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Functional metal-binding proteins by metal-stimulated bacteria for the development of an innovative metal removal technology

T. Antsuki, D. Sano and T. Omura

Department of Civil Engineering, Graduate School of Engineering, Tohoku University, Aoba-yama 06, Sendai 980-8579, Japan (E-mail: *sanodai@mail.cc.tohoku.ac.jp*)

Abstract Heavy metal pollution has become an environmental problem throughout the world because heavy metals can be accumulated into the food chain and bring about serious problems, not only for ecosystems but also for human health. In this study, functional metal-binding proteins (FMBPs) were isolated from a metal-stimulated activated sludge culture with the aim of applying them to an innovative metal removal technology. Activated sludge bacteria was cultured in growth media including copper ion, and the stimulation of protein production by copper ion led to the 14% increase in a quantity of extracted crude proteins per 1 g of bacterial cell pellet (wet). In order to isolate FMBPs, extracted crude proteins were applied to the immobilized metal affinity column in which each of copper, nickel and zinc was used as a ligand. Several FMBPs were succesfully isolated from copper-stimulated bacteria. One of FMBPs (molecular weight of about 40 kDa) exhibited an ability to adsorb all three metals. The multi metal-binding property of this FMBP could be applied to an innovative metal removal technology. Furthermore, isolated FMBPs that could capture only one kind of heavy metal would also be attractive as a metal adsorbent in recovering a specific metal as a resource from wastewater, including several heavy metals.

Keywords Activated sludge culture; copper; functional metal-binding proteins (FMBPs); immobilized metal affinity chromatography; nickel; zinc

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Introduction

The release of large quantities of heavy metals into the natural environment has resulted in a number of environmental problems (Hutton and Symon, 1986; Nriagu, 1988). Although metal removal technologies, such as chemical precipitation and ion exchange, have been developed, a large energy and troublesome treatment for chemical waste is required to employ these conventional technologies. Meanwhile, metal removal technologies using microorganisms have been extensively exploited because this technology is environmentally friendly. From this viewpoint, attempts have been made to utilize the metal removal ability of microorganisms such as yeast (Norris and Kelly, 1979), algae (Volesky, 1994; Matheickal *et al*., 1999) and bacteria (Chang and Hong, 1994) for the removal of heavy metals from polluted water.

The mechanisms of heavy metal removal by microorganisms could be categorized as either metabolic or non-metabolic uptakes. The former is due to the fact that some heavy metals are essential micro-nutrients for microbial growth, while the latter includes organic bindings to cell wall and extracellular biopolymers, such as precipitation and physical sorption in the case of detoxification (Fukushi *et al*., 1996). Usually, the non-metabolic heavy metal uptake is faster than the metabolic uptake. The amount of non-metabolic uptake is deeply related to the characteristics and amount of extracellular biopolymers (Fukushi *et al*., 2001). Biopolymers, such as proteins, lipids, polysaccharides, nucleic acids, lipoproteins, glycocalyxes, and others of bacterial cell membrane and cell wall, can adsorb heavy metals and proteins especially play an important role in binding metal ions (Belly and Kydd, 1981; Fukushi *et al*., 1996).

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Attempts for metal removal by bacterial biopolymers could be divided into two approaches. One approach is to express metal-binding biopolymers including metal-binding proteins (MBPs) that are encoded in bacterial genome DNA. For example, it is well-known that activated sludge bacteria can produce metal-binding biopolymers. A marked reduction in the concentration of soluble metals occurs during the activated sludge process (Lawson *et al.*, 1984). Bacteria have developed resistant mechanisms to toxic metals to make them innocuous. Bacteria with unique abilities of metal adsorption, accumulation or resistance can be searched for among naturally occurring bacteria (Malin and Leif, 2001). The synthesis of MBPs such as metallothioneins is one of the bacterial defense systems, because the toxicity of heavy metals to bacteria is reduced by complexing metals with bacterial extracellular biopolymers. Many researchers have reported that quantities of heavy metals removed from the solution were enhanced by these characteristics of bacteria (Rudd *et al.*, 1984; Ghosh and Bupp, 1991, 1992; Bupp and Ghosh, 1991; Fukushi *et al.*, 1996). Fukushi *et al.* (2001) reported that some amount of copper in growth media stimulated the production of novel proteins that had high enough capacity to bind copper ions. However, since these MBPs have not been sufficiently characterized, molecular biological analysis of MBPs, such as amino acid sequence and structure analysis, would be necessary for further development of the metal removal technology.

The other approach is to utilize MBP which was expressed by *E. coli* transformed with a vector carrying MBP genome. The introduction and/or expression of metal-binding peptides have been widely exploited to increase the metal-binding capacity, and they are applied to removing heavy metals from solution (Malin and Leif, 2001). Several metal-binding peptides, which mainly consist of histidine and cysteine residues, e.g. Gly-Cys-Gly-Cys-Pro-Cys-Gly-Cys-Gly (Kotrba *et al.*, 1999), $[\text{Cys-Gly-Cys-Cys-Gly}]_3$ (Pazirandeh *et al.*, 1998), $[\text{Gly-His-His-Pro-His-Gly}]_2$, (Kotrba *et al.*, 1999), His-Ser-Gln-Lys-Val-Phe (Mejare *et al.*, 1998), His-His-His-His-His-His (Sausa *et al.*, 1998), have been expressed on the bacterial outer membrane of *E. coli* cells with the aim of enhancing the cadmium accumulation. However, there would be a reluctance to use these artificial peptides in water and wastewater treatment processes because these peptides may not exist in natural environments, and their effects on human health and ecosystems in the water environment are not clear. Furthermore, heavy metal removal using artificial peptides are still mainly tested on a laboratory scale and it remains to be seen whether they will be an industrially acceptable concept (Malin and Leif, 2001).

The objective of this paper is to isolate functional metal-binding proteins (FMBPs) from activated sludge that have a potential to be used as a metal removal adsorbent. Since FMBPs from activated sludge bacteria are not artificial, and normally exist in the wastewater treatment process, the applicability of FMBPs to the metal removal technology would be attractive if FMBPs could be isolated, characterized, and cloned. In this study, an immobilized metal affinity column was used to isolate FMBPs from activated sludge bacteria. Then, molecular weights of isolated FMBPs were estimated and their applicability to the metal removal technology was discussed.

Materials and methods

Microbial culture source

A mixed microbial culture was taken from the return sludge of a municipal wastewater treatment plant located in Sendai, Japan. This wastewater treatment plant employs the conventional activated sludge process. The collected culture was harvested on a nutrient broth non-selective media (Eiken, Tokyo, Japan) in the presence of 0.5 mM of copper chloride for 24 hours at 20 ± 5°C in a constant temperature chamber. The control culture was also harvested without copper in the medium. In order to obtain a sufficient dissolved

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oxygen concentration in the medium, the air at a rate of about 80 ml/min was introduced into the media through a diffuser.

Protein extraction from bacteria

The copper-stimulated activated sludge culture was obtained as a pellet by the centrifugation ($3,000 \times g$, 15 minutes). The collected pellet was washed twice with 20 mM of sodium phosphate buffer (pH = 7.2). The cell suspension was processed with a 50 W ultrasonic cell disrupter (UP-5s, Taitec, Tokyo, Japan) for 2 minutes to expel inner materials of cells to the liquid phase. The cell mixture was centrifuged ($20,000 \times g$, 90 minutes, 4°C), and the supernatant was discarded. The cell pellet was resuspended in 10 ml of 20 mM sodium phosphate (pH = 7.2) and 10 ml of *n*-butanol was added and mixed with vortex. In order to enhance the protein extraction efficiency, 50 W ultrasonic was applied for one minute. The slurry was centrifuged at $20,000 \times g$ for 10 minutes at 4°C. After the centrifugation, the slurry was separated to three phases: the solvent phase, the cell solid phase, and the water phase. The water phase was sampled, and the concentration of extracted crude proteins in the water phase was measured with a protein assay kit (Bio-Rad Laboratories, CA, USA). Bovine serum albumin was used as a standard protein to produce the calibration equation between absorbance and protein concentration.

Immobilized metal affinity chromatography

In order to isolate FMBPs, extracted crude proteins were applied to the affinity column in which copper, nickel and zinc ions were immobilized as a ligand (AKTA FPLC with HiTrap Chelating, Amersham Pharmacia biotech, Uppsala, Sweden). Start and elution buffers of affinity chromatography were 0.5 M NaCl in 20 mM sodium phosphate (pH = 7.2) and 0.5 M NaCl in 20 mM sodium phosphate (pH = 3.5), respectively. Flow speed was 0.4 ml/min and the temperature was 23 ± 3°C. An ultraviolet absorbance detector at 280 nm was utilized to determine protein concentrations during chromatography analysis. Two 1 ml aliquot were collected as affinity fractions.

Molecular weight analysis

To identify the presence of FMBPs and estimate molecular weight of FMBPs, the affinity fractions were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). FMBPs were separated in running gel (10%-polyacrylamide) at a constant current (10 mA per gels) for 1.5 hours. The gels were stained with silver stainning kit (Amersham Pharmacia biotech, Uppsala, Sweden).

Results and discussion

Heavy metal stimulation for bacterial protein expression

Table 1 shows amounts of bacterial proteins that were extracted from mixed cultures of activated sludge bacteria. These extracted crude proteins would mainly consist of bacterial membrane proteins, because bacterial inner proteins were removed as many as possible at the protein extraction step. When copper chloride was added in the mixed culture, the amount of extracted proteins per 1 L of growth media decreased, because copper ions were used to inhibit bacterial cell growth. Meanwhile, the quantity of extracted crude proteins per 1 g of pellet of copper-stimulated culture was larger than that of the non-stimulated culture. These results indicate that copper ion in growth media had an adverse effect on the cell growth, but worked as an inducer and stimulated bacteria to express bacterial membrane proteins. The amount of synthesized proteins per bacterial cells was increased by 114%. Since functional proteins that could adsorb metals were considered to be produced by bacterial resistance mechanism against copper ions, it was suggested that FMBPs would be found in the extracted crude proteins from copper-stimulated bacteria.

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Table 1 Quantities of extracted crude proteins from mixed culture of activated sludge bacteria

	Quantities of extracted proteins (mg)	
	Per 1 l of growth media	Per 1 g of pellet (wet)
Test*	17.2 (0.19)**	2.5 (0.02)
Control	21.8 (0.14)	2.2 (0.02)

* Extracted crude proteins from copper-stimulated bacteria
** Values in parentheses are standard deviation

Isolation of FMBPs with immobilized metal affinity chromatography

Extracted crude proteins were processed for immobilized metal affinity chromatography to isolate FMBPs. Figure 1 shows the chromatographic profiles of FMBPs, which were isolated with the copper-immobilized affinity column. Black and gray lines are profiles of FMBPs produced by copper- and non-stimulated bacteria, respectively. Clear peaks were obtained in both profiles, which indicate the successful isolation of copper-binding proteins (CBPs) from both of copper- and non-stimulated bacteria. The amount of CBPs (estimated by peak height) from copper-stimulated bacteria was clearly smaller than that from non-stimulated bacteria because of the toxicity of the copper ion to cell growth. However, one specific peak was obtained in the profile of CBPs from the copper-stimulated bacteria (the arrow in Figure 1). This indicates that CBPs in the peak were expressed inductively by the copper ion in the growth media.

If these CBPs are able to bind other heavy metals, CBPs could be a more promising material than functional metal-binding proteins (FMBPs) in a new technology for eliminating various heavy metals from water. Therefore, extracted crude proteins were applied to each of nickel and zinc-immobilized affinity column for discovering multi metal-binding proteins. Figure 2 shows chromatographic profiles of FMBPs, which were isolated with each affinity column. Interestingly, the peaks in line 2 in Figure 2 indicate that copper-stimulated bacteria produced zinc-binding proteins (ZBPs), while any ZBPs could not be obtained from non-stimulated bacteria. These results imply the expression of ZBPs by activated sludge bacteria requires copper ions as an inducer in the growth media. On the other hand, nickel-binding proteins (NBPs) could be obtained from both copper- and non-stimulated bacteria (lines 1 and 3). As a result, there is a probability that FMBPs from copper-stimulated bacteria could be multi-metal adsorbents for all heavy metals of copper, nickel and zinc.

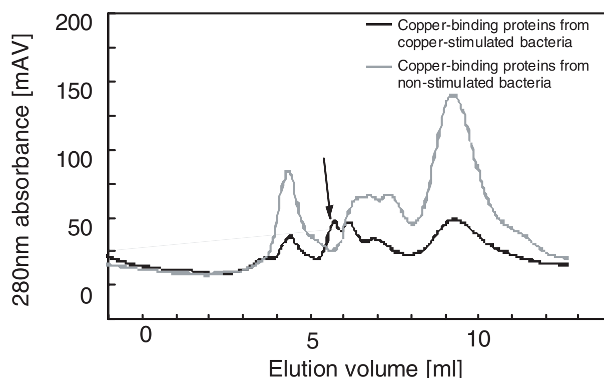

Figure 1 Affinity chromatographic profiles of copper-binding proteins isolated with copper-immobilized affinity column. Black and gray lines are profiles of copper-binding proteins produced by copper- and non-stimulated bacteria, respectively. The arrow indicates a specific peak of copper-binding proteins, which could be obtained from only copper-stimulated bacteria

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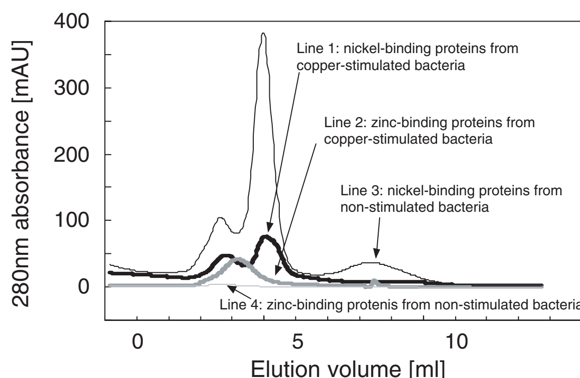

Figure 2 Affinity chromatographic profiles of nickel- and zinc-binding proteins. Line: 1, profile of nickel-binding proteins from copper-stimulated bacteria; 2, profile of zinc-binding proteins from copper-stimulated bacteria; 3, profile of nickel-binding proteins from non-stimulated bacteria; 4, profile of zinc-binding proteins from non-stimulated bacteria

Estimation of molecular weights of FMBPs with SDS-PAGE

In order to estimate molecular weights of FMBPs, affinity fractions were processed for SDS-PAGE, and a silver-stained gel was shown in Figure 3. Many bands were observed in all lanes, which means the presence of FMBPs in all affinity fractions. Although the number of CBPs from non-stimulated bacteria (lane 1) was almost the same as that from the copper-stimulated bacteria (lane 2), paler silver bands in lane 2 indicate that the amount of CBPs from the copper-stimulated bacteria was smaller than that from the non-stimulated bacteria. As for nickel-binding proteins (NBPs), copper-stimulated bacteria (lane 4) produced a smaller number and lower amount of NBPs than the non-stimulated bacteria (lane 3). On the other hand, copper-stimulated bacteria gave a larger number and amount of

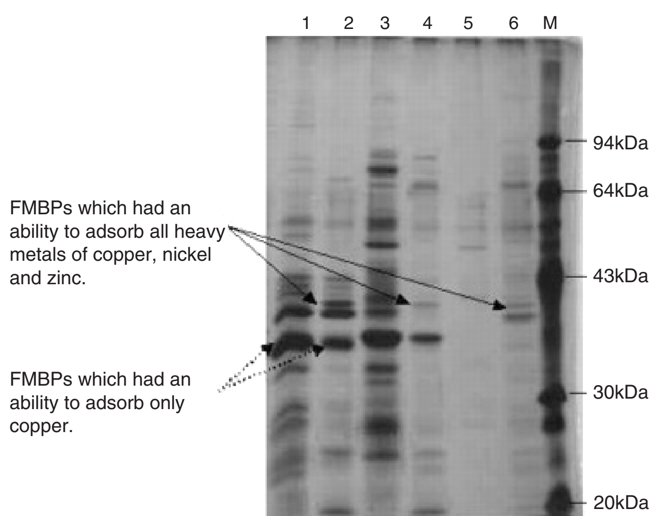

Figure 3 SDS-PAGE analysis of FMBPs. Lane: 1, Copper-binding proteins from non-stimulated bacteria; 2, Copper-binding proteins from copper-stimulated bacteria; 3, Nickel-binding proteins from non-stimulated bacteria; 4, Nickel-binding proteins from copper-stimulated bacteria; 5, Zinc-binding proteins from non-stimulated bacteria; 6, Zinc-binding proteins from copper-stimulated bacteria; M, Molecular weight marker. Solid arrows indicate FMBPs that had an ability to adsorb all heavy metals of copper, nickel and zinc. Dashed arrows indicate FMBPs that had the ability to adsorb only copper

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zinc-binding proteins (ZBPs) (lane 6) compared to the non-stimulated bacteria (lane 5). These results were consistent with the consequences of affinity chromatography of CBPs, NBPs and ZBPs (Figures 1 and 2). It is very interesting that copper ions caused the decrease in the quantity of isolated CBPs (lane 2 in Figure 3) and NBPs (lane 4 in Figure 3), as well as the quantity of extracted crude proteins per 1 l of growth media (Table 1), while the bacterial resistance system to the copper ion enhanced the expression of ZBPs. This enhancement of the expression of FMBPs by copper ion in growth media would also contribute to increasing the quantity of extracted crude proteins per 1 g of pellet (Table 1).

Applicability of FMBPs to metal removal technology

All FMBPs obtained in this study are candidates for heavy metal adsorbents. In particular, the FMBP from the copper-stimulated bacteria (lanes 2, 4 and 6), indicated by the solid arrows in Figure 3, was attractive as a metal adsorbent in an innovative metal removal technology, because this FMBP was able to adsorb all heavy metals of copper, nickel and zinc. Since this FMBP, which had a molecular weight of about 40 kDa, was not produced by non-stimulated bacteria (lanes 1, 3, 5), it is emphasized that the copper ion in the growth media enhanced the expression of this FMBP which had the multi metal-binding property. If this FMBP will be characterized and cloned in a further study, FMBP could be available for multi-metal adsorbent in the new technology for heavy metal removal from water.

Although the multi FMBP would be useful in the metal removal technology, other FMBPs that could adsorb only one kind of heavy metal would be also attractive. For example, the CBP from the copper-stimulated bacteria (lanes 1 and 2 in Figure 3), which was indicated by the dashed arrows, did not appear between lanes 3 and 6. These results mean that this FMBP has an ability to capture only the copper ion. Therefore, this FMBP can be used as an adsorbent for the copper ion when this ion has to be recovered as a resource from wastewater including several heavy metals.

Furthermore, the procedure for isolating FMBPs described in this study would make it possible to get FMBPs which have an ability to adsorb a specific rare metal such as uranium, platinum and gold. These FMBPs could be easily applied to the rare metal recovery in recycle systems. In the future, an innovative metal removal technology using these FMBPs could be truly developed.

Conclusions

Functional metal-binding proteins (FMBPs) were successfully isolated from copper-stimulated activated sludge culture by immobilized metal affinity chromatography. One of the FMBPs (molecular weight of about 40 kDa) had an ability to adsorb all heavy metals of copper, nickel, and zinc. This multi metal-binding property of the FMBP could be applied to the innovative metal removal technology. Furthermore, isolated FMBPs that could capture only one kind of heavy metal would also be attractive, as a metal adsorbent in recovering a specific metal as a resource from wastewater including several heavy metals.

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