Single-Step Purification of Raw Starch Digesting α-Amylase Using Nuvia™ cPrime™ Hydrophobic Cation Exchange Resin

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Tech Note

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Abstract

Raw starch digesting α-amylase (RSDA) is an industrially important enzyme. The kinetics and mode of action of RSDA are not well understood. Further elucidation of this enzyme's mechanism of action, however, requires highly pure RSDA. Here we demonstrate a single-step purification protocol, using the mixed-mode resin Nuvia cPrime, that yields ~96% pure recombinant enzyme from *E. coli*. This level of purity is sufficient for further mechanistic studies. Additionally, the protocol developed here is suitable for large-scale purification of RSDA and similar enzymes.

Introduction

Large-scale purifications of enzymes and other biomolecules typically involve a multicolumn chromatography workflow consisting of capture and polish purification steps (He and Snyder 2012, Fitchmun et al. 2016) or chromatography in conjunction with other techniques, such as precipitation and filtration (Sundarram et al. 2014). Multiple steps are required to ensure proper concentration of the initial feed, minimization of product- and process-related impurities such as endotoxins, host cell proteins (HCPs), genomic DNA, and aggregates, and to achieve a highly pure batch of the target protein. In the case of enzymes, each additional purification step can lead to a proportional loss of enzymatic activity. This makes a single-step purification strategy for enzymes highly appealing.

A single-step purification strategy (Figure 1) was recently developed for the purification of raw starch digesting α-amylase (RSDA) (Lončar et al. 2015) using Bio-Rad's hydrophobic cation exchange mixed-mode resin Nuvia cPrime. Alpha-amylases are one of the most important industrial enzymes (Gupta et al. 2003), with applications in a wide array of industrial areas, including food, fermentation, textile, paper, and pharmaceutical industries (De Souza and de Oliveira Magalhães 2010).

Although many studies have been performed on these enzymes, the molecular basis of their mode of action is still unknown. In order to better characterize this class of enzymes, a downstream purification protocol was designed to isolate highly pure RSDA in the most productive manner with regards to retention of enzyme activity and speed of purification.

Nuvia cPrime is a mixed-mode resin that offers both hydrophobic interaction (HIC) and cation exchange (CEX) interaction capabilities. Proteins can bind by either or both of these modes. Modulating buffer pH and conductivity allows very selective interactions between target molecules and the mixed-mode resin. Since high salt promotes hydrophobic interactions, Nuvia cPrime Resin has the competitive advantage of high salt tolerance relative to other unimodal ion exchange (IEX) resins. By taking advantage of this unique property of Nuvia cPrime we were able to develop a highly efficient and scalable single-step purification protocol for RSDA.

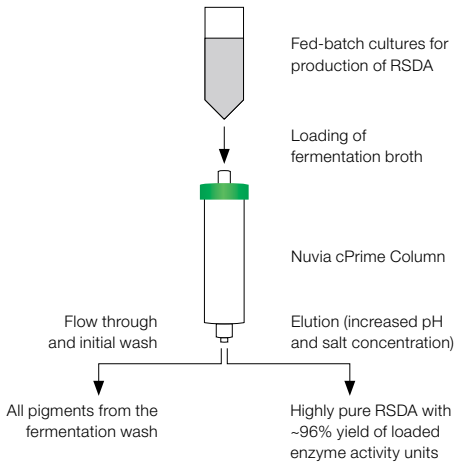

Fig. 1. Single-step purification strategy for RSDA using Nuvia cPrime Resin.

Materials and Methods

Cell culture

Recombinant RSDA was expressed in the C43 (DE3) strain of *E. coli* using the pDA-amy plasmid. Cells were grown in fed batch cultures according to an established protocol for RSDA production (Lončar et al. 2015).

Hydrophobic cation exchange chromatography using Nuvia cPrime Resin

Purification of RSDA was carried out by mixed-mode chromatography on a Pharmacia HPLC System (GE Healthcare Group) using a 1 ml Foresight™ Chromatography Column prepacked with Nuvia cPrime Resin (Bio-Rad Laboratories, Inc.

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catalog # 7324722). Surface response methodology was used to optimize pH and salt concentrations for RSDA binding and elution. The column was equilibrated with 20 column volumes (CV) of 50 mM sodium acetate, pH 5.3, 150 mM NaCl (buffer A). Twenty milliliters of the RSDA-containing *E. coli* extract were then loaded onto the Nuvia cPrime Column at a linear velocity of 360 cm/hr. The column was washed with 20 CV of buffer A. RSDA was eluted with 10 CV of 30 mM Tris-HCl, pH 8.0, 0.5 M NaCl (buffer B).

Activity quantification of purified RSDA

RSDA activity was determined by measuring the formation of reducing sugars released during starch hydrolysis in 50 mM phosphate buffer, pH 6.5 at 75°C. Liberated reducing sugar was quantitated using the dinitrosalicylic (DNS) acid method. The amount of enzyme that released 1 µmol of reducing end groups per minute at 75°C was defined as one unit of amylase activity. D-glucose was used to generate a standard curve. Protein fractions were analyzed by 10% SDS-PAGE using the SE260 Mighty Small II Deluxe System (Hoefer Inc.) and quantified using ImageJ Software (imagej.net).

Results and Discussion

The yield of crude RSDA from fermentation was 1.2 g/L of fermentation broth with a total activity of 9,200 IU. This fraction contained not only RSDA and other cellular proteins, but also some pigment and coloring substances from the fermentation broth. Pigments can bind tightly and sometimes irreversibly to ion-exchangers, thereby compromising resin quality and decreasing its reusability (Levison 2003, Ersson et al. 2011). Anion exchangers are more affected by this issue than their cation exchange counterparts (Janson and Jönsson 2011, Karlsson and Hirsh 2011). Since Nuvia cPrime contains a cation exchange group in addition to hydrophobic interaction groups, strong pigment binding was not anticipated. As expected, applying crude extract to the Nuvia cPrime Column yielded all of the pigments in the flow-through and wash steps. Therefore Nuvia cPrime serves as a more appropriate choice for pigment removal than ultrafiltration membranes or Sepharose-based IEX resins, both of which bind pigments (Karlsson and Hirsh 2011, Scopes 1994).

The behavior of RSDA during purification with Nuvia cPrime cannot be predicted based solely on the protein's pI or amino acid sequence. Nuvia cPrime is designed to present multiple possible interaction modes to resolve complex target proteins and impurities. Therefore, under any specific purification condition, one or more such interaction modes may be involved in the binding or repulsion between the resin and RSDA or impurities. Prior studies have shown that a simple design of experiment (DoE) setup can be used to determine the effects of buffer pH and conductivity on selectivity, recovery, and robustness of protein purification with Nuvia cPrime (He et al. 2016).

A preliminary range of purification variables was determined based on the manufacturer's suggested protocol (He et al. 2016). To identify optimal binding and elution conditions, a central composite design (CCD) or a DoE study was performed with four operation parameters (A, binding pH; B, binding NaCl concentration; C, elution pH; and D, elution NaCl concentration). Five replicates at the center point were included to calculate experimental uncertainty variance (Figure 2).

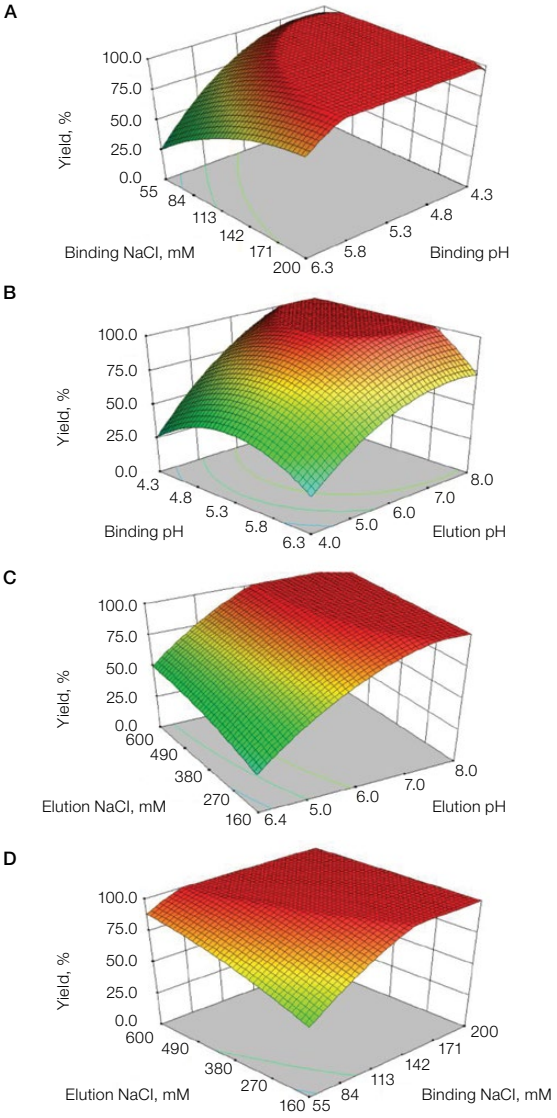

Fig. 2. DoE with Nuvia cPrime Resin. Central composite design with 3-D response surface plots of the effects of interactions between variables when two variables were set to optimal levels. **A**, elution pH = 8.0, elution NaCl = 500 mM; **B**, binding NaCl = 150 mM, elution NaCl = 500 mM; **C**, binding pH = 5.3, binding NaCl = 150 mM; and **D**, binding pH = 5.3, elution pH = 8.0. These data were first published in Lončar et al. (2015) under CC BY 4.0.

Optimal values for binding and elution buffer pH and NaCl concentration were determined by analyzing the response surface contour plots using Design-Expert Software (Stat-Ease Inc.). This analysis indicated the following optimal experimental

conditions: pH 5.3, 150 mM NaCl binding buffer and pH 8, 500 mM NaCl elution buffer (Figure 2). Analyses of variance (ANOVA) of response surface model fitting data are shown in Table 1. Regression analysis demonstrated that the model was significant, as was evident from the calculated F-value of 25.59 and *P* value of 0.0003. Data from CCD were analyzed with a second-degree polynomial equation.

Table 1. ANOVA for response surface quadratic model of CCD.

Source	Sum of Squares	df	Mean Square	F Value	*P* value Prob > F	
Model	30720.74	14	2194.34	25.59	0.0003	significant
A-Binding pH	1512.50	1	1512.50	17.64	0.0057	
B-Binding NaCl	1012.50	1	1012.50	11.81	0.0139	
C-Elution pH	13410.34	1	13410.34	156.37	< 0.0001	
D-Elution NaCl	612.50	1	612.50	7.14	0.0369	
AB	601.07	1	601.07	7.01	0.0382	
AC	552.78	1	552.78	6.45	0.0441	
AD	183.48	1	183.48	2.14	0.1939	
BC	270.28	1	270.28	3.15	0.1262	
BD	263.31	1	263.31	3.07	0.1303	
CD	57.78	1	57.78	0.67	0.4431	
A^2	7990.87	1	7990.87	93.18	< 0.0001	
B^2	1961.50	1	1961.50	22.87	0.0031	
C^2	3936.31	1	3936.31	45.90	0.0005	
D^2	287.43	1	287.43	3.35	0.1169	
Residual	514.55	6	85.76			
Lack of Fit	177.75	2	88.88	1.06	0.4284	not significant
Pure Error	336.80	4	84.20			
Cor Total	31235.29	20				

df, degrees of freedom; Cor total, total corrected for the mean. These data were first published in Lončar et al. (2015) under CC BY 4.0.

In order to determine whether these conditions were ideal for RSDA binding to Nuvia cPrime, the total amylase activity in the flow-through and initial wash fractions was determined. In contrast to the fermentation broth pigments, which did not bind, only ~2.6% of the total loaded enzyme activity units were detected in the combined flow-through fractions, indicating that RSDA binds to Nuvia cPrime very efficiently under the predicted optimal conditions. The dynamic binding capacity (DBC) of Nuvia cPrime under these conditions was calculated to be ~60 mg/ml.

The Nuvia cPrime Resin ligand has three major functional groups: a weak carboxylic acid end group, an aromatic hydrophobic ring, and an amide bond between the two that serves as a potential hydrogen bond donor/acceptor (Figure 3). Amylases have hydrophobic surface patches by which they may bind to starch granules (Cockburn et al. 2014). These

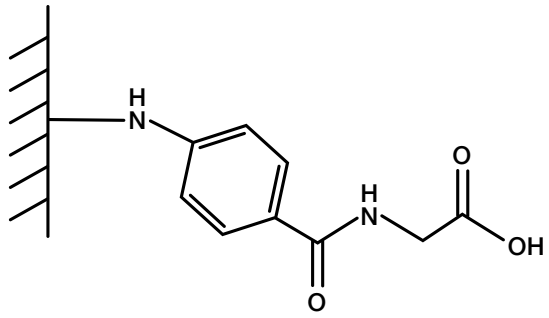

Fig. 3. Structure of Nuvia cPrime Resin ligand.

hydrophobic patches can potentially interact with the Nuvia cPrime ligand's aromatic hydrophobic ring. RSDA was eluted at high salt and pH from the Nuvia cPrime Column. The eluted enzyme showed high activity corresponding to ~96% of the total loaded enzyme activity (Table 2). SDS-PAGE analysis of the eluted sample showed a highly pure eluate fraction (Figure 4).

Table 2. Purified RSDA activity analysis.

Sample type	Total Protein, mg	Total Activity, IU	Specific Activity, IU/mg	Yield, % Based on Total Activity
Fermentation broth	66	9,200	139	100
Eluted sample from Nuvia cPrime Column	23	8,800	382	96

These data were first published in Lončar et al. (2015) under CC BY 4.0.

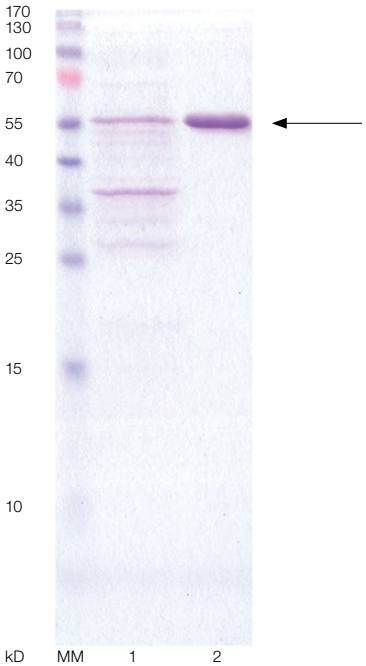

Fig. 4. RSDA purity by SDS-PAGE analysis. Lane MM: molecular markers; lane 1: cell-free extract (fermentation broth); lane 2: purified amylase. These data were first published in Lončar et al. (2015) under CC BY 4.0.

Conclusions

Our analysis demonstrates that Nuvia cPrime can be used to produce highly pure and active enzyme sample in a single step. The use of this resin overcomes the problem of compromised resin reusability caused by strong binding of fermentation broth components, such as pigments, seen with other resins. The process developed here may also be applicable to the purification of other bacterial enzymes and proteins. Nuvia cPrime Resin's multiple interaction modes, high capacity, and high selectivity can also help limit the number of steps required in a purification workflow.

While this single-step strategy meets the purity requirements for nontherapeutic targets, therapeutic enzymes and proteins generally require greater stringency in their purification processes. For such purposes, Nuvia cPrime could be incorporated into a workflow with suitable additional purification steps.

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