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Heavy Metal Removal by Novel CBD-EC20 Sorbents Immobilized on Cellulose

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Heavy metals are major contributors to pollution of the biosphere, and their efficient removal from contaminated water is required. Biosorption is an emerging technology that has been shown to be effective in removing very low levels of heavy metal from wastewater. Although peptides such as metallothioneins or phytotchelatins are known to immobilize heavy metals, peptide-based biosorbents have not been extensively investigated. In this paper, we describe the construction and expression of bifunctional fusion proteins consisting of synthetic phytochelatin (EC20) linked to a *Clostridium*-derived cellulose-binding domain (CBD_{clos}), enabling purification and immobilization of the fusions onto different cellulose materials in essentially a single step. The immobilized sorbents were shown to be highly effective in removing cadmium at parts per million levels. Repeated removal of cadmium was demonstrated in an immobilized column. The ability to genetically engineer biosorbents with precisely defined properties could provide an attractive strategy for developing high-affinity bioadsorbents suitable for heavy metal removal.

Introduction

The problem of coping with the presence of heavy metals has become a top priority in water treatment.[1] The danger that these heavy metals such as cadmium, lead, and mercury pose to humans and the environment has caused more stringent control over the allowable limits in drinking water.[2] Unfortunately, regulation today can only prevent future pollution, and the only answer to the environmental disasters of yesteryear is remediation.

Although conventional technologies such as precipitation−filtration, reverse osmosis, oxidation−reduction, and membrane separation are adequate to remove the bulk of the heavy metal contamination, they are often inadequate to reduce heavy metal concentrations to acceptable regulatory standards.[3] It becomes clear that a cost-effective secondary polishing treatment process is needed for the removal of heavy metals at dilute concentrations. One emerging technology that is receiving more attention is the use of metal-binding peptides. Naturally occurring metal-binding peptides such as metallothioneins (MTs)[4] are the main metal sequestering molecules used by cells to immobilize metal ions, offering selective, high-affinity binding sites. Recently, a new class of metal-binding peptides known as synthetic phytochelatins (ECs) with repetitive metal-binding motif $(Glu\text{-}Cys)_nGly$ were shown to have improved Cd^{2+} binding capability over that of MTs.[5]

Great success in heavy metal removal by microbial-based systems has been achieved by using organisms overexpressing either MTs or ECs.[5,6,7] However, peptide-based systems have so far failed to attract much attention due to the tedious protocol and the high cost associated with purification. In recent years, several affinity-tag systems such as hexahistidines,[8] glutathione S-transferase,[9] or maltose-binding protein[10] have been used for one-step purification and immobilization of enzymes or proteins. These systems, unfortunately, require costly affinity matrixes and are too expensive for large-scale applications.

Cellulose-binding domains (CBDs), which bind specifically to cellulose, have been isolated from a variety of cellulotyic bacteria.[11,12] Because of their high affinity toward cellulose, CBD has been exploited as an affinity tag for the purification and immobilization of heterologous fusion proteins onto cellulose supports.[13] Fusion proteins containing a CBD moiety could be constructed so that little or no alternation in the properties of the fusion partner is observed.[14] The CBD-cellulose affinity system is attractive because it does not require a derivatized matrix, and cellulose is available in a variety of inexpensive forms, such as preformed microporous beads, highly adsorbent sponge or cloth, and microcrystalline powders.

Here we reported the construction, expression, and immobilization on cellulose of fusion proteins consisting of CBD and EC20. We demonstrated that purification and immobilization of CBD-EC20 fusions were easily achieved in a single step onto a variety of cellulose matrixes and that cadmium was efficiently removed by the immobilized CBD-EC20 in both batch and continuous operations.

Experimental Section

Bacterial Strains and Plasmids. *E. coli* XL-1 Blue was used for standard cloning and *E. coli* BL21(DE3) was used

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as the host strain for protein expression. Plasmids pET-34b-(+) and pET-37b(+), used to generate fusions with CBD from *Clostridium cellulovorans* and *Cellulomonas fimi*, respectively, were purchased from Novagen Inc. (Madison, WI).

Construction of pEC20A and pEC20B. The artificial gene encoding for EC20 was amplified from plasmid pVT20[5] by PCR with M13/pUC forward primer (GIBCO BRL, Grand Island, NY) as well as the primer *ec-c*, GCTGGATCCTATGGAATGTG. The amplified *ec20* fragments were digested with *Bam*HI and *Hin*dIII, gel-purified, and subcloned into pET-34b(+) or pET-37b(+) to generate pEC20A and pEC20B, respectively. DNA manipulations and PCR-amplified procedures were performed according to standard procedures.[15]

Protein Expression and Fractionation. Bacterial cells were cultivated in Luria-Bertani (LB) medium or MJS medium supplemented with 30 μg/mL of kanamycin and 1 mM $ZnCl_2$. Cells were grown to an OD(600 nm) of 0.6 at 30 °C when IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM. The cultivation was continued for an additional 2−3 h at 30 °C. Cells were harvested by centrifugation at 4960*g* and resuspended in 50 mM phosphate buffer, pH 6.5, supplemented with 0.1 mM $ZnCl_2$. After two passages through a French pressure cell (15000−20000 psi), crude cell extract was centrifuged for 10 min at 19800*g*. The resulting supernatant was recovered as soluble fraction and used for cellulose binding and cadmium removal studies, and the pellet was taken as an insoluble fraction. Both fractions were subjected to SDS-PAGE analysis. Protein quantification was performed by the BioRad (Bradford) protein assay kit.

Radiolabeling of the target proteins were performed as follows. Cells were cultivated in MJS medium. Radiolabeled cysteine (1075 Ci/mmol, ICN) was added at the time of induction to a final concentration of 5 μCi/ml. Total proteins extracted from cell cultures were analyzed with SDS-PAGE. The gel was dried and exposed to an X-ray film.

Batch Removal of Cadmium by Immobilized CBD_{clos}-EC20. Soluble fraction from crude extract was supplemented with sodium chloride and Triton-X100 to a final concentration of 50 mM and 0.1% (v/v), respectively, and was then exposed to Avicel A1 (Sigma) prewashed with 50 mM phosphate buffer (pH 6.5). After 1 h of gentle agitation at room temperature, Avicel−CBD_{clos}-EC20 complexes were recovered by centrifugation at 3300*g* for 10 min. The slurry was washed three times with the same phosphate buffer and incubated with 0.9 ppm of $CdCl_2$ for 1 h at room temperature. After extensive washing with phosphate buffer, Cd^{2+} was stripped off from Avicel−CBD_{clos}-EC20 complexes by incubating with 25 mM EDTA for 1 h, at room temperature. Retrieved Avicel−CBD_{clos}-EC20 complexes were collected by centrifugation and were ready to be reused after washing. Cd^{2+} was detected with an atomic absorption spectrophotometer (AA-6701, Shimazdu Co., Columbia, MD). The amount of immobilized CBD-EC20 was determined as described previously[16] and by the thiol analysis.[17]

Continuous Removal of Cadmium by Immobilized CBD_{clos}-EC20 Columns. CF11 cellulose (Whatman Inc., Clifton, NJ) was washed twice with 50 mM phosphate buffer (pH6.5), resuspended, and packed into a 0.4 × 8 cm Poly-Prep column (Bio-Rad Lab., Hercules, CA). Cell extracts were loaded onto the above-prepared cellulose column with 50 mM NaCl and 0.1% (v/v) Triton X-100. Cd^{2+} solutions of 0.9 ppm were passed through the CBDclos-EC20 immobilized columns at a rate of 0.2 mL/min. Effluents were collected as fractions of 1 mL, and the remaining Cd^{2+} of each fraction was determined with an atomic adsorption spectrophotometer. Columns saturated with Cd^{2+} were regenerated by a three-column volume of 25 mM EDTA. Repeated removal of Cd^{2+} with an immobilized column was tested for 7 days at room temperature.

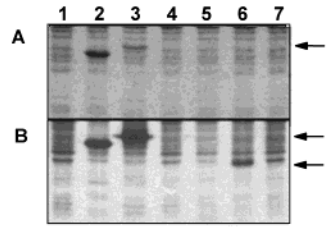

Figure 1. SDS-PAGE analysis (A) and matching autoradiogram of radiolabled proteins (B). Cells were cultivated in MJS minimal medium at 30 °C. IPTG was added to a final concentration of 1 mM when the cultures reached a turbidity (OD_{600}) of 0.6. Cells were allowed to grow for an additional 3 h after induction. Radiolabeled cysteine (^{35}S, 1075 Ci/nmol, ICN) was also added at the time of induction to a final concentration of 5 μCi/mL. Lane 1, BL21(DE3); lane 2, BL21(DE3)/pET-34b(+); lane 3, BL21(DE3)/pEC20A (induced); lane 4, BL21-(DE3)/pEC20A (uninduced); lane 5, BL21(DE3)/pET-37b(+); lane 6, BL21(DE3)/pEC20B (induced); lane 7, BL21(DE3)/pEC20B, uninduced.

Results and Discussion

Production and Immobilization of CBD-EC20 Fusion Protein. The potential of using immobilized CBD-EC20 fusion proteins for heavy metal removal was examined. Initially, two different CBDs from *Clostridium cellulovorans* (CBD_{clos})[18] or *Cellulomonas fimi* (CBD_{cenA})[19] were selected for their ability to generate N-terminal fusion with EC20. These two CBDs, however, have very different affinity to cellulose; CBD_{clos} belongs to the family III CBD and provides essentially irreversibly binding, while CBD_{cenA} can be eluted easily with water or buffer.[20]

Cultures carrying different expression vectors were grown at 30 °C and induced with 0.4 mM IPTG. Maximum production of the fusion proteins was achieved 3 h after induction. Production of the fusion proteins was demonstrated by SDS-PAGE and only the CBD_{clos}-EC20 fusions were produced to an appreciable level (~9% of the total protein). An intense band with an apparent MW of 28 kDa was detected (Figure 1A). The presence of EC20 in the fusion proteins was confirmed by labeling with ^{35}S cysteine (Figure 1B). With this sensitive detection, a band corresponding to the CDB_{cenA}-EC20 fusion proteins was also observed, indicating that they were produced at a very low level. For the CBD_{clos}-EC20 fusions, over 90% of the total fusion protein was recovered in the soluble fraction (Figure 2). This is in contrast with some previous reports showing the

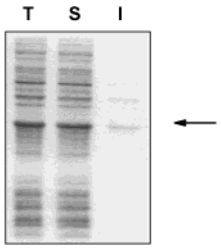

Figure 2. Fractionation of CBD_{clos}-EC20 proteins from *E. coli* strain BL21(DE3)/pEC20A. Cells were cultivated in LB medium at 30 °C. The culture was induced at an OD_{600} of 0.6 by adding IPTG to a final concentration of 0.4 mM, and was grown for an additional 2.5 h after induction. T, total proteins; S, soluble proteins; and I, insoluble proteins.

formation of insoluble inclusion bodies with CBD_{clos} fusions.[14,21] In addition, the CBD_{clos}-EC20 fusion protein did not appear to undergo significant proteolysis as no degraded product was detected.

Purification and immobilization of the CBD fusions were achieved by incubating cell extracts with different amounts of Avicel A1 (Sigma) in the presence of 0.1% Triton-X100. After extensive washing, the bound and unbound fractions were subjected to SDS-PAGE analysis. The percentage of CBD_{clos}-EC20 bound increased with increasing amount of Avicel present. With 240 mg of Acivel added, over 95% of the soluble CBD_{clos}-EC20 fusions was removed from the supernatant and was found to bind to Acivel. Remarkably, nearly all CBD_{clos}-EC20 fusions produced were both soluble and functional. Approximately 22 mg/L of functional CBD_{clos}-EC20 fusion were produced under these conditions.

Cd^{2+} Binding to Immobilized CBD_{clos}-EC20 Fusion. To illustrate the metal-binding functionality of the immobilized CBD_{clos}-EC20, avicel-bound CBD_{clos}-EC20 was prepared as described above. To prevent oxidization of the thiol groups, production and immobilization of CBD_{clos}-EC20 were preformed in the presence of $ZnCl_2$. Because of the large difference in affinity, bound Zn^{2+} ions from EC20 can be rapidly displaced by cadmium. For the binding experiments, cadmium chloride was added to a final concentration of 20 μM and the amount of bound Cd^{2+} was measured after 1 h. Consistent with our reported binding stoichiometry for EC20,[5] a ratio of ~10 Cd^{2+} per immobilized CBD_{clos}-EC20 was observed (Figure 3). In contrast, essentially no Cd^{2+} removal was observed with the Avicel-bound CBD_{clos}, indicating that the functionality of the EC20 alone was responsible for Cd^{2+} removal. The bound Cd^{2+} could be removed by the addition of EDTA; over 99% removal was achieved by incubating with 25 mM EDTA within 30 min. Regenerated sorbents again retained the same metal-binding capability with a similar metal-binding ratio of ~10 Cd^{2+} per immobilized CBD_{clos}-EC20 for two additional cycles.

Continuous Removal of Cd^{2+} by Immobilized CBD_{clos}-EC20 Column. Immobilization of CBD_{clos}-EC20 onto CF11 cellulose was used to investigate the possibility of removal of very low levels of Cd^{2+} in repeated operations. A 0.8 cm diameter column was prepared by loading 560 μg of CBD_{clos}-EC20 onto 0.32 mg of CF11 cellulose. One milliliter fractions of 0.9 ppm solution of $CdCl_2$ were then added to the column, and the solution eluted out of the column was collected and analyzed for Cd^{2+}. Figure 4 depicts the breakthrough curve

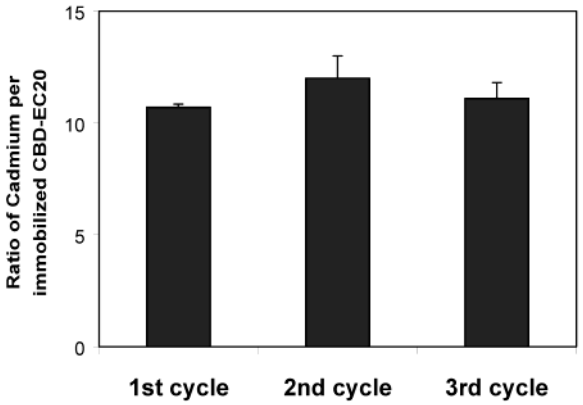

Figure 3. Cadmium removal with Avicel-immobilized CBD_{clos}-EC20 fusions. Avicel−EC20 complexes were incubated with 20 μM $CdCl_2$ solution for 1 h and were washed repeatedly followed by incubation with 25 mM EDTA solution. Three cycles of $CdCl_2$ loading and EDTA stripping were performed. Data are given as mean ± standard deviation for four experiments.

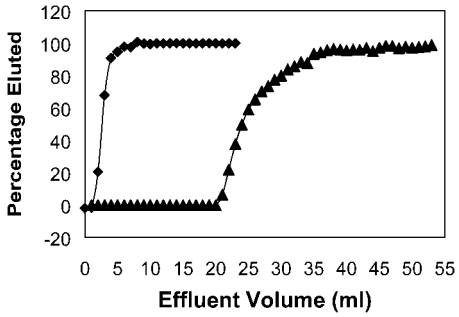

Figure 4. Cadmium removal with columns packed with CF11 cellulose. Proteins in the amount of 560 μg (▲) for CBD_{clos}-EC20 and 734 μg for CBD_{clos} (◆) were loaded onto 0.32 mg of CF11 cellulose. Cd^{2+} solutions of 0.9 ppm were passed through the CBD_{clos}-EC20 immobilized columns at a rate of 0.2 mL/min. Effluents were collected as fractions of 1 mL, and the remaining Cd^{2+} of each fraction was determined with an atomic adsorption spectrophotometer.

of the column. One hundred percent removal of Cd^{2+} was retained for the first 21 fractions, followed by a gradual increase in the effluent Cd^{2+} concentration. Complete breakthrough was not detected until the 43rd fraction. The total amount of Cd^{2+} removed was determined to be 25.6 μg by integrating the breakthrough curve. This corresponds to a ratio of ~11 Cd^{2+} per EC20, a value consistent with the expected stoichiometry. In contrast, breakthrough occurred after only one fraction for a similar column with only CBD_{clos} immobilized (Figure 4). These results clearly indicated the high affinity of EC20 for Cd^{2+} even at parts per million levels. This is precisely what is needed for this strategy to be useful as an efficient polishing process for heavy metal removal. Again, regeneration of the immobilized sorbents was accomplished by adding 25 mM EDTA. All bound Cd^{2+} was removed after the third washing steps.

Regenerated sorbents were then subjected to rebinding of Cd^{2+} as described above. After an initial drop in the efficiency of about 35%, the column maintained around 50% of the original capacity even after 7 days (Figure 5). This drop in efficiency cannot be attributed to the leakage of CBD_{clos}-EC20 from the CF11 cellulose since the amount of immobilized proteins was constant throughout the experiment, demonstrating the effectiveness of CBD_{clos} as an

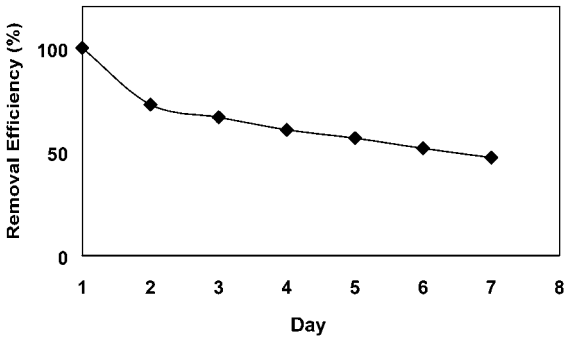

Figure 5. Metal-binding efficiency of the immobilized CBD_{clos}-EC20 column during 7-day repeated cycles. Values are shown as percentages of the value at day one.

immobilization tag (data not shown). It is likely related to a loss of binding capacity of EC20 due to partial oxidation.

Conclusion

In this paper, we have demonstrated the feasibility of a protein-based technology for heavy metal removal. Bifunctional proteins composed of CBD_{clos} and EC20 were generated, enabling single-step purification and immobilization onto different inexpensive cellulose materials. We were able to rectify the inherent insoluble nature of CBD_{clos} by optimizing the culture conditions and produced CBD_{clos}-EC20 fusion proteins exclusively in the soluble forms. The soluble proteins retained both cellulose-binding and metal-binding functionality. An immobilized CBD_{clos}-EC20 column was used to achieve repeated removal of Cd^{2+}. Regenerated sorbents could be reloaded with Cd^{2+} and reused for repeated cycles. These fusion proteins should prove to be invaluable tools for the development of low-cost, cellulose-based materials as a polishing treatment of heavy metal wastes.

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