly decreased the elution yield on both resins (< 55% yield) due to loss of α-amylase activity.

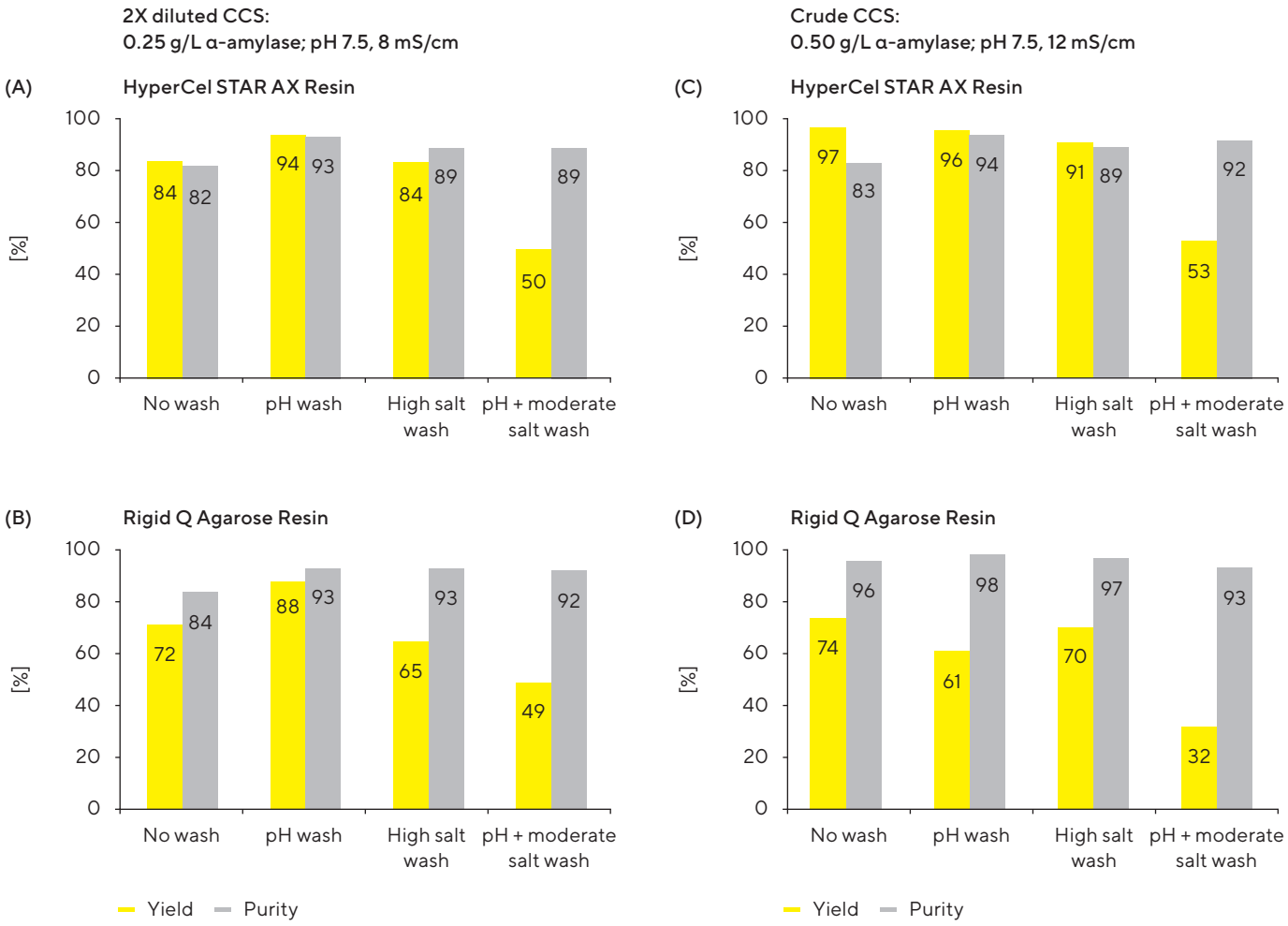
Note: In the runs including "pH + moderate salt wash", total protein assay showed that the total protein recovery was above 95%. Therefore, all α-amylase could be captured on both resins but a significant proportion of the enzyme was inactive. This confirmed data from previous stability study (not shown), that demonstrated that 0.25 M NaCl at pH 4.0 reduces the enzyme activity by 20%. In the present case, a wash at pH 4.5 and a conductivity as low as 5 mS/cm eliminates 50% of the enzyme activity. This may indicate that enzyme bound on the resins is more exposed to denaturation than in solution.

With Crude Feedstock (Figure 4, C and D)

The comparative study with undiluted CCS demonstrated the benefit of using salt tolerant HyperCel STAR AX Resin at 12 mS/cm. As shown on Figure 4, C and D, a 5-fold higher amount of target could be captured and was efficiently eluted on HyperCel STAR AX Resin. The best wash condition was pH 4.5, 2 mS/cm, providing the highest yield (96%) and purity (94%).

On rigid Q agarose Resin, despite a good purity, the yields were low (≤74%). Additionally, the capacity obtained with undiluted CCS is too low to consider a productive scalable process in these conditions.

Figure 4
α-amylase Yield and Purity at Elution vs. Wash Conditions after Capture on HyperCel STAR AX Resin and Rigid Q Agarose Resin During Runs Loaded at 60% of $DBC_{10\%BT2}$

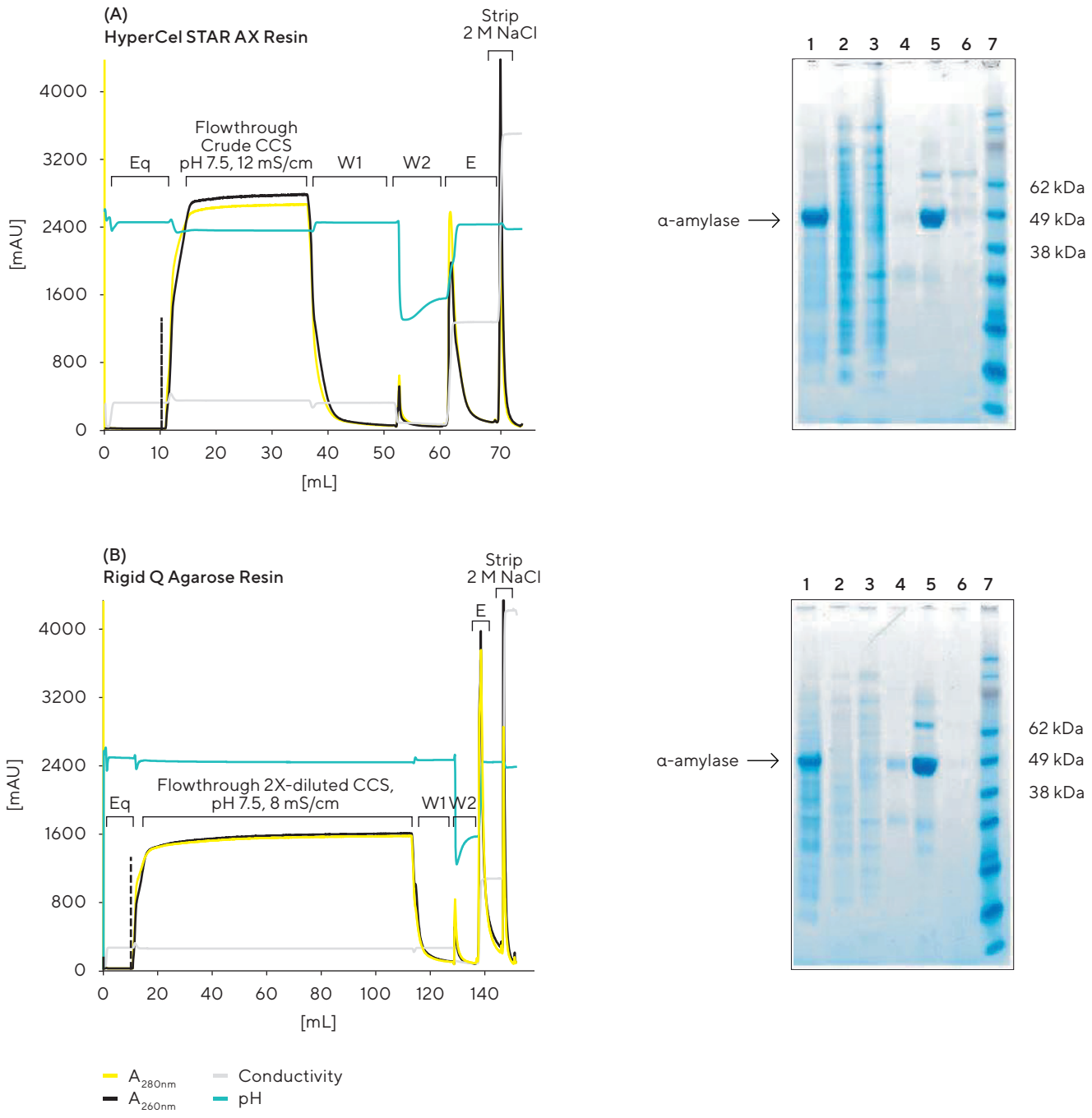


Amount of α-amylase loaded /mL resin: (A) and (B): With 2-fold diluted CCS, HyperCel STAR AX resin: 24 mg/mL, rigid Q agarose resin: 26 mg/mL. (C) and (D): With crude CCS, HyperCel STAR AX resin: 12.5 mg/mL, rigid Q agarose resin: 2.5 mg/mL.
No wash: Equilibration conditions; pH wash: pH 4.5, 2 mS/cm; High salt wash: pH 7.5,15 mS/cm; pH + moderate salt wash: pH 4.5, 5 mS/cm.

As a conclusion, the wash conditions optimization demonstrated that a wash step at a low pH provided the best CHOP removal for both resins while maintaining α-amylase activity. The chromatograms and SDS-PAGE on Figure 5 illustrate the capture of α-amylase in optimized conditions. As suggested earlier (see 4.2.1), the peak in the strip at 2 M NaCl probably consisted mainly of nucleic acids.

Additionally, the A_{260nm} /A_{280nm} ratio is different between the elution fractions from the two resins, with a higher absorbance at 260 nm (red curve) in the elution from rigid Q agarose resin (Figure 5). This supports the hypothesis that nucleic acid elimination during capture is more efficient on HyperCel STAR AX resin than on rigid Q agarose resin.

Figure 5
α-amylase Capture on HyperCel STAR AX Resin and Rigid Q Agarose Resin in Optimized Conditions



(A) Refer to Figure 4 for amounts of α-amylase loaded. Equilibration (Eq): 50 mM Tris-HCl, pH 7.5, 12 mS/cm; Load crude CCS; Wash 1 (W1): 50 mM Tris-HCl, pH 7.5, 12 mS/cm; Wash 2 (W2): 50mM Na acetate, pH 4.5, 2 mS/cm; Elution (E): 50 mM Tris-HCl, pH 7.5, 48 mS/cm. (B) Equilibration (Eq): 50 mM Tris-HCl, pH 7.5, 8 mS/cm; Load 2-fold diluted CCS; Wash 1: 50 mM Tris-HCl, pH 7.5, 8 mS/cm; Wash 2: 50 mM Na acetate, pH 4.5, 2 mS/cm, Elution: 50 mM Tris-HCl, pH 7.5, 34 mS/cm. SDS-PAGE: (1) CCS + α-amylase, (2) Flowthrough, (3) Wash 1, (4) Wash 2, (5) Elution, (6) strip, (7) Molecular weight marker.

Results shown in Table 2 indicate that due to its "salt tolerance", HyperCel STAR AX resin can purify α-amylase from either undiluted or diluted feed with equivalent productivity and purification performance. In contrast, using undiluted feedstock on conventional rigid agarose resin would result in productivity about 4 times lower (data not shown). Therefore, the use of HyperCel STAR AX salt-tolerant resin provides more process flexibility compared to conventional resin.

Table 2
Performance of HyperCel STAR AX Resin for Capture of α-amylase Under Optimized Conditions

Feedstock	$DBC_{10\%BT}$ [mg/mL]	Yield[1]	Purity[1]	Productivity[1] [g/L/hr]
Crude CCS (12 mS/cm)	21	96%	94%	7.9
2-fold diluted CCS (8 mS/cm)	40	94%	93%	8.4

[1] Values obtained with loads of samples at 60% of the DBC.

5. Conclusion

- HyperCel STAR AX "salt-tolerant" anion exchange resin can efficiently capture and purify biologically-active α-amylase, an acid-sensitive enzyme, from both crude and diluted CHO feedstock.
- Crude or undiluted feedstock can be applied with equivalent productivity, bringing process flexibility and robustness.
- DBC of HyperCel STAR AX resin at short residence time of 1 minute with crude CCS is 5-fold higher than that of a conventional rigid Q agarose resin.
- Capture from crude feed eliminates time/buffer consuming operations such as dilution.
- Case could be representative of typical situations encountered with various recombinant protein expression systems.

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