Optimized Homogenization Matrix to Produce High Purity, Tag-less, 160kDa Nuclease Using Ion Exchange and Size Exclusion Chromatography

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ABSTRACT

The discovery of the type II prokaryotic CRISPR "immune system" has allowed the development of an RNA-guided genome editing tool that is simple, easy, and quick to implement. Since then, several approaches to genome editing have emerged. The applications of such nucleases are ever-expanding and were recently validated with the US FDA approval of Casgevy, the first gene editing therapy for the treatment of sickle cell disease. The approval of Casgevy, coupled with the many gene editing programs necessitates versatile, cost-effective enzyme production and purification processes. A well-known strategy to facilitate protein purification is to engineer and express the protein with a Tag (e.g., Histidine Tag). This allows the use of affinity-based purification to achieve high purity. Cleavage of the Tag post-purification may be necessary, as its retention can cause interference with the protein's intended function. In this work, we describe a purification process using Akron's optimized homogenization matrix which allows isolation of the Tag-less nuclease of interest at a relatively high purity after capture chromatography (Cation Exchange Chromatography). A second chromatography step consisting of Size Exclusion Chromatography (SEC) resolves impurities (up to 100 kDa) from the 160 kDa nuclease using an enhanced buffer system. A 97.6% purity, measured by RP-HPLC, was achieved after three chromatography columns. A final chromatography step, designed solely for the reduction of endotoxin, achieved a reduction from ~69.8 EU/mg to levels as low as 1.0 EU/mg. The efficiency of the first CEX was shown to be scalable from a 5 mL column volume (CV) to 5,000 mL CV. This newly developed purification process using cGMP compatible resins, chemicals, and supplies is being assessed for scalability at Akron to establish a cost-effective nuclease purification process that does not require affinity chromatography.

BACKGROUND

The goal of this project is to develop a scalable, cGMP compliant, Tag-less endonuclease purification process. It will utilize homogenization, ion exchange chromatography, size exclusion chromatography, and Tangential Flow Filtration to yield a highly pure, biologically active, 160 kDa endonuclease. Assessment of each unit of operation and optimizations will be performed based upon chromatograms and analytical results.

MATERIALS & METHODS

Materials

- Cell pellet generated from Nuclease Production Platform
- ÄKTA avant and ÄKTAexplorer, Repligen KR2i, GEA Panda Homogenizer, Waters H Class UPLC System (Xbridge Premier Protein BEH C4)
- Clarification filters, Centrifuge
- TFF Cassettes of appropriate size to avoid breakthrough of protein of interest
- HiScale columns with applicable cGMP compatible resins (CEX, SEC, AEX)
- Denovix Spectrophotometer for concentration by UV280 nm as applicable, BioTek Spectrophotometer for quantification of material using Bradford, 4-20% SDS-PAGE for visual in-process assessment, Western Blot (SDS-PAGE, primary and secondary antibody, fluorophore, transfer buffer, PVDF membrane, milk blocking buffer, wash buffer, blotting reagent buffer), ELISA kit, Biological Activity (10X Reaction buffer, agarose gel, nuclease, sgRNA, 1 kb DNA template, thermocycler, Qiagen PCR Purification Kit), Charles River Endotoxin system and cartridges for endotoxin quantification

Methods

- Prepacked columns and loose cation exchange resins were obtained from industry recognized vendors and screened. All resins were sanitized pre- and post-use and were stored in appropriate storage buffers.
- The resins that supported the optimal outcome were selected.
- Buffer compositions were optimized to improve respective unit operations. Chromatography methods were modified to support scalability, resolution, and improvement in yields.
- Densitometry by SDS-PAGE was used for in-process evaluation of fraction relative purities. SDS-PAGE gels were imaged on the GelDoc and fraction purity was assessed using ImageLab 6.1 software. This generated a band % for the protein of interest in each lane, which corresponds to the purity. Average purity from fractions containing the protein of interest are reported. Error bars represent the standard error of the fractions' measured purity.
- For final purity measurement Waters H Class UPLC based in-house method was used.

RESULTS

Homogenization

Variable	Initial Homogenization Matrix	Optimized Homogenization Matrix
Salt Concentration	Low	High
Reducing Agent	A	B
Surfactant	No	Yes
pH Resuspension Buffer	7.2	7.2
Resuspension Solids %	15.5%	13.4%
Homogenization Cycles	5	5
% Reduction OD_{600}	95.3%	94.5%

Table A: Cell lysis parameters showing Salt, Reducing agent, Surfactant and buffer pH conditions comparing base case homogenization matrix (Initial Homogenization Matrix) to the newly designed homogenization matrix (Optimized Homogenization Matrix).

Homogenization output using the GEA Panda Homogenizer shows Resuspended cell pellet concentration (weight:weight, pellet:total weight in %) and homogenization efficiency after 5 homogenization cycles through the measurement of resuspended cell pellet optical density (OD) at 600 nm.

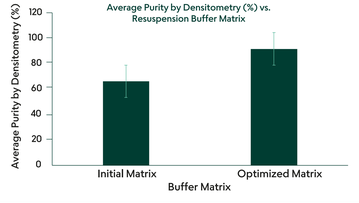


Figure 1: Effect of Homogenization matrix on main elution peak purity. CEX Elution peaks obtained from Clarified lysates of both homogenization matrix were analyzed on SDS-PAGE. The average relative purity of the protein of interest for each CEX eluate was assessed by SDS-PAGE-based Densitometry using ImageLab 6.1. Error bars represent standard error for the average purity of the CEX eluate.

CEX

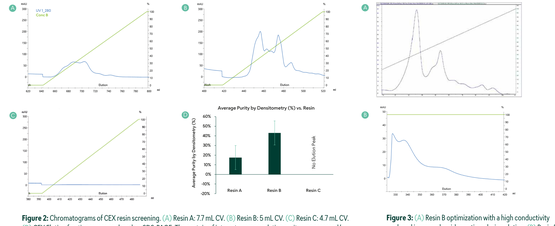
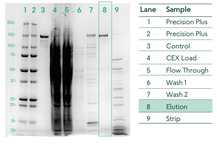
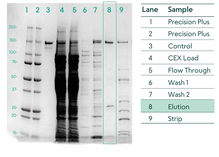


Figure 2: Chromatograms of CEX resin screening. (A) Resin A: 7.7 mL CV. (B) Resin B: 5 mL CV. (C) Resin C: 4.7 mL CV. (D) CEX Elution fractions were analyzed on SDS-PAGE. The protein of interest average relative purity was assessed by SDS-PAGE-based Densitometry using ImageLab 6.1. Error bars represented standard error. No peak was eluted from Resin C under the load, wash, and elution parameters evaluated.

Figure 3: (A) Resin B optimization with a high conductivity wash and increased residence time during elution. (B) Resin B transitioned to a step elution based upon targeted conductivity using 5 mL CV.



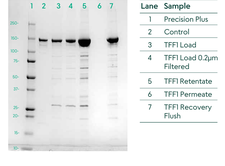
Lane	Sample
1	Precision Plus
2	Precision Plus
3	Control
4	CEX Load
5	Flow Through
6	Wash 1
7	Wash 2
8	Elution
9	Strip



Lane	Sample
1	Precision Plus
2	Precision Plus
3	Control
4	CEX Load
5	Flow Through
6	Wash 1
7	Wash 2
8	Elution
9	Strip

Figure 4: (A) SDS-PAGE confirmation of Resin B optimized run parameters on a 71.14 mL CV (B) SDS-PAGE confirmation of Resin B optimized run parameters on a 5.09 L CV.

TFF



Lane	Sample
1	Precision Plus
2	Control
3	TFFI Load
4	TFFI Load 0.2µm Filtered
5	TFFI Retentate
6	TFFI Permeate
7	TFFI Recovery Flush

Figure 5: SDS-PAGE of CEX elution material ultrafiltration prior to SEC.

SEC

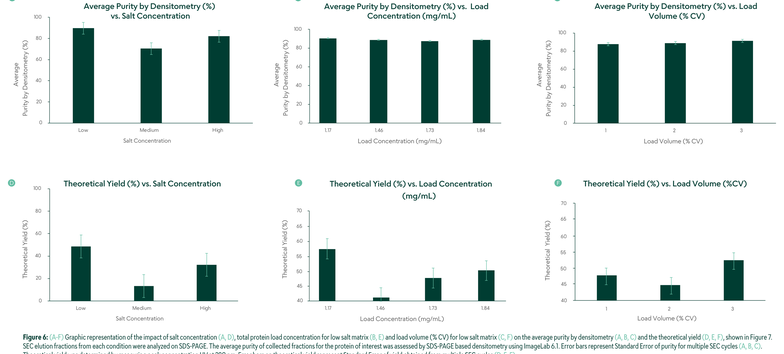
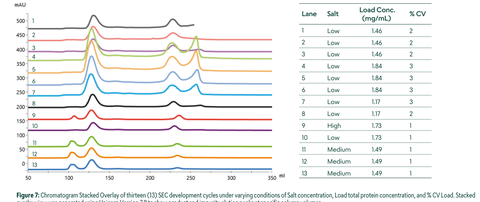


Figure 6: (A-F) Graphic representation of the impact of salt concentration (A, D), total protein load concentration for low salt matrix (B, E) and load volume (% CV) for low salt matrix (C, F) on the average purity by densitometry (A, B, C) and the theoretical yield (D, E, F), shown in Figure 7. SEC elution fractions from each condition were analyzed on SDS-PAGE. The average purity of collected fractions for the protein of interest was assessed by SDS-PAGE based densitometry using ImageLab 6.1. Error bars represent Standard Error of purity for multiple SEC cycles (A, B, C). Theoretical yield was determined by measuring peak concentration UV at 280 nm. Error bars on theoretical yield represent Standard Error of yield obtained from multiple SEC cycles (D, E, F).



Lane	Salt	Load Conc. (mg/mL)	% CV
1	Low	1.46	2
2	Low	1.46	2
3	Low	1.46	2
4	Low	1.84	3
5	Low	1.84	3
6	Low	1.84	3
7	Low	1.17	3
8	Low	1.17	2
9	High	1.73	1
10	Low	1.73	1
11	Medium	1.49	1
12	Medium	1.49	1
13	Medium	1.49	1

Figure 7: Chromatogram Stacked Overlay of thirteen (13) SEC development cycles under varying conditions of Salt concentration, Load total protein concentration, and % CV Load. Stacked overlay view was generated using Unicorn Version 7.8 to show product and impurity elution peaks at specific column volumes.
NOTE: For stacked chromatograms view Y-Axis does not represent an actual mAu scale.

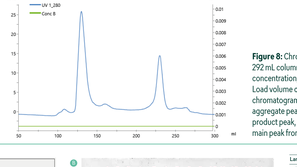


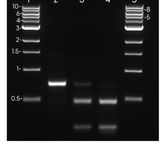
Figure 8: Chromatogram of SEC run on a 292 mL column using optimized Salt concentration, Load concentration, and Load volume conditions. The chromatogram showed reduction of the aggregate peak that eluates before the main product peak, and resolution separating the main peak from the impurity peaks.

AEX

Sample	(EU/mg)	Total Endotoxin in Sample (EU)	LRV	Endotoxin Clearance
Load 11.06 mL	69.8	596.65	1.843	98.32%
Pool 11.06 mL	< 0.1	< 10		
Load 280.78 mL	142	4987.04	0.823	98.66%
Pool 10X 280.78 mL	2.135	~ 67.04		

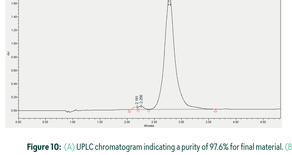
Table B: Data indicating the reduction of endotoxin using anion exchange chromatography.

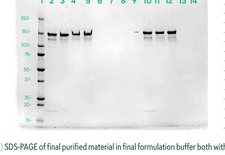
Final TFF



Lane	Sample
1	Linear Ladder
2	Template (Undigested)
3	Control Nuclease
4	Akron Nuclease
5	Linear Ladder

Figure 9: Agarose Gel based Nuclease activity test. The template (787 bp) was incubated in the absence of Nuclease or in the presence of an equivalent concentration of the newly purified Akron and Control Nuclease. Digest mixtures were analyzed using Agarose Gel electrophoresis.





Lane	Sample
1	Precision Plus
2	Control
3	TFF Load
4	Retentate w/ Glycerol
5	Recovery Flush w/ Glycerol
6	UF Permeate w/Glycerol
7	UFDF Permeate w/ Glycerol
8	UFDF Permeate 2 w/ Glycerol
9	Blank
10	Control
11	Retentate no Glycerol
12	Recovery Flush no Glycerol
13	UF Permeate no Glycerol
14	UFDF Permeate w/out Glycerol

Figure 10: (A) UPLC chromatogram indicating a purity of 97.6% for final material. (B) SDS-PAGE of final purified material in final formulation buffer both with and without glycerol.

DISCUSSION

Homogenization

Modification of the resuspension buffer to a higher salt concentration with a surfactant and reducing agent B aided separation of the lower molecular weight bands from the protein of interest in the CEX unit operation (Table A and Figure 1).

CEX

Three CEX resins were screened. Resin B was selected based upon resolution, capture of protein of interest, and attainability (off shelf/minimal lead times). Modifications to residence time during elution enhanced resolution for the peak of interest (Figure 3.A.). Improvements on load duration were obtained by reducing the volume of the load adjustment, while maintaining comparable elution profiles. The CEX elution profile was transitioned from a linear gradient to a step gradient based upon conductivity, allowing scalability and reproducibility (Figure 3). Development was performed on 5 mL, 71.02 mL, and 298.4 mL columns. SDS-PAGE supported capture of the protein of interest from column volumes ranging from 5 mL to 5.09 L (Figure 4).

TFF

A 30 kDa regenerated cellulose cassette allowed concentration of the CEX elution material prior to application on the SEC column without permeate breakthrough (Figure 5).

SEC

The size exclusion columns were loaded with concentrated CEX elution material. Optimizations were performed on load ratio and buffer composition (Figure 6 and Figure 7). Resolution of peaks in the SEC chromatograms improved (Figure 8), allowing a final product purity of up to 97.6% based upon HPLC (Figure 10.A.). Purified material was obtained from SEC columns ranging from 292 mL to 21.04 L.

AEX

The AEX unit operation successfully removed endotoxin from ~69.8 EU/mg to levels as low as <0.1 EU/mg (Table B).
This process flow allowed purification of the endonuclease while maintaining biological activity (Figure 9). The undigested template shows the expected 757 bp band. The digested template was cut into 515 and 242 bp bands as anticipated. This indicates that endonuclease remains active throughout processing.

REFERENCES/ACKNOWLEDGEMENTS

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Thank you to our vendors for supplying resin samples for screening and their support.