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Factors Affecting the Binding of a Recombinant Heavy Metal-Binding Domain (CXXC motif) Protein to Heavy Metals

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Abstract

A number of heavy metal-binding proteins have been used to study bioremediation. CXXC motif, a metal binding domain containing Cys-X-X-Cys motif, has been identified in various organisms. These proteins are capable of binding various types of heavy metals. In this study, heavy metal binding domain (CXXC motif) recombinant protein encoded from *mcsA* gene of *S. aureus* were cloned and overexpressed in *Escherichia coli*. The factors involved in the metal-binding activity were determined in order to analyze the potential of recombinant protein for bioremediation. A recombinant protein can be bound to Cd^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+}. The thermal stability of a recombinant protein was tested, and the results showed that the metal binding activity to Cu^{2+} and Zn^{2+} still exist after treating the protein at 85ºC for 30 min. The temperature and pH that affected the metal binding activity was tested and the results showed that recombinant protein was still bound to Cu^{2+} at 65ºC, whereas a pH of 3-7 did not affect the metal binding. *E. coli* harboring a pRset with a heavy metal-binding domain CXXC motif increased the resistance of heavy metals against $CuCl_2$ and $CdCl_2$. This study shows that metal binding domain (CXXC motif) recombinant protein can be effectively bound to various types of heavy metals and may be used as a potential tool for studying bioremediation.

Keywords: heavy metal binding proteins; bioremediation; heavy metals; binding factors; CXXC motif

1. Introduction

The treatment of hazardous heavy metal contamination in the environment with biological organisms such as plants and microorganisms or their products is known as heavy metal bioremediation (Bonaventura and Johnson, 1997). Microorganisms have an ability to absorb heavy metals by using heavy metal binding-proteins that can degrade, accumulate, or detoxify heavy metals (Huang *et al*., 2003; Wiatrowski and Barkay, 2005; Bondarenko *et al*., 2008). These heavy metal-binding proteins are encoded by the metal regulatory genes and heavy metal transporter genes that function as a heavy metal chaperone, heavy metal transporters or enzymes that detoxify heavy metals (Sitthisak *et al*., 2005; Sitthisak *et al*., 2007; Al Hasin *et al*., 2010). Heavy metal binding proteins have been identified in various organisms with the ability to bind and absorb various types of heavy metals (Kao *et al*., 2008; Al Hasin *et al*., 2010; Zheng *et al*., 2011; Zhu *et al*., 2011). CXXC motif is a metal binding domain that contains Cys-X-X-Cys motif (X is any amino acid). This motif has been found in heavy metal chaperones, in the thiol disulphideoxidoreductase superfamily, in heavy metal transporters from many bacteria, as well as in yeast and in humans (Lutsenko *et al*., 1997; Huang *et al*., 2003; Sitthisak *et al*., 2007; Agarwal *et al*., 2010).

Recently research in bioremediation has focused on genetically engineered bacteria. A number of metal binding proteins from microorganisms are in use to create engineered bacteria for studying bioremediation (Chen *et al*., 1997; Sing *et al*., 2011). However, there are some limitations in bioremediation systems as some can remove only one or two heavy metals. Previous work has shown that metal binding proteins, CopA, CopZ and McsA in *S. aureus* that contains heavy metal binding domain CXXC motifs, are capable of binding various types of heavy metals (Sitthisak *et al*., 2007; Sitthisak *et al*., 2012). Understanding the factors affecting the binding of proteins and heavy metals together will provide information of specificity and selectivity about the heavy metal binding domain CXXC motif, which would then in turn improve analysis about the potential of novel metal binding domain (CXXC motif) recombinant proteins for the purposes of bioremediation. Thus, in this study, an *mcsA* gene that contains four domains of CXXC motifs from *S. aureus* was cloned, expressed, and investigated for various environmental factors that are involved in the binding of recombinant proteins and heavy metals.

2. Materials and Methods

2.1. Cloning and Overexpression of CXXC motif recombinant protein

A PCR was performed using gene coded for CXXC motif metal binding domain (*mcsA* gene). PCR product was cloned into the TA vector and then sub-cloned into a pRSETa (Invitrogen) and overexpressed in *E. coli* BL21 (DE3) PLysS (Novagen) by the method described by Sitthisak *et al*. (2012). To overexpress the recombinant protein, the transformants were grown in LB containing ampicillin 50 µg/ml and chloramphenicol 10 µg/ml at 37ºC until the OD_{600} reached 0.5. Then, the cells were induced to express their proteins by adding 1.0 mM of IPTG for 4 hrs. The induced cells were harvested, washed, and re-suspended in a lysis buffer. Pellets were homogenized with a sonicator and the cell debris was removed by centrifugation at 12,000Xg at 4ºC. Supernatants were applied to nickel-charged agarose affinity columns (Novagen) and eluted with 200-400 mM imidazole. Fractions containing the overexpressed His-tag protein were pooled and dialyzed against dialysis buffer, at a pH of 8.0 (25 mM Tris, 100 mM sucrose, 50 mM NaCl,1 mM of DTT).

2.2. Characterization of the metal binding activity of recombinant protein and E. coli crude extract by IAA chromatography

The metal binding activity of the recombinant protein and crude extract was determined by using a Iminodiacetic acid-agarose (IAA) column as described by Luzenko *et al*. (1997). Columns containing IAA resin were washed thoroughly with a 50 mM Na-Phosphate buffer (pH7.5) and then each separately equilibrated with 10 volumes of the same buffer containing one of various heavy metals ($CdCl_2$, $CuCl_2$, $CoCl_2$, $ZnCl_2$, $Pb(NO_2)_3$, $MnCl_2$, $MgCl_2$, $FeCl_3$) at a final concentration of 1 mM. Excess metal ions were removed by washing again with a PBS buffer. Then, 100 µg of purified protein or 2 mg of *E. coli* crude extract were added to the resin. Columns were centrifuged to remove unbound proteins and washed with a PBS buffer. Bound proteins were eluted from the column with 50 mM of EDTA in a PBS buffer. Both eluted and unbound proteins were analyzed by 15% SDS-PAGE. The amount of total proteins and bound proteins were determined by using a Bio-rad protein assay kit, the percentage of metal binding protein in the *E. coli* crude extract was calculated.

2.3. Analysis of thermal stability and metal binding activity of the recombinant protein

The recombinant protein was incubated at various temperatures (25°C 37°C, 45°C, 65°C and 85°C) for 10 mins. After that, the metal binding activity of the recombinant protein was characterized by using various heavy metals that were bound to the proteins ($CdCl_2$, $CuCl_2$, $CoCl_2$, $ZnCl_2$). This was done by using IAA column chromatography.

2.4. Factors affecting the metal binding affinity

2.4.1. protein concentration affecting the metal binding affinity

The metal binding activities observed through IAA chromatography were characterized by using 25, 50, 100, 150 µg of recombinant proteins. The columns were incubated with various heavy metals that were bound to the proteins ($CdCl_2$, $CuCl_2$, $CoCl_2$, $ZnCl_2$). After that, bound and unbound proteins were collected and the presence of the recombinant proteins was analyzed by 15% SDS-PAGE.

2.4.2. Temperature affecting the metal binding affinity

100 µg of recombinant protein was added to the IAA columns and equilibrated with 10 volumes of the PBS buffer containing various types of heavy metals. The columns were then incubated at various temperatures (37°C, 45°C, and 65°C). After that, bound and unbound proteins were collected and the presence of recombinant proteins was analyzed by 15% SDS-PAGE.

2.4.3. pH affecting the metal binding affinity

The metal binding activities of the recombinant protein were characterized at various levels of pH. Briefly, the columns containing IAA resin were washed with a PBS buffer, and the columns were charged with a heavy metal compound to a final concentration of 1 mM in a PBS buffer with varying pH (3, 5, 7 and 9). After that, bound and unbound proteins were collected and the presence of recombinant proteins was analyzed by 15%SDS-PAGE.

2.5. Physiological characterization of the E. coli expressing heavy metal recombinant protein in the presence of heavy metals

To study the effects of heavy metals in the *E. coli* expressing recombinant protein, the growth characteristics of *E. coli* strains were determined under various heavy metal conditions and compared to *E. coli* harboring overexpressed plasmid (pRset). Bacterial cultures were grown in an LB with 0.1 mM of IPTG, all containing various concentrations of $CuCl_2$, $CoCl_2$, $ZnCl_2$, and

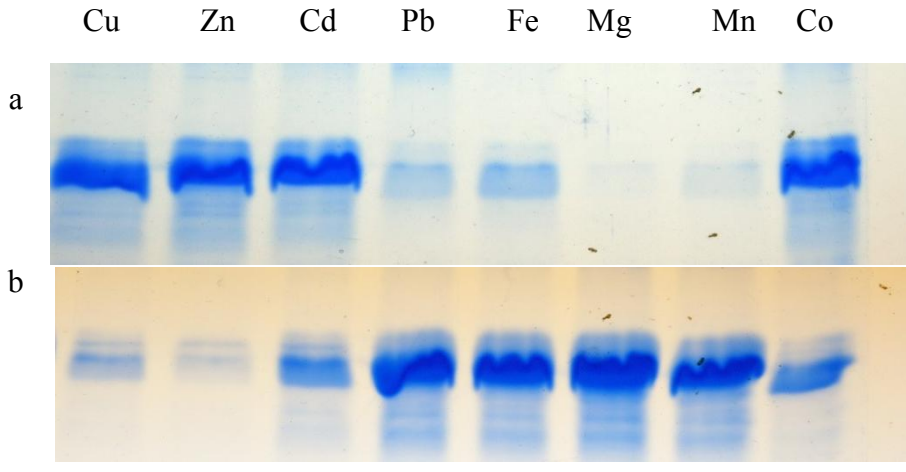

Figure 1. Binding of recombinant protein to IAA resin chromatography. The IAA-resin was charged with different heavy metals as indicated above the respective lane. Bound and unbound proteins were analyzed by 15% SDS-PAGE. Row a, Bound protein; Row b, Unbound protein.

$CdCl_2$ at 37°C with shaking (200 rpm). Growth was measured by optical density determination at 600 nm with a spectronic-20 spectrophotometer.

3. Results and Discussion

3.1. Analysis of metal binding activity of the recombinant protein and crude extract of E. coli

Each protein has different properties to bind and absorb heavy metals. Previous studies have shown that CopA, CopZ and McsA from *S. aureus* and N-WND and N-MNK from humans that contain CXXC motif all bind specifically to copper, cobalt and cadmium (Lutsenko *et al*., 1997; Sitthisak *et al*., 2007; Sitthisak *et al*., 2012). MTs that have cysteine-rich motifs are capable of binding to zinc, copper, cadmium, and mercury (Mejáre and Bülow, 2001; Das *et al*., 2006). In this study, the ability of heavy metal binding domain (CXXC motif) recombinant protein to bind with different heavy metals is shown in Fig. 1(a). The recombinant proteins bind to copper, zinc, cadmium, and cobalt. No binding was observed to the columns charged with lead, iron, magnesium and manganese [Fig. 1(b)]. The protein in the crude extract of *E. coli* that expresses heavy metal binding domain (CXXC motif) can be bound to copper, zinc, cadmium, and cobalt (data not shown). No binding was observed in the crude extract of un-expressed *E. coli* (data not shown). The amount of heavy metal binding protein in the crude extracted *E. coli* was also measured. The presence of heavy metal binding protein in the total protein of the crude extract that can bind to Cd^{2+}, Cu^{2+}, Co^{2+} and Zn^{2+} were 3.86%, 3.30%, 3.53% and 4.63%, respectively.

3.2. Thermal stability of the recombinant protein on metal binding activity

Temperature is an important consideration for protein stabilization. The thermal stability of the recombinant protein was investigated before testing the metal binding activity. The results are shown in Table 1. The metal binding activity of the recombinant protein to Cu^{2+} and Zn^{2+} was still present after treating the protein at 85ºC for 30 min (Table 1). Most proteins lose their enzymatic activity at 55°C, and their protein structure are destroyed when the environmental temperature reaches 80ºC (Huang *et al*., 2010). Since the high temperature did not destroy heavy metal binding activity of the CXXC motif recombinant protein to Cu^{2+} and Zn^{2+}, this data demonstrates that conformational loss does not therefore affect the binding of the CXXC motif to Cu^{2+} and Zn^{2+}.

Table 1. Thermal stability of the CXXC motif recombinant protein.

Temp.	$CdCl_2$	$CoCl_2$	$CuCl_2$	$ZnCl_2$
25°C, 10 min	+	+	+	+
37°C, 10 min	+	+	+	+
45°C, 10 min	+	-	+	+
65°C, 10 min	+	-	+	+
85°C, 10 min	-	-	+	+
85°C, 30 min	-	-	+	+

Thermal stability showed that recombinant protein still bind to heavy metal after treated with various temperatures. All experiments were repeated in triplicate. +; the recombinant protein bound to heavy metals. - ; The recombinant protein do not bound to heavy metals.

3.3. Effect of protein concentration and temperature on metal binding activity

Various amounts of recombinant protein were tested to investigate the optimal concentration of recombinant protein for binding with $CdCl_2$, $CuCl_2$, $CoCl_2$, $ZnCl_2$. The optimal amount was 100 µg. No binding was observed when using less protein than 100 µg (data not shown). In addition, the temperature that affected the metal binding activity was tested and the result was that the recombinant protein was capable of binding Cu^{2+}, Co^{2+}, Zn^{2+} and Cd^{2+} when it was incubated with the heavy metal at 45ºC. However, no binding with any metals, with the exception for Cu^{2+} was detected at 65ºC (Table 2). A previous report by Hinc *et al*. (2010) showed that the temperature (25-45°C) and pH (5-9) did not affect the binding of CotB protein that contain histidine rich metal binding domain with nickel. However, our data demonstrates that the binding of recombinant protein against heavy metals is temperature dependent. High temperature does affect the metal binding activity depending on the type of heavy metals.

Table 2. Temperature that affect the metal binding activity

Temp.	$CdCl_2$	$CoCl_2$	$CuCl_2$	$ZnCl_2$
37°C	+	+	+	+
45°C	+	+	+	+
65°C	-	-	+	-

The effect of temperature on metal binding. All experiments were repeated in triplicate.
+ ; The recombinant protein bound to heavy metals.
- ; The recombinant protein do not bound to heavy metals.

A pH of 3-7 does not affect metal binding (data not shown).

3.4. Increased heavy metal resistance of E. coli expresses heavy metal binding domain CXXC motif recombinant protein

It has been previously proposed to have a role of heavy metal binding protein as a heavy metal chaperone in a stress response against heavy metals (Jordan *et al*.,

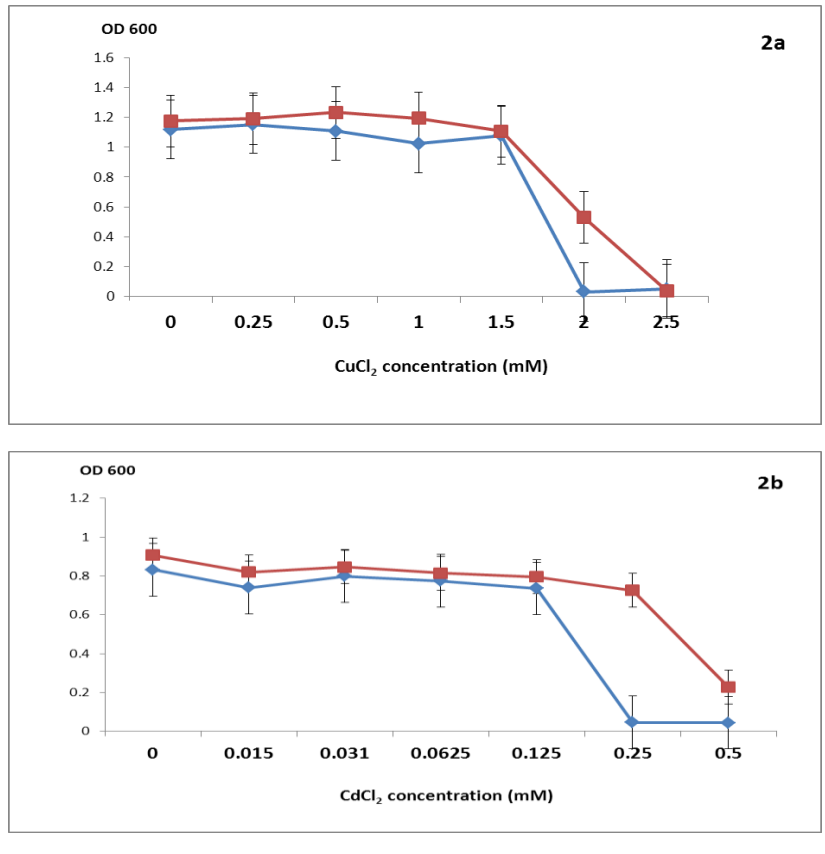

Figure 2. Effects of heavy metals on growth of *E. coli* BL21(DE3) expressing heavy metal binding protein. Overnight cultures were diluted to OD_{600} in LB with 0.1 mM of IPTG and 100 µg/ml at different concentrations of $CuCl_2$ (2a) and $CdCl_2$ (2b). Cultures were incubated at 37°C with shaking. Cell growth was monitored by measuring the optical density at 600 nm for 18 h. Symbols represented: ■; *E. coli* BL21(DE3) expressing CXXC motif heavy metal binding protein, ♦; *E. coli* harboring overexpression vector, pRset (control). Each point represents mean of three experiments. (Standard error of mean represented by bar)

2001; Ettema *et al*., 2003). Engineering an *E.coli* gene involves copper transport on *E.coli* surface enhanced cell resistance and adsorption to copper (Ravikumar *et al*., 2011). To test whether or not heavy metal binding domain CXXC motif has any impact on the heavy metals sensitivity, the effect of heavy metal on the growth of *E. coli* harboring a pRset with and without heavy metal binding domain CXXC motif was compared. The expression of genes encoded for the CXXC motif resulted in increased resistance to copper and cadmium ions. As shown in Fig. 2, the control strain grows slowly in a medium containing 2.0 mM of $CuCl_2$ [Fig. 2(a)] and 0.5 mM of $CdCl_2$ [Fig. 2(b)]. However, there were no significant differences in the growth of the *E. coli* strains in the presence of $ZnCl_2$ and $CoCl_2$ (data not shown). The enhanced resistance is probably due to the binding capacity of the heavy metal binding domain that could detoxify Cu^{2+} and Cd^{2+}.

The results from this study show that metal binding domain recombinant protein can be bound effectively to various types of heavy metals. It is demonstrated that the CXXC motif recombinant protein is rather stable and can be used to generate genetically engineered microorganism. CXXC motif in the recombinant proteins can bind to heavy metals and help genetically engineered microorganism to accumulate the heavy metals. Future work will be focused at cell surface displays of the metal binding domain CXXC motif on *E. coli* or *Saccharomyces cerevisiae* to enhance the absorption of heavy metals.

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