ARTICLE

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Cadmium Removal From Contaminated Soil by Thermally Responsive Elastin (ELPEC20) Biopolymers

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ABSTRACT: Cadmium contamination of soil is a major concern in the biosphere. Beyond the suite of available physico-chemical treatment methods, green and more efficient technologies are desired to reduce cadmium and other heavy metal contaminants to acceptable levels. Elastin-like polypeptides (ELP) composed of a polyhistidine domain (ELPH12) can be used as an environmentally benign chelating agent for ex situ soil washing. However, ELPH12 is relatively non-selective. A biopolymer with metal-binding domains that have stronger affinity, capacity, and selectivity would have distinct advantages. The aim of this work is to investigate the use of a new generation of ELP biopolymer, ELPEC20, containing synthetic phytochelatin (EC) as the metal-binding domain for ex situ soil washing. ELPEC20 was shown to bind cadmium more effectively and selectively than ELPH12. The binding constant of ELPEC20 is an order of magnitude higher and the binding capacity is fivefold higher than ELPH12. In contrast to ELPH12, no decrease in cadmium binding was observed in the presence of other competing metal ions. The improved selectivity and binding capacity provided by ELPEC20 were directly reflected in the enhanced cadmium extraction efficiency from contaminated soil. In batch washing studies up to 62% of the bound cadmium was removed by ELPEC20 while less than 12% was removed by ELPH12. Cadmium was removed not only from the exchangeable fraction but also the oxidizable fraction. The high-affinity binding sites of ELPEC20 also results in very rapid extraction with complete removal achieved within 1 h, suggesting that ELPEC20 could be used as part of a rapid (short retention time) technology with minimum possibility for the biodegradation of biopolymers.

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KEYWORDS: soil washing; ex situ; chelators; EDTA; cadmium

Introduction

Heavy metals are of great concern to environmental safety because of their toxicity and intrinsically non-degradable nature. They tend to accumulate in soil through adsorption and pose a serious health threat to humans and animals. The cleanup of many contaminated sites remains a challenging task but an essential one for site restoration. Different decontamination methods including physical, chemical, thermal, and/or biological processes have been reported for soil treatment. Particularly, soil washing is a technology that extracts heavy metals into a wash solution either by desorption or solubilization (Semer and Reddy, 1996). Several classes of chemical reagents have been investigated including acids, bases, chelating agents, oxidizing agents, and surfactants (Abumaizar and Smith, 1996). However, the use of chemical reagents alters not only the soil characteristics in an adverse manner (Reed et al., 1996) but is also non-biodegradable (e.g. EDTA) and may result in additional pollution.

Biological extractants have been given greater attention recently because they are considered to be environmentally benign and economically acceptable (Herman et al., 1995; Mulligan et al., 1999). Among promising candidates, one emerging technology is the use of tunable, metal-binding biopolymers based on ELPs (Kostal et al., 2001, 2003; Prabhukumar et al., 2004). ELPs are biopolymers derived from natural biological building blocks consisting of a repeating pentapeptide Val-Pro-Gly-Val-Gly. The most interesting feature of ELPs is their ability to undergo a reversible phase transition based on hydrophobic interaction (Urry, 1997) under a range of temperature, pH and ionic strength. The phase transition temperature (T_t) can be precisely controlled by varying the chain length and peptide

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sequence. We have successfully generated ELP biopolymers containing a polyhistidine tail (ELPH12) as the metal chelating domain, and demonstrated the feasibility of easy extraction of cadmium from contaminated soil in ex situ soil washing (Prabhukumar et al., 2004). Although the result was quite encouraging, the overall level of extraction was still lower than the potentially extractable fraction. Moreover, the non-selective nature of the polyhistidine domain resulted in significant co-removal of zinc, rendering it difficult to recover the extracted cadmium by simple precipitation. It should be noted that the flexibility of tailoring the desired metal-binding domain in the ELP biopolymer is a unique property that can be easily exploited for improved affinity and specificity for the target metals (Kostal et al., 2001, 2003). Naturally occurring metal-binding peptides, such as metallothioneins (Valls et al., 2000) are the main metal sequestering molecules used by plants or animals to immobilize metal ions, offering selective, high-affinity binding sites for cadmium due to the repetitive cysteine residues. Recently, a new class of metal-binding peptides known as synthetic phytochelatins (ECs) with the repetitive metal-binding motif $(Glu\text{-}Cys)_n$ Gly were shown to have improved Cd^{2+} binding capability over that of metallothioneins (Bae et al., 2000, 2001). Incorporation of ECs into the ELP biopolymer is expected to significantly enhance ELP's ability to extract cadmium from contaminated soil. The aim of this paper is to present the use of a new generation of ELP biopolymer, which takes advantage of the improved affinity and selectivity of synthetic phytochelatin (EC20), and to demonstrate the utility of ELPEC20 for enhanced extraction of cadmium in soil washing experiments.

Materials and Methods

General Methods

All procedures for DNA manipulation were performed according to standard procedures (Sambrook and Russell, 2001). Protein electrophoresis was performed using 10% (w/v) SDS polyacrylamide gels (Laemmli, 1970) and proteins were detected by silver staining (BioRad, Hercules, CA).

Plasmid Construction

The basic design of the biopolymer is based on 21 repeating units of $(VPGVG)_2VPGKG(VPGVG)_2$. For every five elastin repeats, a valine residue at the fourth position was substituted with a positively charged lysine residue. Synthetic gene coding for $(VPGVG)_2VPGKG(VPGVG)_2$ was used as the basic building block. Polymerization of the synthetic gene was carried out through the compatible cohesive ends generated by the restriction endonuclease *Pfl*MI, followed by subsequent ligation to form the multimeric genes coding for $[(VPGVG)_2VPGKG(VPGVG)_2]_{21}$ as described by Stiborava et al. (2003).

Plasmid pMC20 containing the synthetic gene coding for EC20 was described by Bae et al. (2000). To construct an expression vector for ELP105KEC20, plasmid p08EC20 and p08-ELP105K-EC20 were constructed to facilitate cloning of the fusion protein. A fragment containing EC20 from pMC20 was obtained by digestion with *Nco*I and *Sap*I, and ligated with plasmid pJAN08 digested with *Age*I and *Sap*I to generate p08EC20. Plasmid p08EC20 was digested with *Nde*I and *Xma*I and a 4,070 bp fragment was ligated with a 1,596 bp fragment obtained by digesting pET-ELP105K (Stiborava et al., 2003) with the same enzymes. The resulting plasmid p08-ELP105K-EC20 was digested with *Nde*I and *Hin*dIII, and the gene coding for ELP105KEC20 was inserted into the expression vector pET38b+ (Novagen, Madison, WI), generating the expression vector pET-ELP105K-EC20.

Production and Purification of ELPEC20 and ELPH12

For protein expression, plasmids pET-Ela78H12 and pET-Ela105K-EC20 were introduced into *E. coli* BLR(DE3) (Novagen). All cultivations were carried out in terrific broth (Becton Dickinson, Sparks, MD) media supplemented with either 100 μg/mL ampicillin (pET-Ela78H12) or 30 μg/mL kanamycin (pET-Ela105KEC20) at 300 rpm for 24 h at 30°C. Cells were then harvested by centrifugation, washed in 0.9% NaCl, and resuspended in Tris–Cl (pH 8). After cell lysis with a French press and removal of cell debris by centrifugation for 30 min at 30,000*g*, the biopolymers were purified from the cell extract by three cycles of inverse temperature transition (McPherson et al., 1996). For each cycle, the sample was heated to 30°C and centrifuged at 30,000*g* at 30°C, and the pellet containing the biopolymer was dissolved in ice-cold 50 mM Tris–Cl buffer pH 8. NaCl was added to a final concentration of 2 M in the case of ELP105KEC20 and 1 M for ELP78H12, respectively. Purity of the purified protein was verified by SDS-PAGE electrophoresis followed by silver staining. Purified biopolymers were stored in 50 mM Tris buffer pH 7.4 containing 5 mM dithiothreitol (DTT).

Characterization of Phase Transition Behaviors

The biopolymer concentration was determined by measuring the absorbance at 215 nm using a Beckman DU-60 spectrophotometer. An extinction coefficient of 61.972 μg/mL/A215 was used based on the method described previously (Kostal et al., 2001). The transition temperature of the biopolymers was determined by measuring the solution absorbance at 650 nm between 10 and 70°C. The temperature of transition (T_t) was determined as the temperature at which the optical density reached half of its maximum.

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Metal-Binding Characteristics of ELPEC20

Metal-binding experiments to determine the binding capacity of ELPEC20 or ELPH12 were performed in 250 μL of 50 mM Tris buffer, pH 7.4. Ten nanomoles of biopolymer were mixed with varying amounts of cadmium and incubated 1 h at room temperature. The metal-biopolymer complex was precipitated by the addition of 1.5 M NaCl at 37°C and centrifuge for 5 min at 14,000*g*. The resulting pellets were redissolved overnight in 100 μL concentrated HNO_3. Prior to measurements, each sample was diluted by adding water to the appropriate dilutions. The amount of bound Cd^{2+} was analyzed by atomic flame absorption (AA) spectrometry (Shimadzu AA6701).

From the binding experiments above, the cadmium binding constants of the two biopolymers were determined using the method of Ruzic (1982) by plotting the free Cd^{2+} concentration versus the molar ratio of free Cd^{2+} to bound Cd^{2+}. The assumed stoichiometry for the complexation between the biopolymer and Cd^{2+} was for a single ligand. The stability constant was obtained by dividing the *y*-intercept of the resultant straight-line plot by its slope.

Soil Characterization

Coarse and sandy subsurface soil collected from City of San Bernardino Rapid Infiltration and Extraction (RIX) wastewater treatment facility was used in this study. Grain size distribution was determined by ASTM D422 method (D422-54, 1970). Soil was found to be mostly sand (91%) with low amounts of silt (5%) and clay (2%). Other characteristic were as follows: pH 6.8, 2.4% total organic matter (TOM), and a cation exchange capacity (CEC) of 10.1 mequiv. 100 g^{-1}. Soil pH was determined by adding distilled water to soil in a 1:10 (w/v) ratio and the pH of soil/water mixture was determined using a Fisher Scientific Accumet Model 10 pH meter. The total organic matter content was determined by digesting the soil with hydrogen peroxide. Aliquots of 10 mL hydrogen peroxide (30%) were added to 10 g of soil at room temperature until bubbling was no longer observed. Soil was allowed to dry at 80°C in an oven until no mass changes were observed. The difference in mass was related to the soil organic content.

Soil samples were contaminated by soaking in 1 mM cadmium nitrate for 4 weeks at neutral pH. Samples were then rinsed with distilled water twice and recovered by centrifugation for 15 min at 13,000*g*. The recovered soil was dried in an oven at 80°C until the weight change was less than 5%. Dried cadmium-contaminated soil was stored in a closed plastic container at room temperature until used. The final cadmium content in the soil sample was determined by the USEPA Method 3050 and analyzed by atomic flame absorption (AA) spectrometry (Shimadzu AA67010). A three-step sequential extraction method was carried out to evaluate the different fractions of bound cadmium in the soil (Carapeto and Purchase, 2000). For the exchangeable fraction, soils were extracted for 1 h with 1 M $MgCl_2$ (pH 7) at room temperature. A ratio of 1 g of soil to 10 mL of $MgCl_2$ was used. For the fraction bound to organic matter, soils were extracted with 0.05 M EDTA for 2 h at room temperature. The same v/w ratio was maintained. Finally, for the residual fraction, soils were digested with a mixture of HNO_3:HCl (2:l, v/v). Samples were pre-digested overnight at room temperature, after which the temperature was raised to 125°C over a period of 3 h. The samples were gently boiled and refluxed for 4 h to ensure full leaching of metals.

Soil-Biopolymer Adsorption Characteristics

The adsorption characteristics of ELP biopolymers were determined with an uncontaminated soil. Experiments were carried out with 2 g of soil using a soil–solution (w/v) ratio of 1:10 with ELP biopolymer concentrations ranging from 0.65 to 5 mg/mL. The soil and ELP biopolymer solution were mixed in a mechanical tumbler at 30 rpm for 24 h. The soil was then recovered by centrifugation at 13,000*g*. The ELP biopolymer concentrations in the supernatant after adsorption were determined by measuring the absorbance at 215 nm using a Beckman DU-60 spectrophotometer. Minerals and other constituents release from the soil had no significant interference at this wavelength. The sorption isotherm was described using a Freundlich isotherm:

$$\log\left(\frac{x}{m}\right) = \log K_f + \frac{1}{n}\log C_{eq}$$

where x is the amount of ELP biopolymer adsorbed (mg), m is the mass of soil (g), K_f is the Freundlich adsorption coefficient, n is a measure of nonlinearity, and C_{eq} is the concentration of biopolymer (mg/mL) in solution after adsorption is completed. All experiments were performed in triplicate and the mean values are presented.

Biopolymer–Cadmium Extraction Studies

All batch washing experiments were performed with 1 g of soil using a soil–solution (w/v) ratio of 1:10. Prior to the soil washing experiments, DTT was removed from the ELPEC20 stock solution by heat precipitation and the pellets were redissolved in 50 mM Tris-buffer, pH 7.4. Various concentrations of ELPEC20 biopolymer were added to the contaminated soil and mixed in a mechanical tumbler at 30 rpm for 24 h. Soil samples were recovered by centrifugation at 13,000*g* for 15 min and dried in an oven at 80°C overnight before cadmium analysis. The percentage of cadmium removed was based on the initial metal content of 296.9 mg/kg. All experiments were performed in triplicate and the mean values from the three separate experiments are presented. Comparative experiments were carried out using ELPH12. The three-step sequential extraction method was carried out to evaluate the residual cadmium in the different soil fractions (Carapeto and Purchase, 2000).

Results and Discussion

Production and Characterization of the ELPEC20 Biopolymers

Although ELP-based biopolymers containing a polyhistidine tag have been demonstrated successfully for ex situ soil washing, the use of polyhistidine tag does not offer the desired affinity and selectivity. To overcome these problems, a synthetic phytochelatin domain containing 20 glutamate-cysteine repeats (EC20) with significantly improved affinity and selectivity toward cadmium was exploited as the metal-binding moiety (Bae et al., 2000). To generate a biopolymer programmed with a phase transition response under a wider range of environmental stimuli, a charged lysine residue was incorporated into every five elastin repeats, enabling phase transition to be triggered by switching pH due to the proton-transfer equilibrium of the lysine residues (Urry, 1997).

Production and purification of the ELPEC20 biopolymers was achieved as described previously (Kostal et al., 2001; Stiborava et al., 2003). By taking advantage of the temperature responsive property of the ELP domain, biopolymers were easily purified by inverse temperature cycling to homogeneity as judged by the presence of a single band around 48 kDa on SDS-PAGE (Fig. 1); up to 500 mg/L of purified biopolymers was obtained. The molecular weights of the purified biopolymers were independently verified by MALDI-TOF mass spectrometry (data not shown).

To demonstrate the flexibility to precipitate the ELPEC20 biopolymers over a range of conditions, the transition temperature (T_t) was characterized as a function of biopolymer concentration, salt concentration and pH. As reported previously with other ELP biopolymers containing lysine residues (Stiborava et al., 2003), the T_t value was a strong inverse logarithmic function of the biopolymer concentration. Either the addition of 1 M NaCl or raising the pH to 10 reduced the transition temperature to below 25°C (data not shown), a condition that can be employed for easy aggregation and recovery of biopolymer–cadmium complexes. In all cases, over 98% recovery of the ELPEC20 biopolymer was obtained.

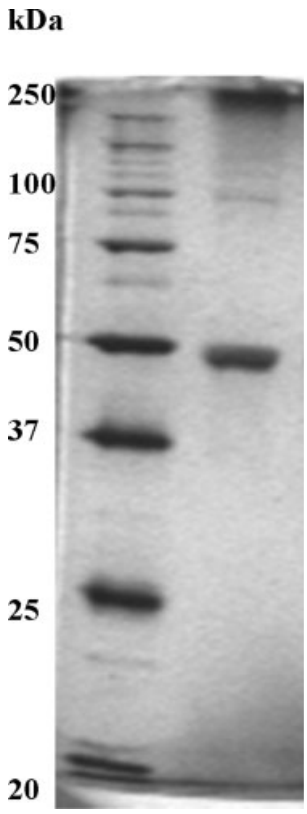

Figure 1. SDS-PAGE analysis of purified ELPEC20 biopolymers. **Lane 1**: Broad range unstained molecular weight standards. **Lane 2**: ELPEC20.

Biopolymer–Metal Complexation

The binding property of ELPEC20 was evaluated by incubating the biopolymers with a range of cadmium concentrations. After 1 h incubation, the biopolymer–cadmium complex was recovered by precipitation and subjected to cadmium analysis. As depicted in Figure 2, cadmium binding increased linearly at lower concentrations with essentially all added cadmium removed under these conditions. A maximum binding ratio of 8.2 Cd^{2+} ions per ELPEC20 was observed, which is substantially higher than the ratio of 2.1 observed for ELPH12. This value is also consistent with the previously reported binding stoichiometry for EC20 (Bae et al., 2000). More importantly, a similar maximum binding ratio was also obtained using

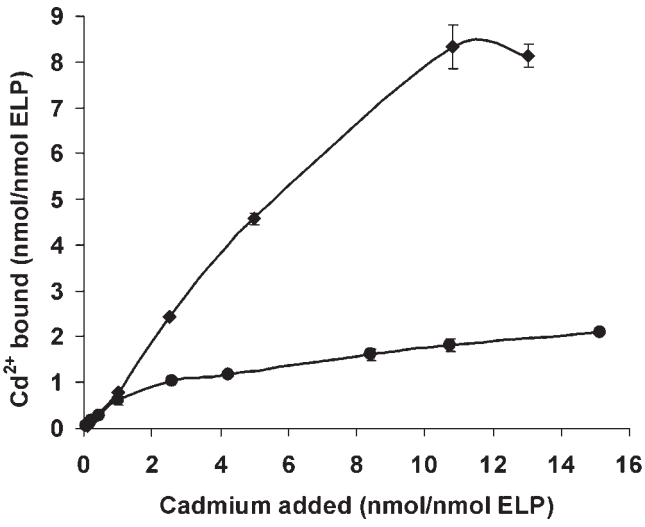

Figure 2. Cadmium binding stoichiometry of (◆) ELPEC20 and (●) ELPH12 in 50 mM Tris–Cl buffer pH 7.4 and 8, respectively. Results represent the average of three different experiments.

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size-exclusion chromatography, indicating that precipitation of the biopolymer–metal ion complex has no effect on cadmium binding (data not shown). The stability constant (K_L) of the biopolymer–cadmium complex was determined using a Ruzic (1982) plot (Fig. 3). The log K_L value of 5.2 for ELPEC20 is one order of magnitude higher than that obtained with ELPH12 (4.0) under the same binding condition. As a result, the use of ELPEC20 offers not only improved binding stoichiometry but also improved affinity over that of ELPH12.

One of the major limitations of the ELPH12 biopolymers is the lack of specificity. Even the presence of a low concentration of zinc was shown to inhibit and displace cadmium from the polyhistidine domain (Prabhukumar et al., 2004). To investigate whether use of the EC20 moiety affords improved selectivity, a competitive cadmium binding experiment was conducted in the presence of equal concentrations (molar) of either Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}or Al^{3+}. In all cases, no decrease in cadmium binding was detected for ELPEC20. In contrast, the presence of Zn^{2+} reduced the Cd^{2+} binding content by 80% for ELPH12, indicating the highly selective nature of ELPEC20 towards cadmium (Fig. 4). This in combination with the improved affinity and binding ratio make this biopolymer an attractive candidate for soil washing applications.

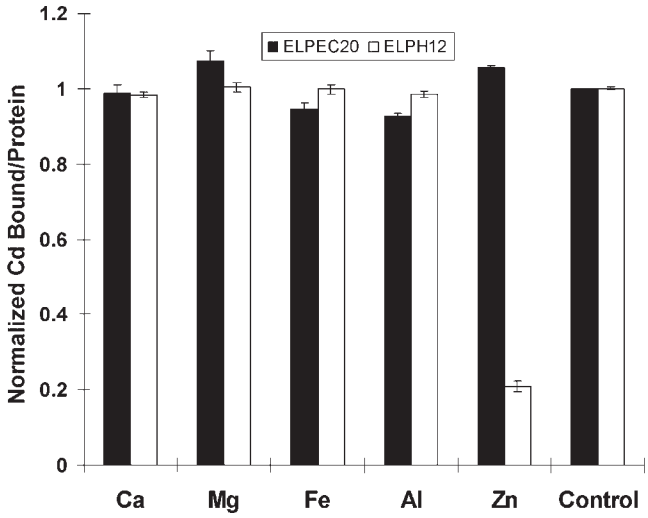

Figure 4. Cadmium binding by ELPH12 and ELPEC20 in the presence of other divalent cationic metals. In the control experiment, 100 μM of Cd^{2+} and biopolymers were added. For the competitive binding experiments, 100 μM of the corresponding metal was also added.

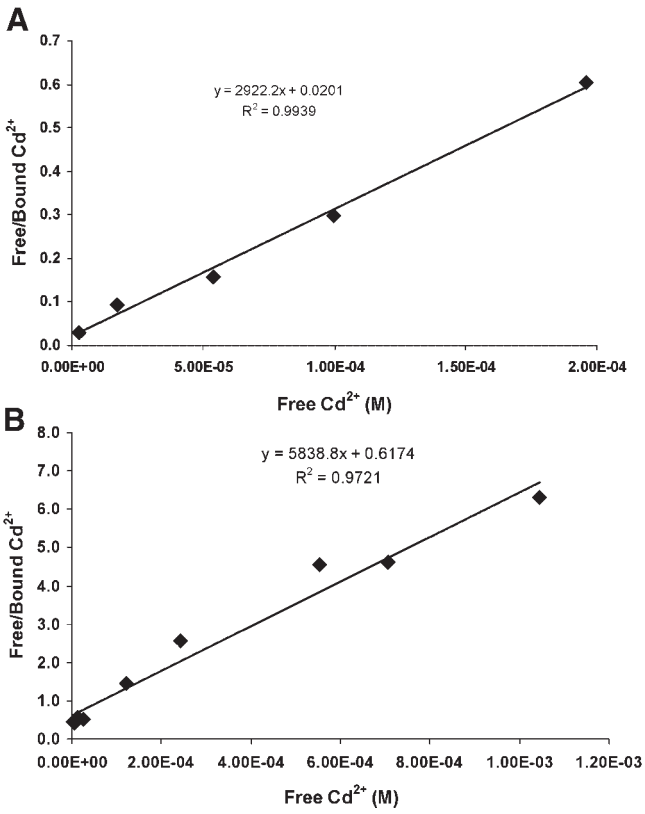

Figure 3. Ruzic plots of free Cd ion concentration/bound Cd ion concentration against free Cd ion concentration for ELPEC20 (**A**) and ELPH12 (**B**). The cadmium binding constant was calculated by dividing the *y*-intercept by the slope for the linear plot.

Soil-Biopolymer Sorption Characteristics

The effectiveness of soil washing is adversely affected by the sorption of biopolymers onto soil particles with lesser availability of the biopolymer for metal complexation at higher adsorption. As shown in Figure 5, the sorption behavior of the ELPEC20 biopolymers can be described by a Freundlich isotherm. A K_f value of 3.4 and $1/n$ value of 0.619 was obtained. The maximum adsorption onto the soil was

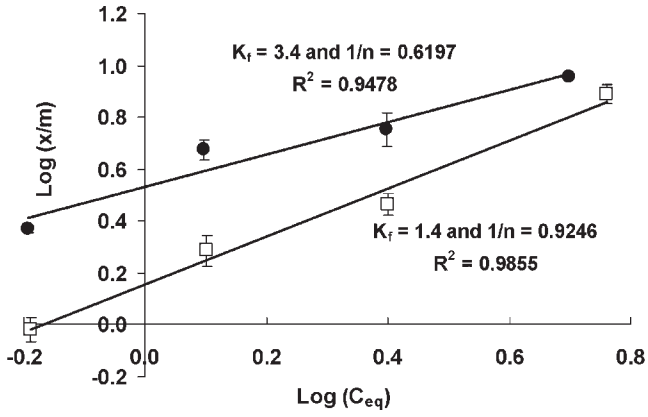

Figure 5. Comparison of Freundlich isotherms for adsorption of ELPEC20 (●) and ELPH12 (□) onto soil. *x* is the amount of ELP biopolymer adsorbed (mg), *m* is the mass of soil (g), and C_{eq} is the concentration of biopolymer (mg/mL) remaining in solution after adsorption.

around 40% at the lowest biopolymer concentration. However, at the working concentration range of 50 μM or higher, only 20% adsorption was observed. The extent of adsorption of the ELPEC20 biopolymer is relatively low compared to biosurfactants such as rhamnolipids, indicating that ELPEC20 could be used more effectively than these alternative bioagents for soil washing applications.

Batch Soil Washing Studies

To test the efficiency of the ELPEC20 biopolymer in soil washing experiments, artificially contaminated sandy soil was used. The final cadmium content was determined to be 297 ± 18 mg/kg with $48.6 \pm 7.6\%$, $29.1 \pm 4.5\%$, and $11.2 \pm 0.80\%$ of the bound cadmium in the exchangeable, oxidizable (bound to organic matter), and residual fraction, respectively. The cadmium content for the contaminated soil used in this study was three times higher than the soil used in our previously study using ELPH12. In addition, it has a higher total organic matter content, which led to the lower level of easily exchangeable fraction.

Soil washing using either distilled water or buffer as controls removed only 2% of bound cadmium (Fig. 6). The use of 50 μM ELPEC20 increased the extraction efficiency substantially with 25% of the bound cadmium removed (Fig. 6). This is double the amount extracted using the same concentration of ELPH12. Increasing the ELPEC20 concentration to 75 μM further improved the extraction efficiency to 40%. In contrast, virtually no increase in efficiency was observed with ELPH12. When the cadmium that was extracted with the ELPH12 biopolymers was analyzed, only 20% of the extracted cadmium was actually bound to the ELPH12 biopolymers; the remaining cadmium was found in solution. Comparatively, more than 80% of extracted cadmium was complexed with the ELPEC20 biopolymer, a result consistent with the selective nature of the EC20 domain towards cadmium.

To explore whether the cadmium removal efficiency could be further improved at higher ELPEC20 concentrations, batch soil washing experiments were repeated using 150, 200, and 250 μM biopolymer. As shown in Figure 6, cadmium removal increased with increasing ELPEC20 concentration; up to 62% removal was achieved at a biopolymer concentration of 250 μM. This corresponds to 100% removal of the ion-exchangeable fraction and 45% of the more tightly bound, less mobile/labile fraction bound to organic matter. These results are comparable to those reported with the use of either EDTA or EDDS for the extraction of soil-bound Cu^{2+} or Zn^{2+}, which were shown to remove mostly exchangeable fraction and some of the organic-bound fraction (Tandy et al., 2004). However, the major advantage of the ELPEC20 biopolymers is the use of a significantly lower concentration (250 μM) of biopolymer as compared to 4 mM of EDTA or EDSS used in the other study, enabling the treatment of more contaminated soil with the same amount of ELPEC20 biopolymer.

The kinetics of the soil washing was investigated to determine whether 24 h incubation is necessary for maximum cadmium extraction. As can be seen in Figure 7, cadmium extraction by ELPEC20 showed a very rapid initial increase with over 90% of the maximum extraction occurring within 1 h. In comparison, less than 70% extraction was achieved with ELPH12 during the same duration. Complete extraction by ELPEC20 was observed within 4 h of contact, demonstrating that this could be applied in a rapid technology (short detention time) with very minimum possibility for the degradation of biopolymers. The improved affinity and selectivity of ELPEC20 resulted in cadmium removal not only from the exchangeable fraction but also the organic-bound fraction within 1 h.

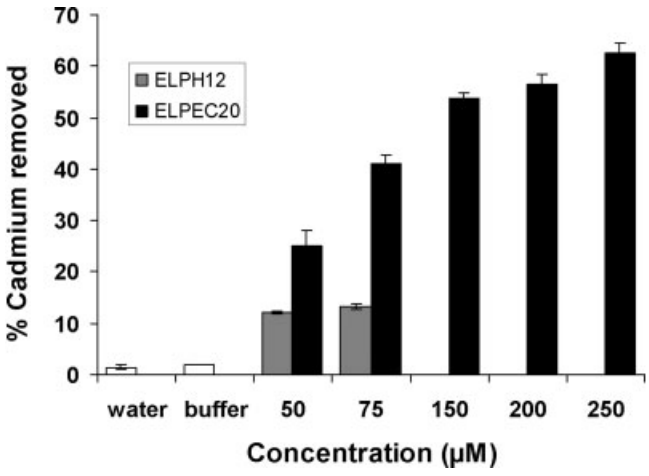

Figure 6. The efficiency of batch soil washing by ELPEC20 and ELPH12. Experiments were carried out with 1 g of soil at a constant 1:10 (w/v) soil to solution ratio. The percentage of cadmium removed was calculated based on an initial cadmium content of 296.9 mg/kg.

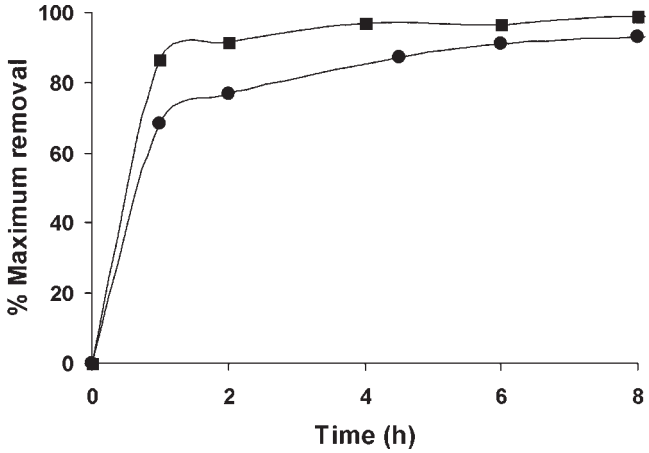

Figure 7. The kinetics of cadmium extraction by ELPEC20 (■) and ELPH12 (●).

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In conclusion, this work successfully demonstrated the feasibility of tailoring ELP biopolymers with enhanced cadmium binding affinity and selectivity by employing the EC20 binding moiety. The metal-binding functionality of EC20 was maintained and an average ratio of 8.2 Cd^{2+} per ELPKEC20 was observed. In contrast to the first generation ELPH12 biopolymer, no interference on binding was found in the presence of all other common metal ions. This new generation of biopolymers was successful for ex situ soil washing applications, enabling the removal of cadmium not only from the exchangeable fraction but also from part of oxidizable fraction due to the high affinity and specificity offered. Comparing to other bioagents such as biosurfactants, ELPEC20 is significantly more cost effective because of the ease of recovery by simple centrifugation and the less than 20% soil adsorption, resulting in a much lower concentration required to achieve higher extraction efficiencies.

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References

Abumaizar RJ, Smith EH. 1996. Heavy metal contaminants removal by soil washing. J Hazard Mater 70:71–86.

Bae W, Chen W, Mulchandani A, Mehra RK. 2000. Enhanced bioaccumulation of heavy metals by bacterial cells displaying synthetic phytochelatins. Biotechnol Bioeng 70:518–524.

Bae W, Mehra R, Mulchandai A, Chen W. 2001. Genetic engineering of *Escherichia coli* for enhanced bioaccumulation of mercury. Appl Environ Microbiol 67:5335–5338.

Carapeto C, Purchase D. 2000. Use of sequential extraction procedures for the analysis of cadmium and lead in sediment samples from a constructed wetland. Environ Contam Toxicol 64:51–58.

Herman DC, Artiola JF, Miller RM. 1995. Removal of cadmium, lead, and zinc from soil by a rhamnolipid biosurfactant. Environ Sci Technol 29:2280–2285.

Kostal J, Mulchandani A, Chen W. 2001. Tunable biopolymers for heavy metal removal. Macromolecules 34:2257–2261.

Kostal J, Mulchandani A, Gropp KE, Chen W. 2003. A temperature responsive biopolymer for mercury remediation. Environ Sci Technol 37:4457–4462.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

McPherson DT, Xu J, Urry DW. 1996. Product purification by reversible phase transition following *Escherichia coli* expression of genes encoding up to 251 repeats of the elastomeric pentapeptide GVGVP. Protein Expr Purif 7:51–57.

Mulligan CN, Yong RN, Gibbs BF, James S, Bennett HP. 1999. Metal removal from contaminated soil and sediments by the biosurfactant surfactin. Environ Sci Technol 33:3812–3820.

Prabhukumar G, Matsumoto M, Mulchandani A, Chen W. 2004. Cadmium removal from contaminated soil by tunable biopolymers. Environ Sci Technol 38:3148–3152.

Reed BE, Carriere PC, Moore R. 1996. Flushing of a Pb(II) contaminated soil using HCl, EDTA, and $CaCl_2$. J Environ Eng ASCE 122:48–50.

Ruzic I. 1982. Theoretical aspects of the direct titration of natural waters and its information yield for trace metal speciation. Anal Chim Acta 140:99–113.

Sambrook J, Russell RW. 2001. Molecular cloning: A laboratory manual, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Semer R, Reddy K. 1996. Evaluation of soil washing process to remove mixed contaminants from a sandy loam. J Hazard Mater 45:45–57.

Special procedure for testing soil and rocks for engineering purpose (D422-54). 1970. Annual book of ASTM standards. West Conshohocken, PA: ASTM. p 101–103.

Stiborava H, Kostal J, Mulchandani A, Chen W. 2003. One-step metal-affinity purification of histidine-tagged proteins by temperature-triggered precipitation. Biotechnol Bioeng 82:605–611.

Tandy S, Bossart K, Mueller R, Ritschel J, Hauser L, Schulin R, Nowack B. 2004. Extraction of heavy metals from soils using biodegradation chelating agents. Environ Sci Technol 38:937–944.

Urry DW. 1997. Physical chemistry of biological free energy transduction as demonstrated by elastic protein-based polymers. J Phys Chem B 101:11007–11028.

Valls M, Atrian S, Lorenzo V, Fernandez L. 2000. Engineering a mouse metallothionein on the cell surface of *Ralstonia eutropha* CH34 for immobilization of heavy metals in soil. Nat Biotechnol 18:661–665.