Purification of an Acidic Enzyme Using Ceramic Hydroxyapatite Chromatography: Effective Removal of Acidic Impurities

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Enzyme Purification

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Abstract

The present study is concerned with the effect of buffer components on the separation of an acidic enzyme from other acidic impurities produced by an *Escherichia coli* strain using ceramic hydroxyapatite chromatography. Low concentrations of calcium cations and sodium chloride in a sodium phosphate (NaPi) buffer at pH 6.5 promote the binding of target protein, which can then be selectively eluted with a more concentrated sodium phosphate buffer. This enzyme can also be purified in flow-through mode, when the CHT Ceramic Hydroxyapatite Column is pre-equilibrated with a buffer containing a low concentration of sodium phosphate at pH 7.0. Both purification strategies offer efficient clearance of acidic contaminants. The possible mechanisms by which these buffer components may modulate the interactions between biomolecules and CHT Media are also discussed.

Introduction

Crystalline hydroxyapatite is a naturally occurring mixed-mode chromatography media. This inorganic material has a chemical composition of $(Ca_5(PO_4)_3OH)_2$ with hydroxyl groups, calcium doublets, and phosphate triplets arranged in a repeating geometric pattern. The phosphoryl groups (P sites) interact with positively charged amino residues of protein molecules electrostatically, while the calcium ions in the crystal lattice (C sites) form a chelation complex with the carboxylic side chains of aspartyl and glutamyl residues (Figure 1). These types of interactions operate synergistically or individually, offering unique selectivity for bioseparation. The ceramic form of hydroxyapaptite, CHT, is a spherical, macroporous, and hydrodynamically stable chromatography media. It has been widely employed for the general preparation of biomolecules as well as the commercial production of biopharmaceuticals.

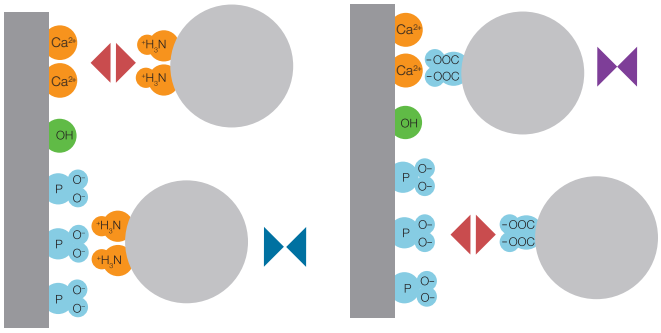

Fig. 1. Schematic representation of CHT interactions with biomolecules.

Chromatography with CHT Media is typically operated in the pH range of 6.5–7.5. At these pH levels, acidic proteins, namely those with a pI lower than 7, are negatively charged. They are likely repelled by the P sites on CHT, while facing competition for the C sites from acidic impurities (such as the nucleic acids and endotoxins in feedstock). The use of buffers containing inorganic phosphate for chromatography may further compromise the binding of acidic proteins by CHT. In the present study, we attempted to use CHT for the polish purification of an acidic enzyme, CDP-D-glucose 4,6-dehydratase (E_{od}) (Thorson et al. 1994). The pI of this enzyme has been estimated as 6 on the basis of its polypeptide sequence (Thorson et al. 1994, He et al. 1996, Vogan et al. 2002). The recombinant enzyme was overproduced in *E. coli* while the crude cell extract contained enormous amounts of endotoxins and nucleic acids that are strong contenders for the C sites on CHT. Using conditions identified in the design of experiment (DOE) screening, we were able to perform scale-up purification of this enzyme on CHT columns to achieve efficient removal of a variety of acidic impurities.

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Material and Methods

General

CHT Ceramic Hydroxyapatite (Type I, 40 µm) is a product from Bio-Rad Laboratories. Chromatography purification was conducted on either a BioLogic DuoFlow QuadTec 10 System (Bio-Rad) or an NGC 10 Medium-Pressure Chromatography System (Bio-Rad) using Mini Bio-Spin Columns at room temperature. Protein fractions were analyzed by SDS-PAGE using Precision Plus Protein Standards and Criterion TGX Stain-Free Precast Gels and the gel images were recorded on a Gel Doc EZ Gel Documentation System (Bio-Rad). The clearance of host cell proteins (HCP) and double-stranded DNA (dsDNA) was determined by *E. coli* HCP ELISA Kit (Cygnus Technologies) and ddPCR *E. coli* Residual DNA Quantification Kit (Bio-Rad), respectively. Endotoxin contamination in protein samples was detected with Endpoint Chromogenic LAL Assays (Lonza). Protein concentration was quantified with the Bradford Protein Assay (Bio-Rad). Chemicals of analytical grade or higher were purchased from commercial vendors such as EMD Millipore or VWR for the preparation of buffers.

DOE screening for the binding and elution conditions

A design of experiments (DOE) study was performed to obtain purification conditions for E_{od} using CHT. The effects of calcium chloride, sodium chloride, sodium phosphate, and buffer pH on the binding of target protein were investigated. For target elution, the effects of sodium phosphate concentration and buffer pH on protein purity and recovery were the focus. A two-level full factorial screening design suggested by JMP Software (SAS Institute) was employed (Table 1). Mini Bio-Spin Columns, each containing 50 µl of CHT Media were pre-equilibrated with the binding buffer to be tested. The E_{od}-containing feedstock, partially purified by anion exchange chromatography, was exchanged into its respective test binding buffer. To each pre-equilibrated spin column 2–2.5 mg of E_{od} were loaded and mixed with resin at room temperature for 10 min with agitation. Unbound materials were removed at the end of the incubation by spinning at 1,600 x g for 30 sec. Elution of target protein was achieved by incubating the loaded resins with respective elution buffers for 10 min with agitation, followed by centrifugation at 1,600 x g for 30 sec. These eluates were analyzed by SDS-PAGE. The absorbance (280 nm) readings of the collected eluates were used to quantify the recovery of the target enzyme, and target protein purity was assessed with Image Lab Software (Bio-Rad). A standard least squares regression was employed to obtain the critical parameters for E_{od} purification using JMP Software.

CHT chromatography in bind-and-elute mode

A 0.56 x 4 cm (1 ml) CHT Column was equilibrated with 10 column volumes (CV) of 5 mM sodium phosphate, 200 mM sodium chloride, and 1 mM calcium chloride at pH 6.5 (buffer A). The partially purified E_{od} in buffer A was loaded at a linear velocity of 300 cm/hr and the column was washed with 10 CV of the equilibration buffer. The bound target protein was eluted by 15 CV of 75 mM sodium phosphate at pH 6.5 (buffer B).

CHT chromatography in flow-through mode

The partially purified E_{od} in 5 mM sodium phosphate and 5 mM sodium chloride at pH 7 (buffer C) was loaded at a linear velocity of 300 cm/hr onto a 0.56 x 4 cm (1 ml) CHT Column pre-equilibrated with 10 CV of the same buffer. The target enzyme was recovered in the flow-through fractions. The bound impurities were stripped with 10 mM sodium phosphate and 1.5 M sodium chloride at pH 7.0 (buffer D).

Table 1. Design of experiments (DOE) setup for binding and elution condition screening.

Experiment	Calcium Chloride Concentration, mM	Sodium Chloride Concentration, mM	Sodium Phosphate Concentration, mM	Binding Buffer pH	Sodium Phosphate Concentration, mM	Elution Buffer pH
	Binding Buffer				Elution Buffer	
1	**0.5**	**100**	**3**	**6.8**	**75**	**6.8**
2	0	200	1	6.5	50	7.1
3	0	200	5	7.1	50	6.5
4	0	0	1	7.1	100	6.5
5	0	0	1	6.5	50	7.1
6	1	0	5	7.1	100	7.1
7	0	0	5	7.1	50	6.5
8	1	200	1	6.5	100	6.5
9	1	0	5	6.5	50	6.5
10	1	200	5	7.1	100	7.1
11	0	200	5	6.5	100	7.1
12	1	0	1	6.5	100	6.5
13	**0.5**	**100**	**3**	**6.8**	**75**	**6.8**
14	1	200	5	6.5	50	6.5
15	0	0	5	6.5	100	7.1
16	1	0	1	7.1	50	7.1
17	1	200	1	7.1	50	7.1
18	0	200	1	7.1	100	6.5

Note: Experiments 1 and 13 are the center points.

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Results

Parameters crucial to the purification of E_{od} were identified by analyzing the DOE data with JMP Software. The binding of this enzyme by CHT benefits from the use of calcium chloride and sodium chloride, which is further enhanced by a lower buffer pH (Figure 2). These results are in good agreement with previously published work by P. Gagnon and coworkers, who demonstrated the binding of protein fragments on calcium-derivatized hydroxyapatite via a calcium coordination mechanism (Gagnon et al. 2009). The highest binding capacity of E_{od} in the presence of 5 mM sodium phosphate was estimated as 17 mg/ml. For the elution of target protein, better yield can be achieved with buffers containing higher concentrations of sodium phosphate at a higher pH (Figure 3A). However, the purity of eluted E_{od} would be compromised under such strong elution conditions (Figure 3B). Good clearance of endotoxin is feasible either with low sodium phosphate concentration at high pH or with high sodium phosphate concentration at low pH (Figure 3C).

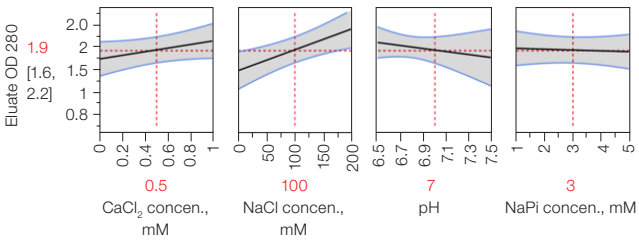
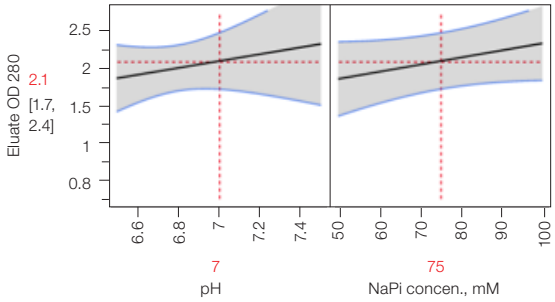
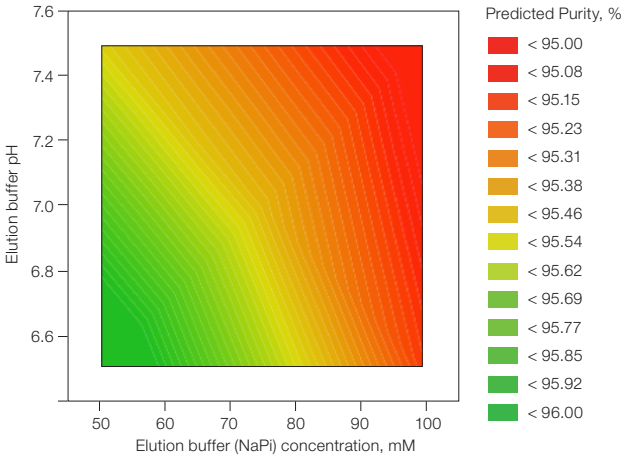


Fig. 2. Prediction profile of the effect of binding buffer components on the yield of E_{od} in eluate as indicated by absorbance (eluate OD 280).

A. Predicted yield profile



B. Predicted purity profile



C. Predicted endotoxin profile

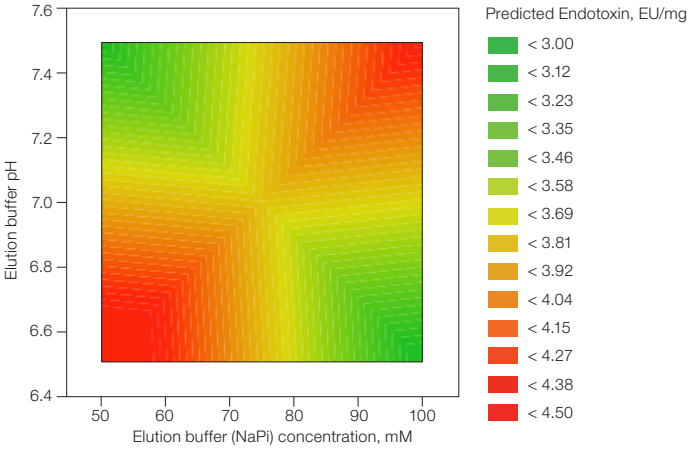


Fig. 3. Effect of elution buffer components on the purity and yield of E_{od} in eluate. A, predicted yield of E_{od} in eluate is profiled per elution buffer conditions. **B** and **C**, a range of purity and endotoxin predictions are profiled against two elution buffer variables.

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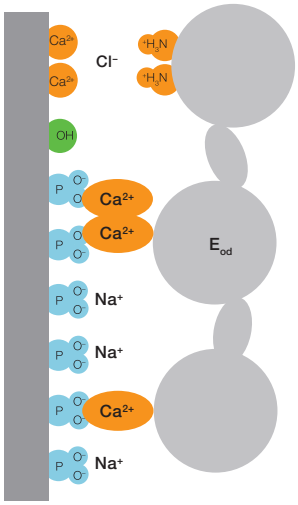

Fig. 4. Enhancing the interaction between CHT and E_{od} with the addition of calcium chloride and sodium chloride in binding buffer.

Scale-up purification in the bind-and-elute mode was performed on a 1 ml CHT Column pre-equilibrated with buffer containing 1 mM calcium chloride and 200 mM sodium chloride at pH 6.5 — two chemicals that have been shown to enhance the binding of E_{od}. The calcium ion in the buffer may be absorbed by the P sites on the surface of CHT, forming more metal affinity chelation complexes with the carboxyl residues of the target protein molecules. Moreover, the negatively charged phosphoryl groups are shielded by the sodium cations, suppressing the electrostatic repulsion between the chromatography media and the acidic enzyme (Figure 4). The bound E_{od} was eluted well within 3 CV with a buffer containing 75 mM sodium phosphate at pH 6.5, a condition that balances the needs of target recovery and the resolution of the acidic impurities such as endotoxins and nucleic acids from the expression host cells (Figure 5).

As predicted by the DOE screening, E_{od} was not effectively bound by CHT at higher pH due to the strong repulsion between the P sites and the negative surface charges of the acidic target protein in the absence of a high concentration of sodium chloride. Therefore, the purification of E_{od} was also performed in the flow-through mode, with a CHT Column equilibrated with 5 mM sodium phosphate and 5 mM sodium chloride at pH 7.0. Under this chromatography condition, species with stronger affinity for the calcium metals (such as nucleic acids, endotoxins, and contaminating proteins from the expressing host cells) were effectively captured and remained on the column until stripping with a buffer containing 1.5 M sodium chloride (Figure 6). Flow-through operation is simple and effective, requiring minimum manipulation of feedstock and buffer preparation. The impurity clearance efficiency of the two chromatography processes is summarized in Table 2. They both offered excellent removal of impurities.

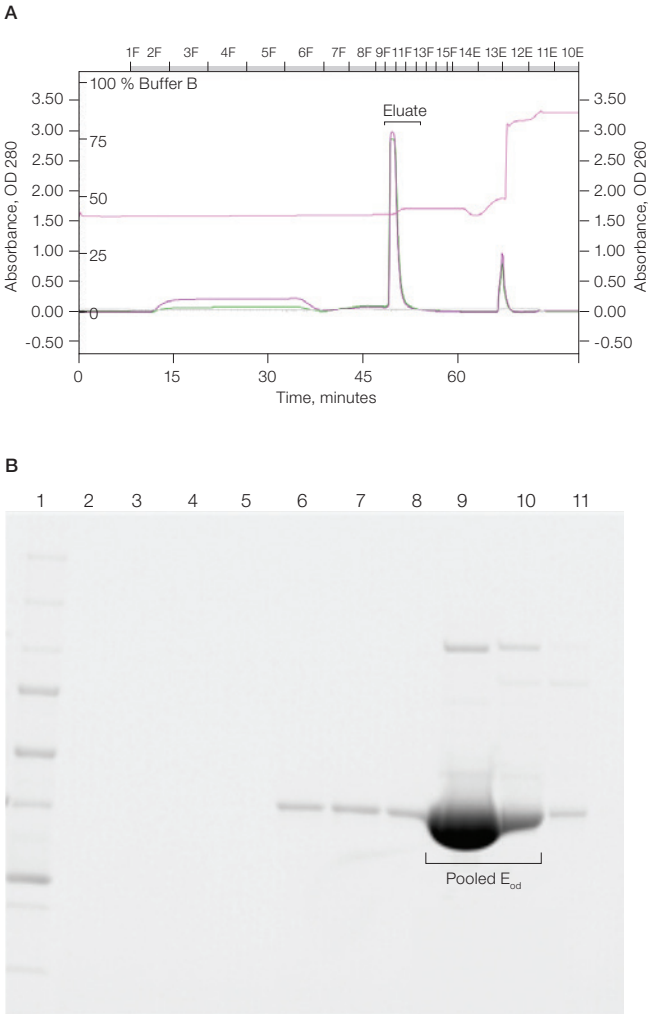

Fig. 5. Purification of E_{od} on a 1 ml CHT Column (0.56 x 4 cm) in bind-and-elute mode. A, column separation chromatogram. OD 280 (—), OD 260 (—), and buffer pH (—). **B**, SDS-PAGE analysis of column fractions. Lane 1, Precision Plus Protein Standards; lanes 2–5, column fractions 2F–5F; lanes 6–11, column fractions 8F–13F. Target protein-containing fractions 9F and 10F were pooled for purity and yield analysis.

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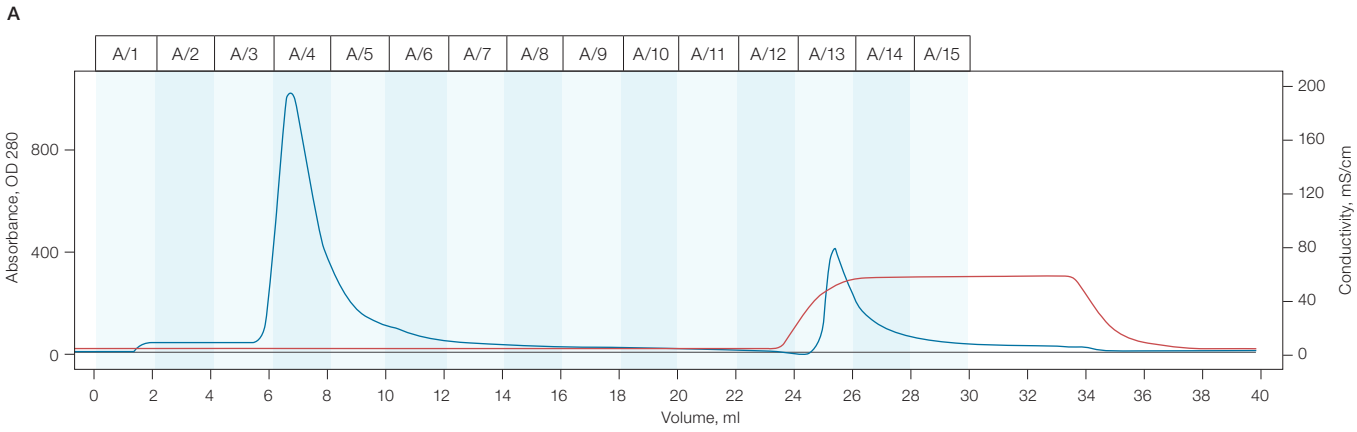

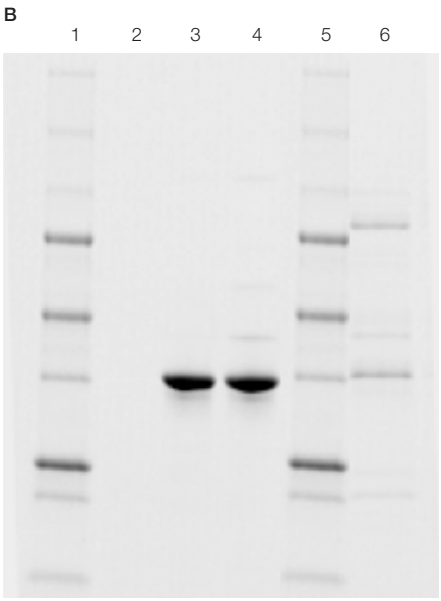

Fig. 6. Purification of E_{od} on a 1 ml CHT column (0.56 x 4 cm) in the flow-through mode. A, column separation chromatogram. OD 280 (—), buffer conductivity (—). **B**, SDS-PAGE analysis of column fractions. Lanes 1 and 5, Precision Plus Protein Standards; lanes 2–4, column fractions A3–A5; lane 6, column fraction A13. Target protein-containing fractions A3 and A4 were pooled for purity and yield analysis.

Table 2. Comparison of impurity clearance efficiency.

Samples	Endotoxin, EU/mg*	dsDNA, ppm**	HCP, ppm***
Load	4,317	1,028	1,599
E_{od} purified in flow-through mode	11	2	225
E_{od} purified in bind-and-elute mode	3	4	142

* LAL assay, per milligram of protein.
** ddPCR analysis, per milligram of protein. Detection limit is 1.2 pg/ml.
*** ELISA. Detection limit is 1.6 ng/ml.

Conclusion

We have successfully employed DOE for the development of CHT chromatography methods. The present results demonstrate that the binding capacity and selectivity for the target acidic enzyme is dependent on the composition of the chromatography mobile phase. Our study reveals that the binding of acidic proteins benefits from the use of low calcium chloride concentration with sufficient tolerance to inorganic phosphate ions in buffers as well. Addition of calcium and phosphate to the process solution is recommended for "significant positive effect on CHT stability" (for more detail on this, see bulletin 6068). Finally, scale-up preparation of E_{od} can be achieved in either bind-and-elute or flow-through mode, which showcases the robustness of chromatography separations using CHT. Both purification strategies are effective, offering high product yields and excellent clearance of acidic impurities from the expression host cells.

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