

Isolation of copper-binding proteins from activated sludge culture

K. Fukushi, S. Kato, T. Antsuki and T. Omura

Department of Civil Engineering, Graduate School of Engineering, Tohoku University, Aoba-yama 06, 980-8579, Japan

Abstract Six copper-binding microbial proteins were isolated from activated sludge cultures grown on media containing copper at various concentrations. Molecular weights among isolated proteins were ranged from 1.3k to 174k dalton. Isolated proteins were compared for their copper binding capabilities. Proteins isolated from cultures grown in the presence of copper in the growth media exhibited higher copper binding capabilities than those isolated from the culture grown in the absence of copper. The highest metal uptake of 61.23 (mol copper/mol protein) was observed by a protein isolated from a culture grown with copper at a concentration of 0.25 mM. This isolated protein (CBP2) had a molecular weight of 24k dalton. Other protein exhibited copper binding capability of 4.8–32.5 (mol copper/mol protein).

Keywords Copper; metal-binding proteins; heavy metals; activated sludge; chromatography

Introduction

A significant number of investigations have been conducted on the microbial uptake of heavy metals. Ruchoft (1949), Cheng *et al.* (1975), Neufeld *et al.* (1975), Rossin *et al.* (1982), Brown *et al.* (1982), Petrasek *et al.* (1983), Rudd *et al.* (1984), Lawson *et al.* (1984) and Shumate *et al.* (1985) reported on the ability of a mixed biological culture to remove a number of heavy metals such as Fe, Cu, Pb, Ni, Zn, Hg, Cr, Ag, Cd, U, Pu, Mg, Co, and Mo in significant quantities.

Mechanisms of metal removal in biological systems can be considered to be both metabolic and non-metabolic uptakes. The metabolic heavy metal uptake occurs because microorganisms need certain kinds of heavy metals for their metabolic activities. The non-metabolic heavy metal uptake includes organic binding to cell wall, membrane, and extracellular biopolymers, precipitation, and physical sorption. The non-metabolic heavy metal uptake is usually faster than metabolic uptake. The amount and kinetics of non-metabolic uptake are deeply related to the characteristics and amount of cellular biopolymers.

Microorganisms produce extracellular biopolymers to protect themselves from the outer environment which contains toxic substances such as heavy metals (Shumate *et al.*, 1985; Rudd *et al.*, 1984; Lion *et al.*, 1988; Beveridge *et al.*, 1981, 1982; Remacle 1981). It has been suggested that extracellular biopolymers play an important role in removing metal ions from solution (Brown and Lester, 1979). Heavy metals complexed with the anionic ligands found in biopolymers of the cell membrane, cell wall, and extracellular biopolymers (e.g., capsules, microcapsules, and loose slime). Since metal ions complex with anionic sites, the amount of metals bound to cells is related to the amount of biopolymers in each cell. Tenney and Verhoff (1973) showed that an increase in the negative charge on the surface of activated-sludge bacteria was directly related to increasing extracellular biopolymer concentration. They also indicated that an increase in the negative charge of biopolymers enhanced the uptake of metal ions.

As mentioned above, microorganisms produce extracellular biopolymers to preserve inner-cellular materials from toxins in the outer environment. Heavy metal toxicity to microorganisms changes according to their species. Generally, the biopolymer complexes

with heavy metals have a lesser toxicity than those in ionic form. Bitton and Freihofer (1978) reported that the toxicity of a metal to bacteria is reduced by complexing it with extracellular biopolymers. Johnes (1971) and Corpe (1975) indicated that the chelation of metal by extracellular biopolymers reduces the toxicity of metal ions to microorganisms.

The stimulation of heavy metal uptake was investigated by including a simple biochemical in the growth media along with trace amounts of heavy metal (Ghosh *et al.*, 1990; Ghosh and Bupp, 1991; Bupp and Ghosh, 1991; Ghosh and Bupp, 1992). The trace amount of heavy metal induces the production of heavy metal-complexing biopolymers, and simple biochemicals stimulate the synthesis of such biopolymers; the inducing heavy metal and the simple biochemicals were called "inducer" and "biopolymer stimulators", respectively. In their study, cadmium was selected as an inducer to trigger the genetic information to synthesize metal-complexing biopolymers. Inclusion of the sulfur-containing amino acids, cystine and cysteine (biopolymer precursors), and cadmium (biopolymer inducer) for biopolymer synthesis enhanced copper, cadmium, and lead uptake efficiencies and kinetics. The inclusion of peptone and β -glycerophosphate are also suggested to increase copper and cadmium uptakes significantly (Ghosh *et al.*, 1995).

The work of Belly and Kydd (1981) suggests that a very large fraction of removed metals may be sequestered by extracellular proteins and lipoproteins. Methallothioneins (MTs) are well known as one kind of metal-binding proteinaceous biopolymers. Metallothioneins are low-molecular weight proteins which are rich in the sulfur-bearing amino acids, cystine and cysteine. These low molecular-weight proteins and other biopolymers are inducible and part of the microbial defense arsenal. These proteinaceous biopolymers are considered to play an important role in removing heavy metals from aqueous solution (Ghosh and Bupp, 1992; Ghosh *et al.*, 1995; Fukushi *et al.*, 1996). However, the isolation and characterization of metal-removing proteins from activated sludge are not fully investigated although it is quite beneficial in order to develop efficient, yet economical, metal-removing processes.

Objectives of this paper are to isolate copper-binding proteins from activated sludge culture and to investigate the effect of medium composition on the production of copper-binding proteins.

Materials and methods

To accomplish proposed objectives, a mixed microbial culture was sampled from a municipal wastewater treatment plant and was harvested on non selective growth media. The harvested cell mass was processed for the crude membrane protein extraction, and the extracted proteins were incubated with copper. The copper-complexed proteins were then isolated according to their molecular weights.

Microbial culture source

A mixed microbial culture was collected from the secondary clarifier of a municipal wastewater treatment plant located in Sendai, Japan. This wastewater treatment plant employs the standard activated sludge process. The collected culture was harvested on a nutrient broth non-selective media (Eiken, Tokyo, Japan) in the presence of 0.5 and 2.5 mM of cupric chloride for 48 hours at $20 \pm 5^\circ\text{C}$ in a constant temperature chamber. The control culture was also harvested without including copper in the medium. In order to obtain sufficient dissolved oxygen concentration in the medium, the air at a rate of about 80 ml/min was introduced into the medium liquid through a diffuser. The culture identification was shown in Table 1.

Table 1 Copper concentration in the growth media

Culture identification	Copper concentration	
Control	0	mM
Test 1	0.5	mM
Test 2	2.5	mM

Protein extraction procedure

The cell suspension was centrifuged ($3000 \times g$, 10 min) to obtain a cell pellet. The collected pellet was resuspended in 0.05 M of phosphate buffer solution (PBS) and centrifuged ($3000 \times g$, 10 min). This cell-washing procedure was repeated twice to remove a soluble fraction of growth media or undesired soluble substances.

The cell suspension was processed with a 50-watt ultrasonic cell disrupter (UP-5s, Taitec, Tokyo, Japan) for two minutes to expel inner materials of cells to the liquid phase. The cell mixture was centrifuged ($20,000 \times g$, 90 min). The supernatant of centrifuged cell mixture was discarded, and the pellet containing solid cell components was resuspended in 15 ml of 0.05 M PBS. The same volume of n-butanol was mixed with the slurry on a vortex mixer. In order to enhance the protein extraction efficiency 50-watt ultrasonic was applied for one minute. The slurry was centrifuged at $20,000 \times g$ for 10 minutes at 4°C . After centrifugation, the slurry was separated to three parts: the solvent phase, the cell solid phase, and the water phase. Membrane proteins are expected to exist in the water phase.

Metal binding experiment

Collected crude proteins were incubated in the presence of 0.05 M of cupric chloride for 20 minutes. The copper-mixed proteins was loaded into a size-exclusion chromatography (AKTA FPLC with Superdex 75 10/30, Amashamphalmacia biotech, Uppsala, Sweden). A fraction of each protein peak was collected and analyzed for copper concentrations. The metal binding capability of proteins was expressed mole copper per mol protein or mg copper per mg protein.

Copper and protein quantification

Copper-complexed proteins are analyzed for their metal concentrations. In order to remove organic fraction from samples a nitric acid digestion was performed accordingly with the *Standard Methods* (APHA *et al.*, 1992). Digested samples were then analyzed for metal concentrations with ICP-MS metal analyzer (Series 4500, Hewlet Packard, CA, USA). All glassware and plasticware was washed as per procedures in *Standard Methods* (APHA *et al.*, 1992).

Protein concentrations in samples were measured with 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.

Results and discussion**Molecular weight distribution of cultures**

Extracted crude proteins were incubated with copper and analyzed for the molecular weight distribution. Chromatograms for the Control, Test 1 (0.5 mM of copper in the media), and Test 2 (2.5 mM of copper in the media) are shown in Figure 1. Molecular weights of isolated membrane proteins were between 1.3k to 170k dalton (Da). A protein corresponding to each major absorbance peak in the chromatogram was identified to be

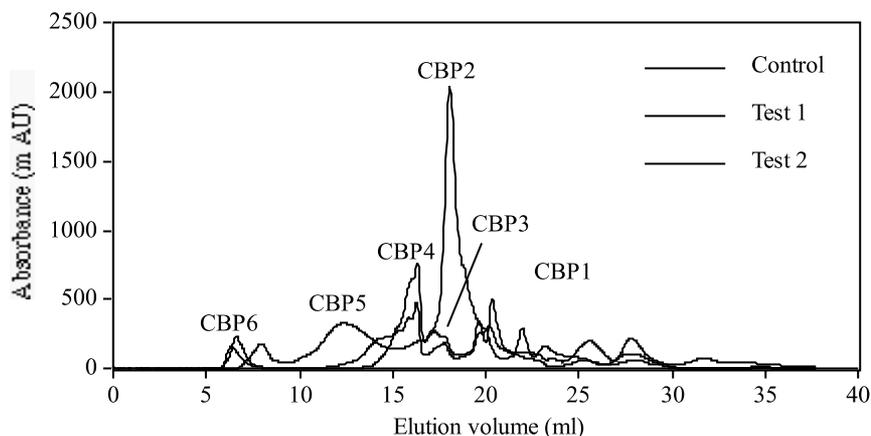


Figure 1 Size exclusion chromatograms. Elution volume is a cumulative volume of carrier buffer at a flow rate of 0.5 ml/min

CBPs. The molecular weight distributions of the control and Test 1 cultures were quite similar to that for the Control culture. On the other hand, the molecular weight of cellular membrane proteins for Test 2 culture was quite different. Since a distribution of molecular weight is closely related to the microbial composition of species, 2.5 mM of copper in the growth media changed the composition of microorganisms. The presence of 0.5 mM of copper in the media did not have any effect on the structure of the microbial fauna. It is generally known that a high concentration of heavy metals changes microbial composition drastically. Molecular weights of CBP 1, CBP 2, and CBP 3 are quite small to be protein. Although it may be appropriate to call them as “polypeptide”, the authors would like to call them “protein” in this paper for simplicity.

Metal binding capability of isolated proteins

Six isolated proteins (CBP 1-CBP 6) were tested for copper binding capabilities. Results are summarized in Table 2. The copper binding capabilities of isolated proteins are expressed by mole of copper per mole of protein as well as weight of copper per weight of protein. The copper complexation capability of proteins varies between 4.80 to 61.23 mole/mole. In general, proteins obtained from test cultures exhibited higher copper uptakes than those from the control culture. CBPs 1, 3, 4, and 6 are found commonly in the control and Test 1 cultures. CBP 4 from the control culture and Test 1 culture also exhibited a quite different copper-binding capability. These results indicated that copper binding capabilities of CBP 4 are dependent on the composition of growth medium. The inclusion of copper in the growth medium increased copper-binding capability of CBP 4 by 416%. These results indicate that CBP 4 extracted from the control and Test 1 cultures may be different proteins with a different copper-binding capability although they had a similar molecular weight. In contrast, CBP 1, CBP 3, and CBP 6 from the control and Test 1 cultures exhibited similar copper-binding capabilities. These proteins were probably the same protein from a common species in both cultures. Proteins from Test 1 culture (CBP 3 and CBP 6) exhibited a slightly higher copper-binding capability. The reason for this is that the protein produced by microorganisms in Test 1 culture complexed copper molecules that are initially complexed by these proteins.

Deans and Dixon (1991) tested numerous biopolymers and synthetic polymers for lead and copper ion uptake capabilities. They tested 11 biopolymers and two non-biopolymeric substances. The copper uptake efficiency of each biopolymer ranged 0 to 0.08 mg copper

per one mg of biopolymer. Table 3 is a comparison of copper-binding capability of selected biopolymers presented in Deans and Dixon's paper and our isolated copper-binding proteins. In Deans and Dixon's paper, Chelex-20 was used as a reference metal removing materials and exhibited the highest copper removing capability of 0.094 mg copper per mg of resin. In our study, CBP 2 exhibited the highest copper uptake capability of 1.61 mg copper per mg of protein. This complexation capability was about 17 times of that of Chelex-20. Chelex-20 resin (Bio-Rad Laboratories Inc, USA) is designed as an efficient metal-removing artificial resin. CBPs 3, 4, and 6 also exhibited high copper complexation capabilities compared to those of biopolymer presented in the paper by Deans and Dixon (1991). This capability is also higher than other commercial cationic exchange resins such as Amberlite (Sigam chemicals, USA: exchange capacity of 10 meq/g). Other biopolymers (e.g., carboxymethyl cellulose, alginic acid, chitosan) exhibited much lower copper-removing capability than isolated proteins in our study. This comparison clearly indicates that isolated copper-binding proteins have extremely high copper binding capabilities.

If one kilogram of CBP 2 was applied to a mining wastewater treatment process, this process is able to treat about 32,200 litres of copper containing wastes at a concentration of 50 mg/l. In addition, this removed copper can be recovered with salt solution for ultimate reuse. The isolated proteins may be manufactured biologically with a molecularbiological technique.

There are several metal-binding proteins isolated from biological systems such as human serum, tissue or microbial components. A well known metal-binding protein isolated from human blood and urine is metallothionein, a low-molecular weight protein.

Table 2 Molecular weights and copper binding capabilities of isolated proteins

Protein ID	Molecular weight			Copper binding capability					
	Control	Test 1	Test 2	Control mol/mol	Test 1 mol/mol	Test 2 mol/mol	Control mg/mg	Test 1 mg/mg	Test 2 mg/mg
CBP1	(1353)*	(1353)	–	4.80	3.58	–	0.225	0.168	–
CBP2	–	–	(2419)	–	–	61.23	–	–	1.610
CBP3	3718	3718	–	26.78	32.52	–	0.460	0.560	–
CBP4	4727	4727	–	4.80	24.77	–	0.065	0.330	–
CBP5	–	–	20,440	–	–	4.92	–	–	0.020
CBP6	(173,652)	(173,652)	–	9.07	12.82	–	0.003	0.005	–

* Numbers within the parentheses are estimated values since the recommended isolation range of Superdex 75 size exclusion chromatography column was 3k to 70k dalton

Table 3 Comparison of copper-complexation capability by various materials

Name of materials	Cu uptake mg Cu/mg absorbent
Carboxymethyl cellulose	0.035*
Alginic acid	0.036*
Carboxymethyl chitin-NHOH	0.043*
Chitosan	0.057*
Telephthalic acid	No uptake*
Bio-Rad Chelex-20	0.095*
CBP3	0.56**
CBP6	1.61

* Copper uptakes were recalculated from data by Deans and Dixon (1991). The initial copper concentration of copper uptake experiment was 1.5 mM

** Isolated from Test 1 culture

Metallothionein removes heavy metals via binding to its sulfhydryl functional groups. Freedman (1986) reported that metallothionein complexes seven to eight equivalents of copper per molecule (0.50–0.57 mmol copper per one gram of protein; provided molecular weight of metallothionein is 7,000). In Table 2, isolated proteins from activated sludge complexed up to 61 mole of copper per one mole of protein. The complexing capacity of CBP 2 exhibited about 300% higher value comparing to that of metallothionein.

Conclusions

This paper presented the experimental research findings of isolation of novel proteins from activated sludge culture. Six proteins were isolated from activated sludge culture harvested in the presence/absence of copper in the growth media. One of the isolated proteins (CBP 2) with a molecular weight of about 2,500 dalton complexed 61.23 mole of copper per one mole of protein. This complexation capability was about 17 times of that of commercial chelating resins. This result indicates that activated sludge culture contains a protein which has an extremely high copper complexation capability.

References

- American Public Health Association, American Water Works Association, and Water Environment Federation (1992). *Standard methods for the examination of water and wastewater* (Greenberg, A.E., Clesseri, L.S. and Eaton, A.D. eds.). 18th ed. APHA. Washington, D.C.
- Belly, R.T. and Kydd, G.C. (1981). Silver resistance in microorganisms. *Dev. Ind. Microbiol.*, **23**, 567.
- Beveridge, T.J. and Koval, S.F. (1981). Binding of metals to cell envelopes of *Escherichia coli* K-12. *Appl. Environ. Microbiol.*, **42**, 325–335.
- Bitton, G. and Freihofer, V. (1978). Influence of extracellular polysaccharides on the toxicity of copper and cadmium toward *K. aerogenes*. *Microb. Ecol.*, **4**, 119–125.
- Brown, M.J. and Lester, J.N. (1979). Metal removal in activated sludge: the role of bacterial extracellular polymers. *Water Res.*, **13**, 817–837.
- Brown, M.J. and Lester, J.N. (1982). Role of bacteria extracellular polymers in metal uptake in pure bacterial culture and activated sludge-I, Effect of metal concentration. *Water Res.*, **16**, 1539–1560.
- Bupp, S. and Ghosh, S. (1991). Heavy metal uptake by microbial protein complexation. Paper presented at the Research Symp., Annual WPCF Conf., Toronto, Canada, Oct.
- Chang, D., Fukushi, K. and Ghosh, S. (1995). Stimulation of activated sludge cultures for enhanced heavy metal removal. *Water Environment Research*, **67**(5), 822–827.
- Cheng, M.H., Patterson, J.W. and Minear, R.A. (1975). Heavy metal uptake by activated sludge. *JWPCF*, **47**, 362.
- Corpe, W.A. (1975). Metal-binding properties of surface materials from marine bacteria. *Dev. Ind. Microbiol.*, **16**, 249–255.
- Deans, J.R. and Dixon, B.G. (1992). Uptake of Pb^{2+} and Cu^{2+} by novel biopolymers. *Wat Res.*, **26**, 469.
- Freedman, J.H., Powers, L. and Peisach, J. (1986). Structure of the copper cluster in canine hepatic metallothionein using X-ray absorption spectroscopy. *Biochem.*, **25**, 2342–2349.
- Fukushi, K., Chang, D. and Ghosh, S. (1996). Enhanced heavy metal uptake by activated sludge cultures grown in the presence of biopolymer stimulators. *Water Science and Technology*, **34**(5–6), 267–272.
- Ghosh, S., Bupp, S. and DeBirk, L. (1990). Removal of heavy metals by biopolymers. *Proc. Conf. on Hazardous Waste Research*, Erickson, L.(Ed.), Engineering Extension, Kansas State Univ., Manhattan, KS, 449.
- Ghosh, S. and Bupp, S. (1991). Uptake of heavy metals by unacclimated aerobic culture. *Proc. Conf. on Hazardous Waste Research*, Erickson, L.(Ed.), Engineering Extension, Kansas State Univ., Manhattan, KS, 30.
- Ghosh, S. and Bupp, S. (1992). Stimulation of biological uptake of heavy metals. *Wat. Sci. Tech.*, **26**(1–2), 227–236.
- Johns, G.E. (1971). The fate of freshwater bacteria in the sea. *Dev. Ind. Microbiol.*, **12**, 141–151.
- Lawson, P.S., Sterritt, R.M. and Lester, J.N. (1984). Adsorption and complexation mechanisms of heavy metal uptake in activated sludge. *J. Chem. Tech.*, **34B**, 253.
- Lion, L.W., Shuler, M.L., Hsieh, K.M. and Ghiorse, W.C. (1988). Trace metal interactions with microbial

- biofilm in natural and engineered systems. *CRC Critical Reviews in Environ. Control.*, **17**, 273–306.
- Neufeld, R.D. and Hermann, E.R. (1975). "Heavy metal removal by acclimated activated sludge." *JWPCF*, **47**, 310–329.
- Petrasek, A.C. Jr. and Kugelman, I.J. (1983). "Metals removal and partitioning in conventional wastewater treatment plants." *JWPCF*, **55**, 1183–1190.
- Remacle, J. (1983). Cadmium uptake by freshwater bacteria communities. *Water Res.*, **55**, 1183–1190.
- Rossin, A.C., Sterritt, R.M. and Lester, J.N. (1982). The influence of process parameters on the removal of heavy metals in activated sludge. *Water, Air, and Soil Pollut.*, **17**, 185.
- Ruchoft, C.C. (1949). The possibilities of disposal of radioactive wastes by biological treatment methods. *Sewage Works J.*, **21**, 877.
- Rudd, T., Sterritt, R.M. and Lester, J.N. (1984). "Complexation of heavy metals by extracellular polymers in the activated sludge process." *JWPCF*, **56**, 1260–1268.
- Shumate, S.E. II and Stranberg, G.W. (1985). Accumulation of metals by microbial cells. In: *Comprehensive Biotechnology* (Moo-Young, M., et al. Eds.), **4**, 235, Pergamon Press, New York.

