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Simple protein purification through affinity adsorption on regenerated amorphous cellulose followed by intein self-cleavage

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ABSTRACT

A simple, low-cost, and scalable protein purification method was developed by using a biodegradable regenerated amorphous cellulose (RAC) with a binding capacity of up to 365 mg protein per gram of RAC. The recombinant protein with a cellulose-binding module (CBM) tag can be specifically adsorbed by RAC. In order to avoid using costly protease and simplify purification process, a self-cleavage intein was introduced between CBM and target protein. The cleaved target protein can be liberated from the surface of RAC by intein self-cleavage occurring through a pH change from 8.0 to 6.5. Four recombinant proteins (green fluorescence protein, phosphoglucomutase, cellobiose phosphorylase, and glucan phosphorylase) have been purified successfully.

1. Introduction

Developing simple, low-cost, and environmentally friendly methods for recombinant protein purification on a large scale remains challenging [1,2]. Affinity chromatography by using various affinity tags on various resins is popular in laboratories and biotechnology companies [1,3,4], but it cannot be applied to low-selling-price non-therapeutic proteins, such as industrial enzymes [5]. A low-cost and scalable method for large-scale protein purification is prerequisite for commercialization of industrial scale biocommodity production mediated by enzymes or synthetic enzymatic pathways [6–8].

A cellulose-binding module (CBM) tag has been used for recombinant protein purification on commercial cellulose matrix or powder (Avicel, microcrystalline cellulose or Sigmacell) [9–14], because of (i) highly specific binding for the CBM-tag protein, (ii) low non-specific binding for other proteins, (iii) low-cost affinity matrix (cellulose), (iv) enhanced protein folding [13], and (v) increased protein yields [14]. But commercial crystalline cellulose is a low binding capacity porous matrix, most of whose binding surface is internal [15]. The bound protein cannot be removed efficiently due to enzyme entrapment effect [16], resulting in lower protein recovery yields. In addition, protease could not efficiently work on entrapped protein to release cleaved target proteins [15].

Regenerated amorphous cellulose (RAC), which is made from Avicel through phosphoric acid dissolution followed by regeneration [17], has a greater than 20-fold surface area of Avicel [15]. In addition, the entire binding surface of RAC is externally accessible to the target protein, and protein binding on RAC is faster than on Avicel.

Inteins – protein introns – can excise themselves and/or rejoin two fragments together [18,19]. In order to avoid using costly peptide-specific protease and simplify purification process, self-cleavage intein can be used to replace costly peptide-specific proteases through the changes in pH or thiol reagent concentration [18–20].

Here we developed a generic, low-cost, scalable, protein purification method based on affinity adsorption on a low-cost, biodegradable, high adsorption capacity adsorbent, RAC. The high-

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purity target proteins can be separated through self-cleavage by intein that linked CBM and target protein.

2. Materials and methods

2.1. Chemicals and strains

All chemicals were reagent grade, purchased from Sigma–Aldrich (St. Louis, MO, USA) and Fisher Scientific (Pittsburgh, PA, USA), unless otherwise noted. Microcrystalline cellulose – Avicel PH105 (20 μm) – was purchased from FMC (Philadelphia, PA, USA). *Escherichia coli* DH5α was used as a host cell for DNA manipulation. *E. coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA, USA) containing a protein expression plasmid was used for producing the recombinant protein. Luria-Bertani (LB) medium was used for *E. coli* growth and protein expression with 100 μg/mL ampicillin. *Clostridium thermocellum* genomic DNA was gifted from Dr. Mielenz at Oak Ridge National Laboratory (Oak Ridge, TN, USA). The oligonucleotides were synthesized by Integrated DNA Technologies (Coraville, IA, USA) (Table 1).

2.2. Regenerated amorphous cellulose preparation [17]

Approximately 0.2 g of microcrystalline cellulose (FMC PH-105) was added to a 50-mL centrifuge tube, and 0.6 mL distilled water was added to wet the cellulose powder to form a cellulose-suspended slurry. Ten millilitres of ice-cold 86% H_3PO_4 (i.e., commercial 85% grade) was slowly added to the slurry with vigorous stirring so that the final phosphoric acid concentration was approximately 83.2%. Before the last 2 mL of phosphoric acid was added, the cellulose suspension solution was evenly mixed. The cellulose mixture turned transparent after addition of last 2 mL concentrated phosphoric acid within several minutes, and stood for ca. an hour on ice with occasional stirring. Approximately 40 mL of ice-cold water was added at a rate of approximately 10 mL per addition with vigorous stirring between additions, resulting in a white cloudy precipitate. The precipitated cellulose was centrifuged at ~10,000 × *g* and 4 °C for 20 min. The pellet was suspended by ice-cold water, followed by centrifugation to remove the supernatant containing phosphoric acid four times. Approximately 0.5 mL of 2 M Na_2CO_3, and 45 mL of ice-cold distilled water were used to suspend the cellulose pellet. After centrifugation, the pellet was suspended and centrifuged by distilled waters twice or until pH 5–7. The carbohydrate concentration of RAC was calibrated by the phenol–H_2SO_4 method [21]. No detective amount of cellulose (<1 wt.%) was lost during the treatment most times. Sigmacell or other cellulose powders can be used to replace FMC PH105. The RAC slurry can be stored as a ~10 g RAC/L suspension solution at 4 °C in the presence of 0.2% (w/v) sodium azide for a long time (e.g., 1 year).

Table 1
Primers needed to construct the recombinant protein expression plasmids

Primer	Sequence
CBM-F1	5′ GGTGGTCATATGCCGGTATCAGGCAATTTGAAGGTTGAATTC 3′
CBM-R2	5′ *CCCTCGAGGCCGCCAGGCCT* GGGTTCTTTACCCCATACAAGAACACCG 3′
Intein-F3	5′ GGTGGTAGGCCTAACAACGGTAACAACGGTCTCGAACTGC 3′
Intein-R3	5′ AGAGCCCTCGAGGAATTCGCGGCCGC 3′
GFP-F2	5′ *AGGCCTGGCGGCCTCGAGGGC*TCTTCCATGGTGAGCAAGGGCGAGGAGCTGTTC 3′
GFP-R1	5′ GGTGGTCTGCAGTTACTTGTACAGCTCGTCCATGCCGAG 3′
CBP-F2	5′ ACTGCTCGAGATACCGCCGTCAGATGATCCGATGAAGTTCGGTTTTTTTGATGATGC 3′
CBP-R1	5′ ACTGCTGCAGTTAGGTACCACTAGTATCGATTCCCATAATTACTTCAACTTTGTGAGTC 3′
GNP-F1	5′ TGGTGGCTCGAGATGTATCTTTTTGGAAAAATTAC 3′
GNP-R1	5′ AAGAAGGGATCCTTACTGTACAATCCATCTGATAAGTCC 3′
PGM-F	5′ GCATCGCTCGAG GGCTCTTCC ATGCGAAGTAGCGCGCTTTAT 3′
PGM-R	5′ ACGTGC GGAT CCTCAGTCTT TAAGAAGCGG TTCTATAAC 3′

Underline, the restricted enzyme site; italic: sequence for overlay PCR.

2.3. Recombinant protein expression plasmids

The pCIG plasmid encoding the CBM-intein-GFP (CIG) fusion protein was constructed based on the New England Biolabs plasmid pTWIN1 (Ipswich, MA USA). Three *cbm*, Ssp DnaB intein, and *gfp* DNA fragments amplified by PCR amplification by the primers of CBM-F1/CBM-R2, Intein-F3/Intein-R3, and GFP-F2/GFP-R1, followed by double digestion by NdeI/StuI, StuI/XhoI and XhoI/PstI, respectively, were ligated into the NdeI/PstI digested pTWN1 in one step to produce the pCIG plasmid. The three other target genes included the *C. thermocellum* cellobiose phosphorylase (CBP) [22,23], phosphoglucomutase (PGM, CT1265), and putative α-glucan phosphorylase (GNP, CT0932).

2.4. Recombinant protein expression

The protein expression plasmids were transformed into the strain *E. coli* BL21 (DE3). Two hundred millilitres of LB medium supplemented with 100 μg/mL ampicillin in 1-L Erlenmeyer flasks were incubated with a rotary shaking rate of 160 rpm at 37 °C until the A_{600} reached between ~0.6 and 0.8. The recombinant protein expression was induced by adding IPTG (0.20 mM, final), and then the cultures were incubated at the decreased temperature of 18 °C for 9–12 h. The cells were harvested by centrifugation at 4 °C, washed once by 50 mM Tries–Hal buffer (pH 8.5), re-suspended by ~30 mL of 50 mM Tries–Hal buffer (pH 8.5). The cell pellets were lysed in an ice bath by ultra-sanitation by Fisher Scientific Sonic Dismembrator Model 500 (3-s pulse, total 90 s, at maximum strength). After centrifugation, the supernatant of cell lysate was used for protein purification.

2.5. Self-cleavage efficiency of intein at different temperature and pH

1.2 mL of 10 mg of RAC/mL was mixed with 8 mL of cell lysate containing 0.246 mg CIG/mL at room temperature for 30 min. After centrifugation and washing once in 6 mL of 50 mM Tris–HCl, 0.5 M NaCl, and 1 mM EDTA buffer (pH 6.5 or 7.0) at 4 °C, the RAC pellet with adsorbed CIG was suspended in 8 mL of 50 mM Tris–HCl, 0.5 M NaCl, and 1 mM EDTA buffer (pH 6.5 or 7.0). The suspension solution was incubated at different temperatures (4 °C, 18 °C, 23 °C, 30 °C, 40 °C, 50 °C, and 60 °C). The fluorescence of cleaved GFP in the supernatant was measured and calculated for self-cleavage efficiency.

2.6. Protein purification

Thirty-five millilitres of cell lysate (1.65 mg of crude protein containing 0.33 mg of CIG per mL) was mixed with 5.25 mL of 10 mg RAC/mL at room temperature for 30 min. After centrifugation, the pellet was suspended in 27 mL of 50 mM Tris–HCl buffer (pH 6.5) containing 0.5 M NaCl and 1 mM EDTA. After centrifugation, a 35 mL of the cleavage buffer (50 mM Tris–HCl, 0.5 M NaCl, and 1 mM EDTA buffer, pH 6.5) was added to suspend the pellet. After incubation at 40 °C overnight or for 4–8 h followed by centrifugation, the cleaved GFP was obtained in the supernatant. For heat-labile proteins, lower temperatures (e.g., 4 °C or 18 °C or room temperature) could be used for intein self-cleavage. Similarly, the crude proteins from the cell