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Original article

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Recombinant Protein Purification using Composite Polyacrylamide-Nanocrystalline Cryogel Monolith Column and Carbohydrate-Binding Module Family 64 as Affinity Tag

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Abstract

Background: In the field of recombinant protein production, downstream processing, especially protein purification, is critical and often the most expensive step. Carbohydrate binding module 64 (CBM64) was shown in 2011 to bind efficiently to a broad range of cellulose materials.

Methods: In this study, we developed a protein purification method using nanocrystalline cellulose embedded in a polyacrylamide monolith cryogel and CBM64 affinity tag linked by intein to PD1 as a model protein. The CBM64-Intein-PD1 gene cassette was expressed in *E. coli*. Following cell lysis, CBM64-Intein-PD1 protein bound to the monolith PA-NCC cryogel. After washing and reducing the pH from 8.0 to 6.5, the intein underwent self-cleavage, resulting in the release and elution of pure PD1 protein.

Results: The synthesized monolith column had a porous structure with an average pore size of 30 µm and a maximum binding capacity of 497 µg per gram of dried column. The yield of this purification method was 84%, while the yield of the His tag-acquired CBM64-Intein-PD1 method was 89%.

Conclusions: We used cellulose as support for affinity chromatography, which can be used as a cost-effective method for protein purification.

Keywords: Affinity tag, CBM64, PD1, Protein purification.

Introduction

The purification of bioactive and valuable recombinant proteins is an important aspect of biotechnology processes (1). The growth and development of biotechnology industries, especially in the field of drug production, have increased demand for simpler and more cost-effective purification methods than are currently available (2). The purification step in recombinant protein production is challenging due to high fermentation, extraction, and purification costs, as well as the need for high final product purity. Affinity chromatography is one of the most used of the various extraction and purification methods developed for recombinant protein production (3). Affinity chromatography purification relies mainly on the affinity and binding of attached tags linked or expressed with the target protein, and a ligand fixed on the matrix in the chromatography column (4). One well-known affinity chromatography system is based on the binding of a repetitive histamine tag to a nickel column (5). Similar to the other purification methods, His-tag affinity chromatography has some drawbacks. For example, when this system is used to purify proteins produced by insect or

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mammalian cells, the efficiency and the purity of the final product is decreased. It was hypothesized that this phenomenon is due to the high percentage of histidines in the proteins synthesized in the insect or mammalian cells, which interferes with the purification mechanism (6). To reduce such drawbacks and inefficiencies, researchers investigate novel methods or affinity tags for recombinant protein purification.

One example of an efficient material for column development is cellulose (7). Cellulose is abundant, biocompatible, inert, inexpensive, and tolerant of high external tensile forces (8). Nanocrystalline cellulose is the nano-scale form of cellulose in which amorphous regions are hydrolyzed while the crystalline regions remain. This form of cellulose is 5-70 nm in width and 100 nm in length (9). Nanocrystalline cellulose particles have some desired physiochemical properties that make them suitable for many biological applications; they have high surface-to-volume ratio, disperse in the water efficiently, and for the production of mixed polymeric columns, embed well in the final structure (10). To use cellulose as both ligand and column filler for affinity chromatography, a tag that binds the cellulose must be attached to the target protein.

One group of tags used for affinity chromatography is carbohydrate-binding modules (CBMs). To date 88 CBM families have been identified and their structures and functions determined (11). CBM tags, including those that bind cellulose or starch, have been used in industry for protein purification (12).

In 2011, CBM64, another member of this family, was investigated (13). In 2015, CBM64's structure was determined and some of its physical, chemical, and biological properties characterized (14). Pires and et al. evaluated binding features of CBM64 to different cellulose forms in various pH and temperature conditions and compared the results with other CBM family members. It was shown that CBM64 had greater adsorption to Sigma-cell 20 cellulose powder than to other cellulose forms, but also bound efficiently to microcrystalline cellulose and paper, indicating the ligand plasticity of this module. Another attractive feature of CBM64 is its ability to bind and retain attachment in various pHs and temperatures ranging from 4 to 9.5 and 4 to 90℃, respectively (15).

Considering these beneficial features of CBM64, and the abundance, tensile strength, and biologically inert nature of its ligand cellulose, in this study we examined the efficiency CBM64 as a binding tag and nanocrystalline cellulose as its ligand, for recombinant protein purification.

Materials and Methods

Strains and Plasmids

Escherichia coli (*E. coli*) XL1-Blue cells were provided by Pasteur Institute, Tehran, Iran, and cultured in Luria-Bertani (LB) medium at 37 °C for 16 hr. The pUC57 plasmid harboring the CBM, PD1, and intein fragment was designed and purchased from ProteoGenix (Paris, France). The pET26+ vector (Novagen, Germany) was used to express the CBM64-Intein-PD1 recombinant genes.

Design of the CBM64-Intein-PD1 Cassette

Peptide sequences of CBM64, intein, and PD1 were retrieved from the PBD database. The DNA sequences were determined using the EBML Peptide to Sequence online module and the sequences were codon-optimized for expression in *E. coli*. The expression cassette was designed using GeneSnap software. PD1 was placed at the C-terminus of the gene cassette because intein delta is a C-terminal excising intein.

DNA Manipulation and Cloning of Recombinant Genes

The expression cassette was synthesized and purchased from ProteoGenix (Paris, France). The lyophilized pUC57 plasmid containing the expression cassette was resuspended in 50 µl of deionized water and transferred into the competent *E. coli* XL1-Blue cells using the standard $CaCl_2$ method (16). The transformed cells were incubated for 16 hr at 37 °C and the plasmid was extracted. To sub-clone the CBM64-Intein-PD1 gene fragment into pET-

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26b (+), the plasmid was digested with restriction enzymes *Bam*H I and *Hin*d III (ThermoFisher, USA) at 37 °C for 12 hr. pET-26b (+) and the digested plasmid were ligated using T4 DNA ligase at 37 °C for 12 hr.

Expression of the CBM64-Intein-PD1 Recombinant Gene

The *E. coli* XL1-Blue cells harboring pET26+ CBM64-Intein-PD1 were incubated at 37 °C in LB medium pH 7.0 containing 50 µg/ml of kanamycin. An overnight culture of these cells was transferred to 500 ml of LB medium pH 8.0 and 50 µg/ml kanamycin. When the OD_{600} reached 0.9, isopropylthio-β-galactoside (IPTG) (ThermoFisher, USA) was added to 10 mM to induce recombinant protein expression. After 24 hr of incubation, the cells were harvested by centrifugation at 10,000×*g* for 3 min at 4 °C. The supernatant was decanted, and cells were re-suspended in 100 mM phosphate buffer (75 mM Na_2HPO_4, 24 mM NaH_2PO_4; pH 8.0) for lysis by sonication. The resulting slurry was centrifuged at 10,000 ×*g* for 5 min at 4 °C to pellet cell debris, and the supernatant containing the protein was collected. Proteins were electrophoresed in a 12% SDS-polyacrylamide gel and stained with 0.2% Coomassie Brilliant Blue R-250. Bovine serum albumin (BSA) was used as the protein standard. The total and target protein concentrations were determined by the Bradford assay. For all sample and standard solutions, 0.1 ml of the supernatant was added to 1 ml of Bradford solution. Solutions were incubated for 30 min at room temperature and absorbances measured at 595 nm on a spectrophotometer.

Nano-crystalline Cellulose Preparation

Cellulose powder in the form of microcrystalline cellulose (Avicel 101) was purchased from Ziochemical Co, China. Six grams of microcrystalline cellulose were added to 5 ml of distilled water and placed in an ice bath with magnetic stirring. Seventeen ml of 99% sulfuric acid were added dropwise to prevent the temperature from rising above 45 ^{o}C. To remove the sulfuric acid, the slurry was pelleted at 10,000 ×*g* for 3 min at room temperature and washed 3 times. The purified nanocrystalline cellulose was re-suspended in 10 ml of distilled water.

Polyacrylamide-Nanocrystalline Cellulose Monolithic Column Preparation

Acrylamide polymer was used to entrap nanocrystalline cellulose particles into the cryo-mediated formed structure. Ten µg of acrylamide powder were added to 1 ml of distilled water. One ml of 60% nanocrystalline cellulose was added followed by APS (1.2%) and TEMED (0.6%). The resulting mixture was poured into 1 cm diameter glass tubes, which were placed in 96% -20 °C ethanol. After 24 hr, the polyacrylamide-nanocrystalline cellulose (PA-NCC) cryogel was centrifuged for 3 min at 5,000 ×*g* and the excess solution decanted. The flow rate was calculated by measuring the time needed for 5 ml of distilled water to pass through the column by gravity. To study re- and de-swelling of the column contents, the cryogel monolith column was immersed in deionized water. The swollen cryogel was weighed, dried in the oven at 80 °C, and weighed again.

Polyacrylamide-Nanocrystalline Cellulose Characterization

Scanning electron microscopy (SEM) was performed to evaluate the structure and pores of the yielded polymer. The SEM images were obtained on a field-emission scanning electron microscope (FESEM, Mira3-TESCAN, Czech Republic) at 15 kV. Samples were coated with gold before observation.

Purification using Microcrystalline Cellulose

The Avicel 101 cellulose product was used as microcrystalline cellulose (MCC). Twenty mg of MCC powder were transferred into 2 ml micro-tubes above a filter layer with a 10 µm pore size. A stabilizing polystyrene ring was placed on the top of the MCC layer. The cell lysate was loaded onto the purification column and allowed to stand for 1 hr to allow completion of the binding process. After centrifugation, to break the CBM-Intein link, the intein self-splicing mechanism was

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activated by reducing the pH from 8 to 6.5 by adding 8 ml of 50 mM Tris-HCl, 1mM EDTA, and 0.5 M NaCl (pH 6.5), and incubating at 40 °C for 4 hr. Purified PD1 protein was obtained by centrifuging the samples at 10,000 ×*g* for 5 min. The flow-through was transferred to a sterile tube and stored at 4 °C.

Purification using Nanocrystalline Cellulose Monolith Columns

Nanocrystalline cellulose has a high surface-to-volume ratio and thus is suitable as a bed for binding of the cellulose-binding domain, while nanocellulose and regenerated amorphous cellulose (RAC) cannot be readily used as powder or slurry for purification in commercial applications. Composite cryogel monolith of polyacrylamide and nanocrystalline cellulose can meet this requirement. PD1 protein was used as a model protein and intein was used as a self-cleaving linker to release PD1 from the bed (Fig. 1).

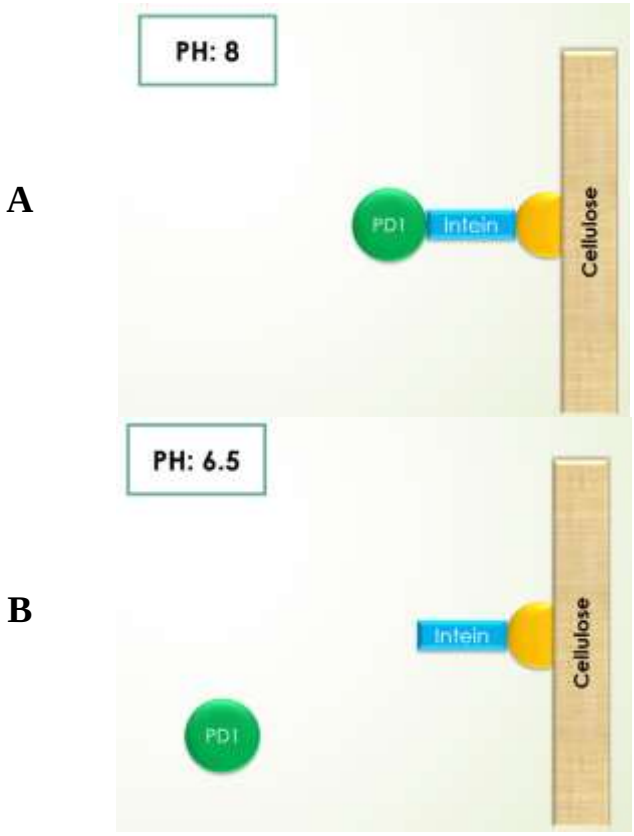

Fig. 1. Mechanism of protein purification in the CBM-Cellulose purification system. A: At pH 8.0, the CBM64-Intein-PD1 is attached to the cellulose bed. B: Reducing the pH from 8.0 to 6.5 results in the activation of the intein self-splicing mechanism, which leads to dissociation of PD1 from the intein-CBM complex and allows elution of purified PD1.

One hundred microliters of recombinant *E. coli* were used to inoculate 100 ml of LB medium containing 100 µg/ml of kanamycin. This culture was incubated for 16 hr at 37 °C with 180 rpm shaking. Five hundred ml of LB medium with kanamycin was inoculated with 500 µl of the *E. coli* culture and incubated for 24 hr at 30 °C. The culture was centrifuged to pellet the cells, which were lysed as previously described. Ten microliters of the cell lysate were added to the vertical stand column and incubated for 1 hr. After incubation, the binding buffer was discharged. The column was filled with 8 ml of elution buffer (50 mM Tris-HCl, 1mM EDTA, and 0.5 M NaCl, pH 6.5) and incubated at 4 °C for 4 hr. Then, the elution buffer containing purified PD1 was collected. Image processing technology was used to determine the yield

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efficiency. The purified PD1 and crude sample band intensities were compared using CLIQS software (Total-Lab Co.).

Determination of Column Protein Binding Capacity

The cell lysate from 1 liter of recombinant *E. coli* culture was used to evaluate the cryogel monolith column binding capacity. About 2.5 ml of cell lysate were added to the column. After binding of CBM64-Intein-PD1 to the cellulose, the column was washed, CBM64-Intein-PD1 eluted from the column, and the concentration measured by Bradford assay. The loaded cell lysate volume was increased stepwise by of 2.5 ml to a final volume of 50 ml.

Determination of Column Reused Maximum Binding Capacity

The used column was treated with 8 M urea followed by three washes with distilled water. The amount of CBM64-Intein-PD1 protein equal to the maximum binding capacity of the column was used to evaluate the binding capacity of the column after column regeneration.

Purification of CBM64-Intein-PD1 Protein using Immobilized Metal Affinity Chromatography (IMAC)

Recombinant *E. coli* were incubated at 37 °C for 24 hr with addition of 50 mM IPTG at OD_{600} of 0.9. After harvesting the cells by centrifugation, the pellet was re-suspended in phosphate buffer (20 mM sodium phosphate, 0.5 M NaCl, 30 mM imidazole, and 2 mM DTT, pH 8.0), and the cells were disrupted by sonication. The lysate was centrifuged, and the supernatant loaded onto a nickel column. The column was equilibrated with binding buffer and the protein was eluted with 20 mM sodium phosphate, 0.5 M NaCl, 100 mM imidazole, and 2 mM DTT, pH 8.0.

Results

Polyacrylamide-Nanocrystalline Cellulose Characteristics

The nanocrystalline cellulose particles were 50-70 nm in size and had the colloidal state in nature. The resulting polyacrylamide cryogel had a similar appearance to the pure polyacrylamide column. The synthesized cryogel column flow rate was 4.7 ml per min and the water retention capacity was calculated to be 8 times the dried weight. The water absorbance capacity of the column was 7.3 times the dry weight.

The internal morphology of the composite cryogel is shown in Figure 2. The cryogel had a porous appearance and the average diameter of pores and canals was 30 µm. The column had rough pore edges and the walls of the pores and canals had complete integrity. The external surface of the column showed complete integrity with no opening lacerations.

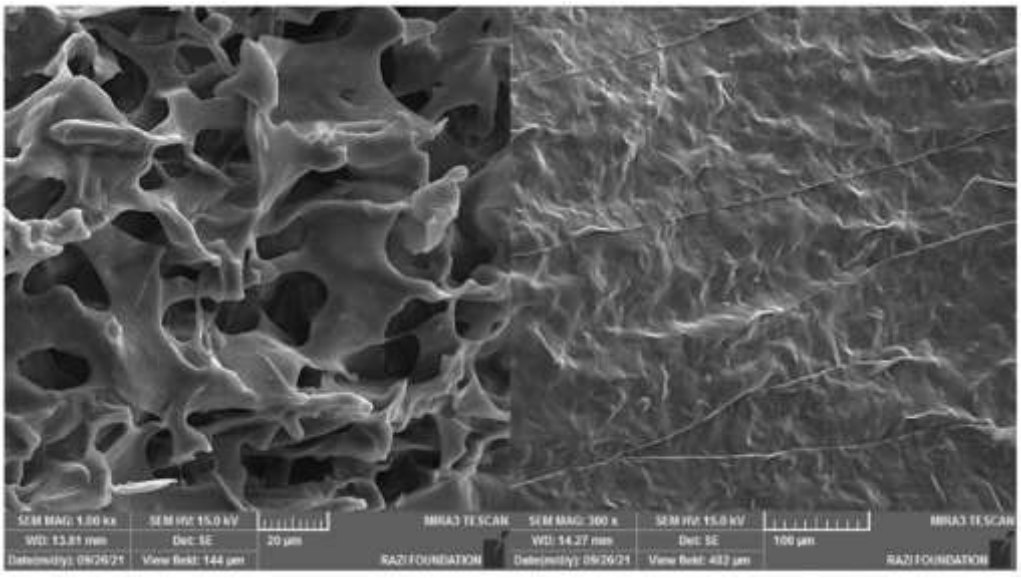

Fig. 2. FESEM image of composite polyacrylamide-nanocrystalline cellulose cryogel. The magnification was 1000 x.

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Recombinant Gene Expression and PD1 Purification

The optimum expression condition was determined by changing the IPTG concentrations and incubation times. Expression was optimum 24 hr after inoculation of cells at OD_{600}= 0.9 with 10 mM IPTG. The expressed recombinant protein had a molecular weight of 48 kDa and an isoelectric point of 5.69.

The molecular weight of the eluted PD1 protein fragment was 17.7 kDa. The molecular weight of the nickel-purified histidine-tagged full-length recombinant protein was 48 kDa. The PD1 yield and purity using MCC, PA-NCC, and IMAC were determined; both yield and purity were greatest with IMAC, followed by PA-NCC, and then MCC (Table 1).

Table 1. PD1 protein yield and purity by the three purification methods used in this study.

Purification Method	Yield (%)	Purity (%)
MCC Pre-Packed Column	76	81
PA-NCC Monolith Column	84	87
IMAC (Nickel)	89	94

SDS-PAGE was performed to determine the purity and yield of the purification method (Fig. 3).

The concentrations of purified PD1 and the CBM64-Intein-PD1 fragment were calculated to be 442 and 307 µg/ml, respectively, while the yields for the two products were 84 and 74%, respectively.

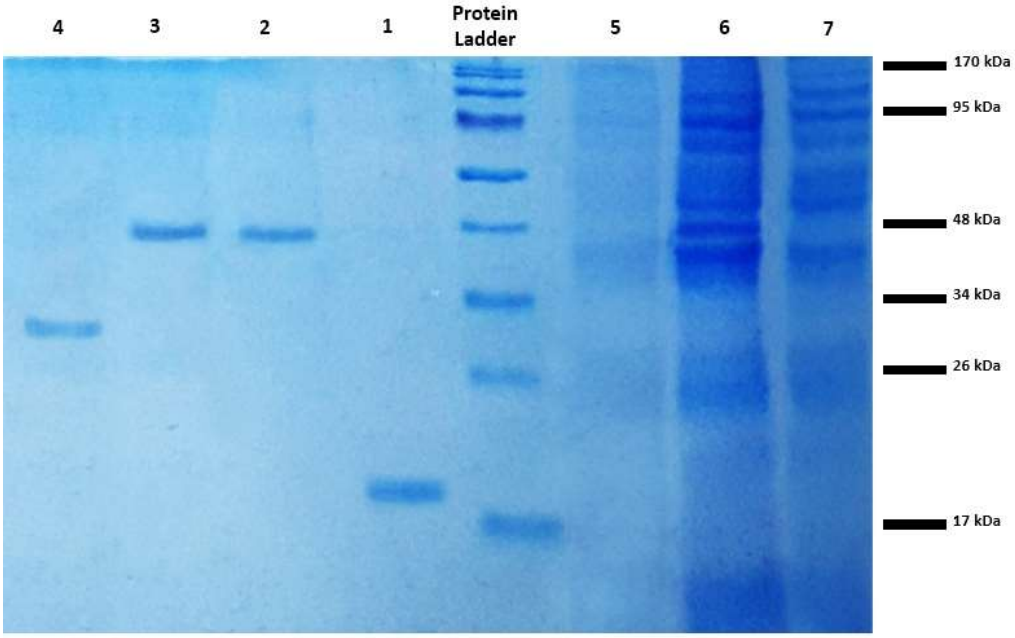

Fig. 3. Acrylamide gel result of PD1 purification using cryogel monolith column and CBM64 as a binding tag. 1: PD1 protein, released from the CBM-Intein complex attached to the cellulose 2: CBM64-Intein-PD1 eluted from the cellulose bed 3: CBM64-Intein-PD1 purified on a nickel column 4: CBM64-Intein fragment eluted after PD1 purification 5: Washing flow-through 6: Crude lysate 7: Binding flow- through.

Functional Characterization of the Monolith Column

The maximum binding capacity of the monolith column was 497 µg per gram of the dried column (Fig. 4). The column was regenerated to evaluate the reused column efficacy. In the first three reuse cycles the efficacy changes were minor; however, after

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regenerations 4-6, the binding capacity decreased considerably. The efficacy changes were minor again after the 7th column regeneration, demonstrating the maximum binding capacity reduction following column regeneration (Fig. 5).

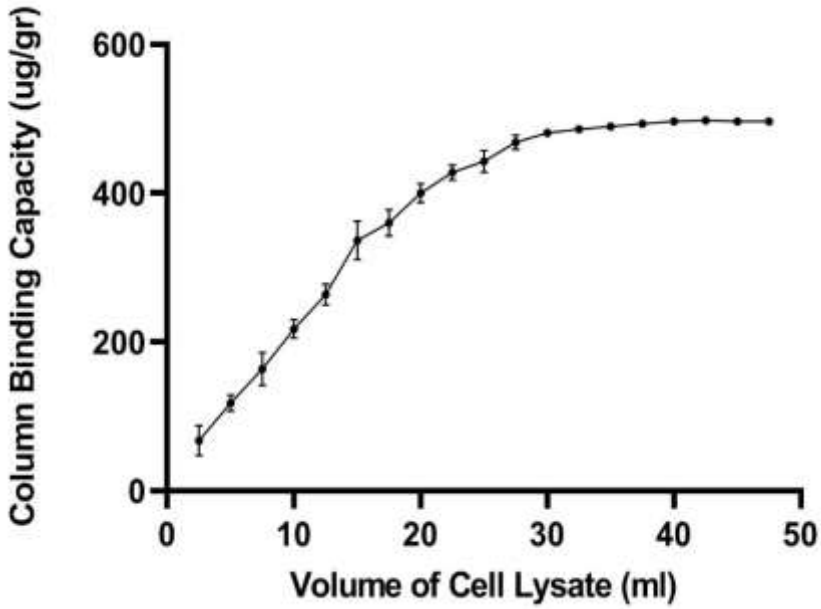

Fig. 4. Protein binding capacity of the monolith column. The maximum binding capacity of the column was determined to be 497 µg per gram of the column. The data are presented as means of triplicate assays±standard deviation.

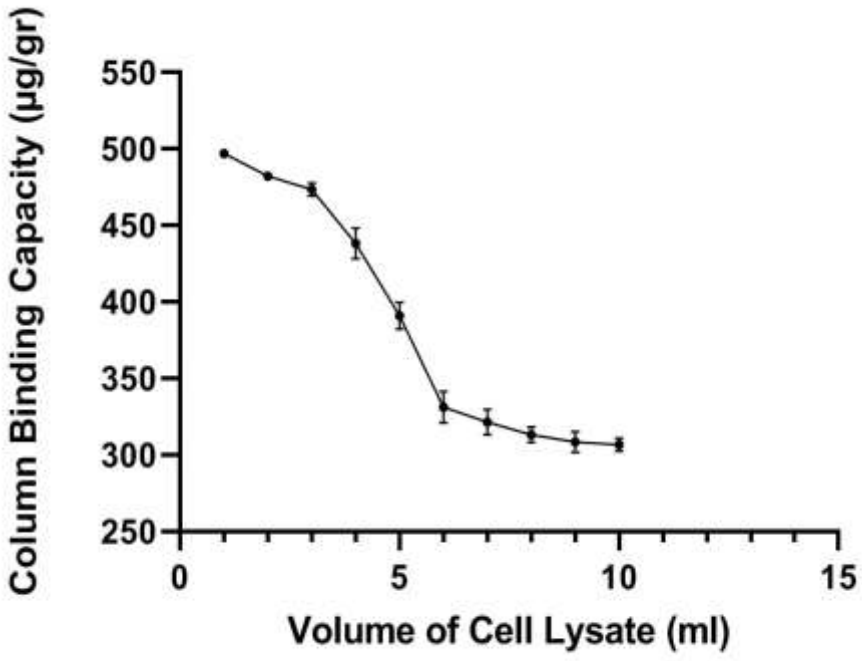

Fig. 5. Maximum binding capacity of the regenerated column. After the 7th regeneration of the column the binding capacity of the column was reduced and remained around 310 µg per gram of the column. The data are presented as means of triplicate assays±standard deviation.

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Discussion

Development of novel purification methods for recombinant proteins is the topic of many studies (17). Using affinity chromatography techniques, recombinant proteins can be isolated and purified (18). Features that make CBMs attractive tools for protein purification include: 1) high specific affinity and binding of CBM tags to cellulose, 2) low non-specific binding of this tag to the cellulose, 3) efficient release of contaminant proteins from the cellulose column during washing, 4) folding and function of the target proteins are rarely altered, and 5) the protein yield is satisfactory (19). In this study we proposed an affinity chromatography technique based on the affinity of the designed peptide tag to a polyacrylamide-nanocrystalline cellulose column. Early studies using cellulose-binding domains as affinity tags for recombinant protein purification date to 1992. In that year, Greenwoods proposed to fuse the gene fragments encoding the cellulose-binding domain of endoglucanase CenA and exoglucanase Cex from *Cellumonase fimi* to coding sequences of the interest protein to purify it by affinity chromatography (20). The cellulose-binding domain is used in a variety of applications from purification to immobilization. For example, Shpigel and his colleagues used the cellulose-binding domain of *Clostridium celluvorans* to immobilize protein A on the surface of a cellulose bed (21). As another example, Liu et al. used a cellulose-binding domain to stabilize carbonic anhydrase on cellulose to capture CO_2 from flue gasses (22). The CBM1 family consists of a fungal cellulose-binding domain. In 2012, Sugimoto et al. used this family of cellulose-binding domains as an affinity tag to purify recombinant proteins. Their research indicated that CBM1 family members can be used as affinity tags to purify both hydrophilic and hydrophobic proteins. The resulting final purity for most recombinant proteins was greater than 80% (23). Most of the previous works regarding the application of CBMs for affinity purification were focused on using CBM families 2 and 3. Examples of proteins studied for affinity purification with these CBM families include antibodies (24), protein A (25), and phytochelatin (26). Here we used the CBM64 family for the first time for recombinant protein purification.

The binding of CBM to cellulose in most CBM families is irreversible. Some researchers tried to overcome this challenge to protein purification by cleaving the CBM-target protein linkage. This method, however, increases purification costs by adding the enzymatic digestion step, and then an additional step is needed to remove the enzyme from the final solution (27). In this work, we used the irreversible nature of CBM binding to the cellulose as a beneficial feature that makes the cleavage and dissociation of the CBM-PD1 relatively easy and eliminates the need for further purification of PD1 from the remaining CBM fragments. The cleavage reaction with the irreversible CBM64 binding to the cellulose ensures the purity of PD1 with no contaminating CBMs in the final mixture. In industries relating to human health, it is often essential to achieve pure target protein with no contaminating tags or attached chains. Hong et al. also used intein-F3 and -R3 to cleave the CBM-GFP peptide chain. They concluded that this method can be used as a simple and low-cost method to purify recombinant proteins with no remaining additional chains. They reported a target protein yield of 74% (28). In our study the yield was 84%.

Protein purification is an essential step in many industrial and pharmaceutical applications. Here, we introduced a novel and cost-effective bio-affinity-based purification method using CBM64 and a composite cellulose column. Further studies are needed to commercialize this method for protein purification.

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The authors declare there is no potential conflict of interest regarding this research and its publication.

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