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Expression of metallothionein of freshwater crab (*Sinopotamon henanense*) in *Escherichia coli* enhances tolerance and accumulation of zinc, copper and cadmium

Yongji He · Wenli Ma · Yingjun Li · Jinping Liu · Weixin Jing · Lan Wang

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Abstract Metallothioneins (MTs) are ubiquitous metal-binding, cysteine-rich, small proteins and play a major role in metal homeostasis and/or detoxification in all organisms. In a previous study, a novel full length MT gene was isolated from the freshwater crab (*Sinopotamon henanense*), a species widely distributed in Shanxi and Henan Provinces, China. In this report, the gene for the crab MT was inserted into a PET-28a-6His-SUMO vector and recombinant soluble MT was over-expressed as fusions with SUMO in *Escherichia coli*. The recombinant fusion protein was purified by affinity chromatography and its biochemical properties were analyzed. In addition, on the basis of constructing SUMO-MT, two mutants, namely SUMO-MTt1 and SUMO-MTt2, were constructed to change the primary structure of SUMO-MT using site-directed mutagenesis techniques with the amino acid substitutions D3C and S37C in order to increase metal-binding capacity of MT. *E. coli* cells expressing SUMO-MT and these single-mutant proteins exhibited enhanced metal tolerance and higher accumulation of metal ions than control cells. The results showed that the bioaccumulation and tolerance of Zn^{2+}, Cu^{2+} and Cd^{2+} in these strains followed the decreasing order of SUMO-MTt1 > SUMO-MTt2 > SUMO-MT. *E. coli* cells have low tolerance and high accumulation towards cadmium compared to zinc and copper. These results show that the MT of *S. henanense* could enhance tolerance and accumulation of metal ions. Moreover, we were able to create a novel protein based on the crab MT to bind metal ions at high density and with high affinity. Therefore, SUMO-MT and its mutants can provide potential candidates for heavy metal bioremediation. This study could help further elucidate the mechanism of how the crab detoxifies heavy metals and provide a scientific basis for environment bioremediation of heavy metal pollution using the over-expression of the crab MT and mutant proteins.

Keywords Metal accumulation · Metal tolerance · Metallothionein · Mutant · *Sinopotamon henanense*

Y. He · W. Ma · Y. Li · J. Liu · W. Jing · L. Wang (✉)
School of Life Science, Shanxi University, 92 Wucheng Road, Taiyuan 030006, Shanxi, People's Republic of China
e-mail: lanwang.sxu@qq.com

Introduction

As we know, many heavy metal pollution in water become more and more serious along with the rapid development of agricultural, industrial and military operations. Heavy metals are, unlike other pollutants, difficult to be removed from the environment and cannot be chemically or biologically degraded (Mejáre and Bülow 2001). They can move into food chains and do great harm to animals and eventually to human health (Chen et al. 1999). Among the heavy metals that pollute the aqueous system, cadmium (Cd) is one of the most toxic and can affect the reparability of DNA double-strand breaks, lead to genome instability, and cause many diseases including stroke, bone damage and cancer, etc. (Ma et al. 2011). Other metal ions, such as zinc (Zn) and copper (Cu) are essential for normal growth and development of an organism. However, higher concentrations of essential metals are known to cause toxic effects, similar to those caused by no-essential metals like cadmium (Wu et al. 2006; Van Dyk et al. 2007; Green et al. 2010). Thus, bioremediation and phytoremediation strategies to deal with metal contamination are very attractive and have been attempted in comparison to physicochemical methods owing to their low cost and high efficiency

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(Sauge-Merle et al. 2012). In bioremediation and phytoremediation bacteria and plants could be used to clean up metal contamination not only by removing metals and sequestering metal from contaminated water but also by solubilizing metals and enhancing the bioavailability of metals to facilitate their extraction from soils and sediments. Many researchers attempt to enhance the metal content of bacterial and plant cells by over-expressing metal-binding peptides or proteins such as poly-histidines (Sousa et al. 1996; Mejáre and Bülow 2001), poly-cysteines (Wu et al. 2006) and metallothioneins (MTs) (Sekhar et al. 2011; Sauge-Merle et al. 2012). In these attempts, MTs are of special interest because MTs have a high metal-binding capacity and can bind metal ions of both, essential (Zn, Cu, Ni, etc.) and toxic (Cd, Hg, Pb, etc.) heavy metals (Mejáre and Bülow 2001; Sauge-Merle et al. 2012).

MTs, found in a large number of phylogenetically diverse organisms, are a class of low-molecular-weight, cytoplasmic, metal-binding proteins that are rich in cysteine residues (Kagi and Schaffer 1988). MTs are involved in essential-metal homeostasis and impart protection against heavy metal toxicity by sequestration (Mir et al. 2004; Hassinen et al. 2009; Huang and Wang 2010). Despite the recent efforts focused on the function of MTs in the development of mammals and aquatic vertebrates (Knapen et al. 2007; Kusakabe et al. 2008; Asselman et al. 2012), the information on invertebrate MT is still scarce. Compared with vertebrate MTs, invertebrate MTs have a great diversity of metal speciation and quantity (Lerch et al. 1982; Huang and Wang 2010). There are multiple isoforms of MT with different amount and different kinds of metal ion to probably play different physiological roles in invertebrates compared to mammals (Amiard et al. 2006). Only a few studies have examined the applied potential of invertebrate MTs in bioremediation (Turchi et al. 2012; Chaturvedi et al. 2012; Pérez-Rafael et al. 2012). Especially, information on mutant MTs constucted by site-directed mutagenesis to investigate the physiological roles and metal-binding abilities is still scarce. The freshwater crab *Sinopotamon henanense* is commonly found in southeast Shanxi Province, an area that has endured the most serious environmental pollution. It lives in the sediment and faces heavy metals directly via its integument and via food. In our previous report, *S. henanense* showed a strong capability of accumulating heavy metals (Ma et al. 2008). A novel MT from *S. henanense* has been purified and its full length gene has been isolated from the cDNA library of *S. henanense*. This MT cDNA (633 bp) contains 18 cysteine residues arranged in 5 Cys-Xaa-Cys-, 2 Cys-Cys- and 3 Cys-Xaa-Yaa-Cys-motifs (Ma et al. 2009). It implied that the *S. henanense* MT protein may be have a higher metal-binding capacity and the unique properties of metal affinity.

In the present work, we prepared and analyzed recombinant MT from *S. henanense* as a fusion protein with small ubiquitin-related modifier (SUMO) to obtain a stable over-expressed protein. SUMO is an ubiquitin-related protein that functions by covalent attachment to other proteins. The advantages of fusion proteins with a SUMO tag include reduced proteolytic degradation of the target protein, increased protein folding and solubility, and simplification of purification and detection in agreement with other traditional fusion systems. Especially, SUMO fusions are superior to the other tags for expression and solubility of the protein (Butt et al. 2005; Satakarni and Curtis 2011). Many complications concerning the expression of MTs intracellularly could be solved by SUMO fusion technology. Furthermore, we constructed two mutants including SUMO-MTt1 and SUMO-MTt2 by site-directed mutagenesis techniques on the basis of constructing SUMO-MT and investigated that the *S. henanense* metallothionein and its mutants bestowed enhanced metal tolerance as well as increased accumulation of Zn, Cu and Cd. Therefore, the genetically modified *E. coli* has the potential to be used for the bioremediation of heavy metal pollution. Also, the present study could help to elucidate the mechanism of how the crab MT is effective in heavy metal detoxification and provide a scientific basis for bioremediation of heavy metal pollution by over-expressing the crab MT and mutant proteins.

Materials and methods

Construction of the expression vector for MTs

To construct the expression plasmid, *S. henanense* MT was engineered with restriction enzyme sites proximal to the coding region of the gene, to facilitate cloning into the expression vector. This was performed by PCR amplification with the sense primer L1 and the anti-sense primer L2 (Table 1) (Sangon, China) containing *Bam*HI and *Hin*dIII sites. The cloning plasmid (pGEM-T-MT) that was constructed in a previous study (Ma et al. 2009) and served as a PCR template. PCR amplifications were performed using one cycle at 94 °C for 5 min; 30 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min; followed by one cycle at 72 °C for 10 min, and hold 4 °C. The purified fragment was digested with *Bam*HI and *Hin*dIII (TAKARA, China), ligated to the pET-28a-6His-SUMO expression vector (GeneCopoeia, USA) for constructing recombinant proteins, and transformed into DH5α competent cells. After sequencing the positive clones in order to ensure in-frame insertion, the recombinant pET-28a-6His-SUMO-MT vector was extracted and used to transform into *E. coli* BL21 (DE3) strain (Novagen, Germany) for protein expression.

Selection of transformed colonies was performed on LB agar plates containing 50 μg/ml kanamycin. Simultaneously the PET-28a plasmid (without SUMO) was introduced into the same strain as a control.

Expression and purification of the fusion protein SUMO-MT

Successfully transformed *E. coli* cells were picked from a single colony and grown overnight at 37 °C in LB medium with 50 μg/ml kanamycin. The culture mixture was diluted 1:100 (v/v) in fresh LB medium supplemented with 50 μg/ml kanamycin. Cells were grown at 37 °C under continuous shaking (200 rpm), until the A_{600} of 0.6 was reached, and expression of the recombinant protein was induced by isopropyl-beta-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. During induction, 1 mM $ZnSO_4$ was added to increase SUMO-MT stability.

Prior to purification, the induced bacteria were harvested by centrifugation at 6,000 g for 20 min at 4 °C and resuspended in five times of the quantity of bacteria (v/w) of lysis buffer (20 mM Tris–HCl, pH 7.8, 0.5 M NaCl, 0.2 mM PMSF, 10 mM imidazole and 0.1 % Triton). Cells were disrupted at 800 bar for 3 min by high-pressure homogenizer (ATS Engineering Inc., Canada) and cell debris were removed by centrifugation at 14,000 g for 25 min at 4 °C (Hitachi CR22G III, Hitachi Inc., Japan). The supernatant was filtered through a 0.45 μm filter and purified by immobilized metal affinity chromatography (IMAC) using a Ni sepharose™ 6 Fast Flow (GE Healthcare, USA) to selectively bind the 6His tag of the fusion protein. Recombinant protein 6His-SUMO-MT was eluted from the resin containing 20 mM Tris–HCl (pH 7.8), 200 mM imidazole, and 0.5 M NaCl. Then the purity and concentration of the recombinant protein were assessed on a 12 % SDS-PAGE.

Production of antiserum and evaluation of antibody activity by indirect ELISA

Imidazole was eliminated from the purified protein through dialysis using a 3000 MWCO membrane (Millipore, USA) against 0.01 M Tris–HCl (pH 7.8) containing 0.2 mM PMSF. Then the recombinant SUMO-MT was rapidly mixed with an equal volume of Quick Antibody (Kangbiquan Inc., China), a novel water-solubility immunoadjuvant without emulsification to distinguish from the Freund's adjuvant. The resulting mixture was transferred to a 1 mL-syringe and was then injected into the hind leg muscle of a mouse. After 3 weeks, the antigen was dissolved in Quick Antibody adjuvant to conduct a boosting injection in the same way. At the 5th week, the blood was collected from the eyeball and centrifuged. It was set still at room temperature for 1 h and centrifuged at 3,000 rpm for 5 min. The supernatants were transferred to new test tubes and set still at 4 °C overnight. After removing precipitated residues, the blood sera were added with 0.02 % NaN_3 and stored at −80 °C until use.

Antibody activity was determined for the prepared antisera according to the indirect ELISA method (Ryu et al. 2009; Kim et al. 2011). To each well of a 96 well microplate, 100 μL of the pure SUMO-MT solution (10 μg/mL) dissolved in the coating buffer (0.5 M $Na_2CO_3/NaHCO_3$, pH 9.6) was added and the microplate was set still at 4 °C overnight. The antiserum was diluted in 200–102,400 folds with 1 × pH 7.4 PBS. The incubation with the secondary antibody was carried out with 100 μL of a 5,000 fold diluted horseradish peroxidase-conjugated goat antibody (GeneTex, USA) diluted with PBSTB (1 × pH 7.4 PBS, 1 % BSA, 0.1 % Tween). Then added 100 μL substrate mixture of o-phenylenediamine (OPD) (Amresco, USA) per well and incubated for 30 min at room temperature. Finally, 100 μL of 2 M H_2SO_4 was added to stop the reaction and quantification was performed with a microplate reader (TECAN Infinite M200, Switzerland) at 492 nm.

SUMO-MT protein analysis

The purified fusion proteins were digested by SUMO protease (GeneCopoeia, USA) at 30 °C for 2 h on an orbital shaker and subsequently heated to 80 °C for 20 min. The denatured proteins were precipitated at 20,800 g for 20 min and the supernatant was concentrated. SUMO-MT fusion proteins and the supernatant MT sample were identified by Western blot analysis using the mouse antiserum and an anti-6His antibody (GeneTex, USA), respectively. The

Table 1 Oligonucleotide primers used for constructing SUMO-MT, SUMO-MTt1 and SUMO-MTt2 plasmid

Primer	Length	Sequence (5′–3′)	Remark
L1	23	CGGGATCCATGCCTGATCCTTGC	*BamHI* site
L2	24	CCAAGCTTTTATCAGGGGCAGCAG	*Hind*III site
L3	30	AAGGATCCATGCCTTGTCCTTGCTGCACAG	Mutation site
L4	30	CTGTGCAGCAAGGACAAGGCATGGATCCTT	–
L5	30	GCGAGAAATGCAAATGCGAGTGCAAGTGCA	Mutation site
L6	30	TGCACTTGCACTCGCATTTGCATTTCTCGC	–

Fig. 1 Amino acid sequences of metallothionein from SUMO-MT and SUMO-MTt1 and SUMO-MTt2 are aligned. The mutant site are indicated by *shaded boxes*

MT region of SUMO-MT

1 M P D P C C T E G T C E C A E G K C K S

21 G C K C T S C R C S P C E K C K S E C K

31 C S T A E E C A K N C T K P C S C C P

MTt1 region of SUMO-MTt1(D3C)

1 M P C P C C T E G T C E C A E G K C K S

21 G C K C T S C R C S P C E K C K S E C K

31 C S T A E E C A K N C T K P C S C C P

MTt2 region of SUMO-MTt2(S37C)

1 M P D P C C T E G T C E C A E G K C K S

21 G C K C T S C R C S P C E K C K C E C K

31 C S T A E E C A K N C T K P C S C C P

SUMO-MT and MT proteins were run on 15 % SDS-PAGE and subsequently transferred to a PVDF membrane (Bio-Rad, USA). Following transfer, the membrane was blocked with 5 % skim milk at pH 7.4 PBS for 60 min at 4 °C and was incubated with primary antibody, mouse antiserum (1:500) or anti-6His antibody (1:1,000) for 2 h at 4 °C and then incubated with 1:5,000 diluted goat anti-mice IgG conjugated with alkaline phosphatase (Sangon, China) (Yudkovski et al. 2008; Gao et al. 2009). The bands were visualized by NBT/BCIP substrates (Amresco, USA).

Site-directed mutagenesis and construction of expression plasmids

On the basis of constructing SUMO-MT site-directed mutagenesis was performed according to the manufacturer's instructions with QuickChange Site-Directed Mutagenesis kit (Stratagene, USA) (Wang et al. 2006; Liu and Lomonossoff 2006). Two mutants named SUMO-MTt1 and SUMO-MTt2 were constructed with the following amino acid substitutions: D3C and S37C (Fig. 1), respectively, in order to turn aspartic acid into cysteine and add Cys-Xaa-Cys. The SUMO-MT plasmid served as a PCR template and L3/L4 primers and L5/L6 primers (Table 1) (Sangon, China) were used for mutagenesis SUMO-MTt1 (D3C) and SUMO-MTt2 (S37C), respectively. Two mutants were also confirmed by DNA sequencing (Sangon, China) and transformed into *E.coli* BL21 (DE3) strain.

Metal tolerance assays and determination of metal ion accumulations in *E. coli*

Overnight cultures (500 μL) were inoculated into fresh liquid LB medium (50 mL) containing 50 μg/mL kanamycin with *E. coli* BL21 expressing SUMO-MT or SUMO-MTt1 or SUMO-MTt2. When the cultures were grown to OD_{600} 0.6, Zn^{2+} (1.0 mM $ZnSO_4$), Cu^{2+} (2.0 mM $CuSO_4$) or Cd^{2+} (0.5 mM $CdCl_2$) was added, respectively. This was followed by the induction of gene expressions by the addition of IPTG (1 mM). Cultures were grown under rotary shaking (200 rpm) at 37 °C and growth (absorbance OD_{600}) was measured up to 8 h for a metal tolerance assay.

To measure the metal content in *E. coli* cells, the overnight cultures of the three recombinant bacteria and control cells (PET-28a) were diluted to 1:100 in 50 mL fresh LB medium supplemented with 50 μg/mL kanamycin. Until the OD_{600} reached 0.6, 1 mM IPTG and metal ions with the same concentration (300 μM $ZnSO_4$, $CuSO_4$ and $CdCl_2$) were added and cells were cultured for 6 h at 37 °C under shaking (200 rpm). Cells were recovered by centrifugation at 6,000×g for 15 min at 4 °C. The cell pellets were washed twice in fresh LB medium and dry weight of cells was measured after dehydration at 80 °C for 48 h. The metal ion accumulation was determined by air-acetylene flame atomic emission spectrometry (SHIMDZU AA-6300, Japan), measuring Zn at 213.9 nm, Cu at 324.8 nm and Cd at 228.8 nm. Metal ion accumulation was expressed as μmol g^{-1} dry weight bacterial cells.

Statistical analysis

All the data were represented as the mean ± standard deviation. Statistical analysis was performed using SPSS 19.0 software. Data from recombinant *E. coli* cells with SUMO-MT or SUMO-MTt1 or SUMO-MTt2 were compared with those from control *E. coli* cells with PET-28a.

Moreover, data from recombinant *E. coli* cells with SUMO-MTt1 or SUMO-MTt2 were compared with those from recombinant *E. coli* cells with SUMO-MT. Statistical analysis was carried out using one-way ANOVA followed by LSD. A probability value of less than 0.05 was regarded as significant.

Results and discussion

Expression and purification of recombinant SUMO-MT

DNA sequencing confirmed that the pET-28a-6His-SUMO-MT expression plasmid included no nucleotide substitutions, and that the MT gene was cloned in the correct frame after the 6His-SUMO coding sequence.

In order to enhance the metal tolerance and accumulation of bacteria, the high metal-binding capacity of MT has been widely exploited. So far MTs from various sources have been expressed intracellularly in *E. coli* (Sauge-Merle et al. 2012; Sekhar et al. 2011; Chaturvedi et al. 2012; Zhang et al. 2012). However, there are many complications concerning the expression of MTs intracellularly. The high cysteine content of MTs interferes with the redox pathways in the cytosol and the intra-molecular reaction of SH groups can possibly result in the formation of inclusion bodies in the recombinant *E. coli* expressing MT. Moreover, MTs are quite unstable with a short half-life owing to its low molecular weight (Mejáre and Bülow 2001; Suleman and Shakoori 2012). Many researchers overcome these difficulties by preparing fusion proteins with host proteins such as GST and MBP proteins (Ma et al. 2011; Morris et al. 1999; Sauge-Merle et al. 2012; Suleman and Shakoori 2012). Nevertheless, sometimes there are still some problems, e.g., a partial proteolysis of recombinant MT fusion with MBP (Sauge-Merle et al. 2012) and the formation of inclusion bodies in the recombinant *E. coli* expressing GST-MT (Chen and Wilson 1997; Yang et al. 2007; Ma et al. 2011). Actually, we have surveyed the expression performance of the crab MT with GST by constructing recombinant pGEX-6p-1-MT vector. Although we could obtain the recombinant GST-MT, a large portion of the fusion protein was still expressed as inclusion bodies (data not show). In the present study, we inserted the *S. henanense* MT gene downstream of the SUMO gene to express the stable fusion protein SUMO-MT. SUMO fusions are superior to the other tags for expression and solubility of the protein (Butt et al. 2005; Satakarni and Curtis 2011). In addition, unlike other proteases, SUMO proteases recognize the tertiary structure of the SUMO tag and cleave specially to yield a native-like protein without extraneous residues in comparison with other traditional fusion systems.

As shown in Fig. 2, SUMO-MT can be induced by adding IPTG compared to the control and a high amount of soluble SUMO-MT fusion protein migrated in SDS-PAGE with an apparent molecular weight of 26 kDa (Fig. 2, lane 3). The overexpressed protein was not detected in an uninduced extract (Fig. 2, lane 2). The results showed that recombinant SUMO-MT had a higher level of expression in agreement with GST and MBP. Moreover, it was observed primarily in the soluble fraction and less easily digested. The effect that SUMO can enhance protein solubility may be explained in part by the structure of SUMO. SUMO has an external hydrophilic surface and inner hydrophobic core and may exert a detergent-like effect on otherwise insoluble proteins (Butt et al. 2005). In addition, it is noteworthy that it is necessary to add metal ions (zinc or cadmium) to the medium after induction in order to stabilize MT clusters and prevent their self-aggregation as reported by Lim et al. (2010). SDS-PAGE results showed multiple bands of SUMO-MT in the absence of metal ions (not shown), while a single band of SUMO-MT was observed in the presence of metal ions after purifying. Because the SUMO constructs bear an N-terminal 6His tag, expressed SUMO-MT proteins can be rapidly purified by Ni–NTA affinity chromatography (Fig. 2, lane 5). Recombinant SUMO-MT was separated from the 6His-SUMO-tail by enzymatic cleavage using SUMO protease (Fig. 2, lane 4). The digestion products of SUMO-MT were heated and concentrated. The supernatant contained the about 7 kDa MT without the SUMO tag.

Production of antiserum and western blot

The antibody production lasted for 5 weeks and used three injections. Although the specificity of the antiserum was good, when using the crab digestion MT as antigen, the antiserum activity was generally poor in the case of an antigenic protein with a molecular mass below 10 kDa (Kim et al. 2011). So the fusion SUMO-MT was chosen as the antigen instead of the crab MT from digestion of SUMO-MT. To measure the antibody activities, an indirect ELISA was carried out. As shown in Fig. 3, when the anti-SUMO-MT serum was diluted for 1,02,400 times, the absorption value was 0.47 at 492 nm. This was more than 0.1 and 2.1 times of the negative pre-immune serum. The titer of SUMO-MT antiserum was more than 1: 1,02,400 by indirect ELISA.

Recombinant SUMO-MT and MT from digestion of SUMO-MT were examined by Western blotting analysis using the mouse antiserum and an anti-6His antibody, respectively. As shown in Fig. 4, the 26 kDa purified recombinant SUMO-MT protein and the 7 kDa MT were detected by the antiserum. However, between the two protein samples only the SUMO-MT with 6His tag was

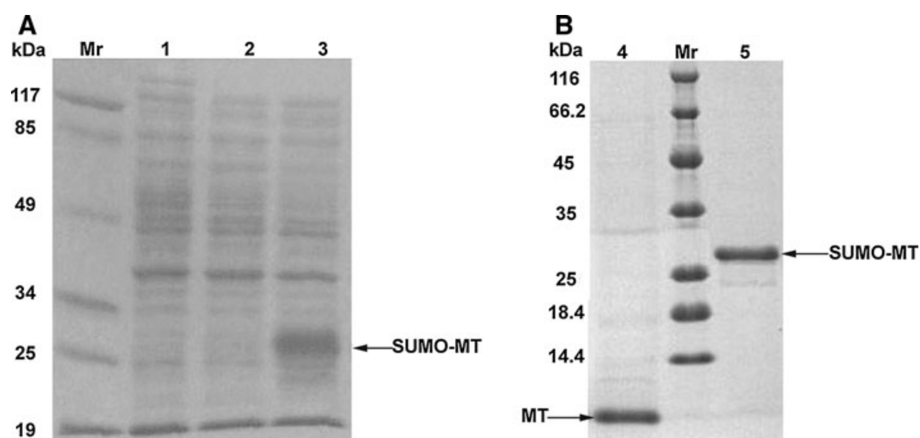


Fig. 2 Expressing of SUMO-MT fusion protein in *E. coli* induced by IPTG (1 mM) and isolated on 12 % SDS-PAGE. *Mr* protein molecular-weight markers; *lane 1* total protein extracts of the control bacterial culture (including PET-28a vector); *lane 2* total protein extracts of the bacterial culture expressing SUMO-MT without IPTG; *lane 3* total protein extracts of induced culture expressing SUMO-MT with 1 mM IPTG; *lane 4* MTs in the supernatant after protease cleavage (about 7 kDa); *lane 5* purified SUMO-MT after Ni-NTA affinity chromatography

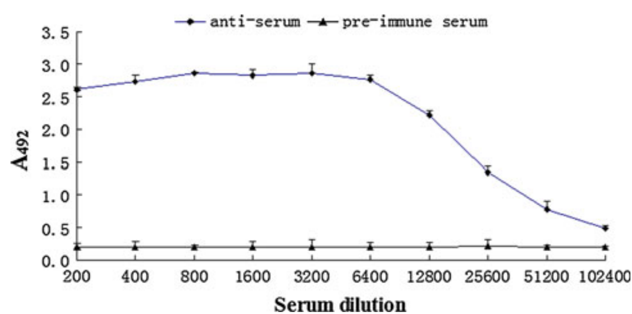


Fig. 3 Results of indirect ELISA to determine antibody activities of anti-SUMO-MT serum

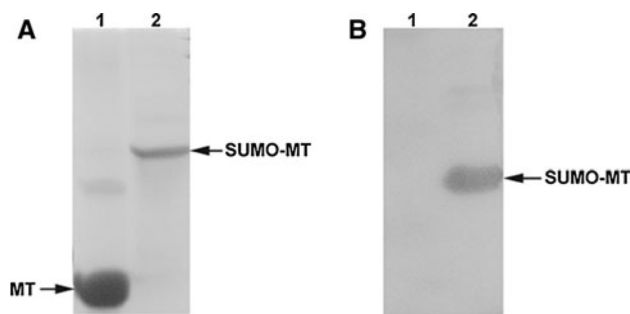


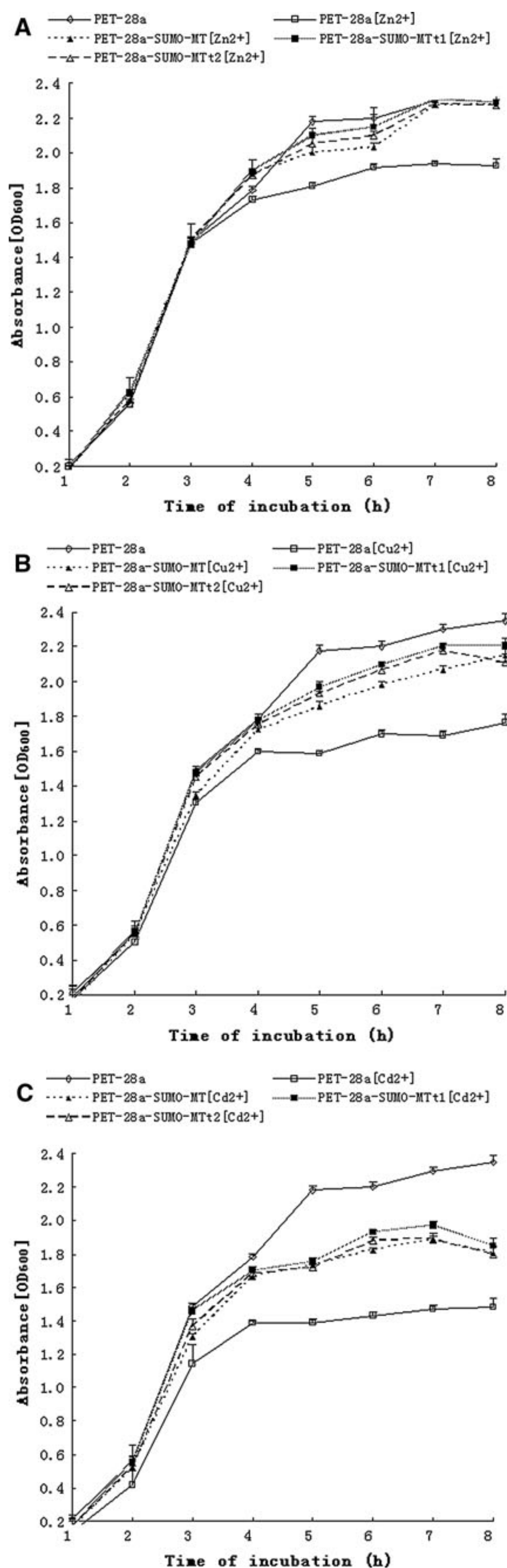
Fig. 4 Western blot of recombined SUMO-MT fusion protein and MTs of a cleavage product with anti-SUMO-MT serum (**a**) and anti-6His antibody (**b**), respectively. *Lane 1* MTs of cleavage product; *lane 2* recombined SUMO-MT

detected by the anti-6His antibody. The results further confirmed the antiserum prepared corresponds to the MT domain of SUMO-MT.

Metal tolerance and ion accumulation in *E. coli* expressing SUMO-MT and its mutants

Despite the efforts on examining the metal-binding capacity of MT taking *E.coli* as a model, information on genetically modified MTs is still scarce. There are only a few researchers who have attempted to increase the metal-binding capacities of the particular MT by over-expression oligomeric metallothioneins (Ma et al. 2011; Hong et al. 2000). The induction of oligomeric metallothioneins increases the stability of recombinant MT and heavy metal bioaccumulation capacity of the recombinant *E. coli* cells. However, the number of the integrated MT molecules is limited as inclusion bodies tend to be formed when excessive MT molecules are integrated (Ma et al. 2011; Hong et al. 2000). Thus, the increase of the metal-binding capacity is limited. In our study, we changed the primary structure of MT to increase the metal-binding capacities by site-directed mutagenesis techniques instead of the introduction of oligomeric metallothioneins.

To test for the effect of SUMO-MT, SUMO-MTt1 and SUMO-MTt2 fusion protein expression on metal tolerance of *E. coli,* the three recombinant strains and control strain were subjected to 1.0 mM $ZnSO_4$ or 2.0 mM $CuSO_4$ or 0.5 mM $CdCl_2$. In the absence of metal, the growth curve of cells containing PET-28a-SUMO-MT was similar to the cells containing PET-28a (data not show). *E. coli* cells expressing SUMO-MT or SUMO-MTt1 or SUMO-MTt2 showed a significant tolerance for Zn^{2+}, Cu^{2+} and Cd^{2+} ions compared to the cells transformed with PET-28a vector; however, a low tolerance was observed for cadmium compared to zinc and copper. It may be related to the fact that zinc and copper are essential for growth (Wu et al. 2008) and cadmium is a nonessential metal and can lead to higher toxicity. The tolerance of zinc was higher compared to copper and this may be due to copper being more toxic



◀ **Fig. 5** Metal tolerance in *E. coli* cells expressing SUMO-MT or SUMO-MTt1 or SUMO-MTt2 subjected to metal stress. *E. coli* cells expressing SUMO-MT or SUMO-MTt1 or SUMO-MTt2 on medium containing $ZnSO_4$ (1.0 mM) (**a**), $CuSO_4$ (2.0 mM) (**b**) or $CdCl_2$ (0.5 mM) (**c**) showed a higher tolerance to metal than the control cells (PET-28a). The tolerance of Zn, Cu and Cd in various strains followed the following order: SUMO-MTt1 > SUMO-MTt2 > SUMO-MT > control

than zinc. Figure 5 showed that the tolerance of Zn^{2+}, Cu^{2+} and Cd^{2+} in these strains followed the decreasing order of SUMO-MTt1 > SUMO-MTt2 > SUMO-MT. Our results prove that changing primary structure can lead to increased tolerance of metal ions of recombinant *E. coli* cells.

At the same concentration of the metal ion, metal accumulation in *E. coli* cells expressing SUMO-MT or SUMO-MTt1 or SUMO-MTt2 was significantly higher compared to the control (*E. coli* transformed with PET-28a) ($p<0.01$). The higher metal accumulation in *E. coli* expressing SUMO-MT is due to the metal binding protein expressed by these cells. The cadmium accumulation was higher than zinc and copper in these recombinant strains (Fig. 6). In these strains the accumulation of metal ions showed a similar trend as did the metal tolerance. Sequestration of metal ions except Cu^{2+} in *E. coli* cells expressing SUMO-MTt1 was considerably higher compared to SUMO-MT and SUMO-MTt2 ($p < 0.01$). Especially, the accumulation of cadmium reached 4.67×10^{-5} mol/g DW (dry weight) in *E. coli* cells expressing SUMO-MTt1 (Fig. 6 c). It was 4-fold higher with SUMO-MT and was 2-fold higher with SUMO-MTt2. Sequestration of zinc ions was higher compared to copper while maximum sequestration occurred with cadmium. Similar results have been observed previously (Chaturvedi et al. 2012; Sauge-Merle et al. 2012). It may be because the induction by cadmium may have enhanced the expression of SUMO-MT and the respective mutants besides the activity of its native gene compared with zinc and copper (Chaturvedi et al. 2012). Furthermore, the single-mutant proteins, SUMO-MTt1 and SUMO-MTt2 showed a higher accumulation and greater tolerance than SUMO-MT because they changed SUMO-MT structure and may have provided additional metal-binding sites (Chen et al. 1999; Carpenè et al. 2007). The results show that the bioaccumulation capacity of metal ions of the recombinant *E. coli* cells could be increased by the introduction of mutations (additional metal-binding sites) as well as the overexpression of oligomeric MTs (Ma et al. 2011). However, the finding that sequestration of Cu^{2+} in *E. coli* cells expressing SUMO-MTt2 was higher compared to SUMO-MTt1 shows the introduction of mutations by addition metal-binding sites

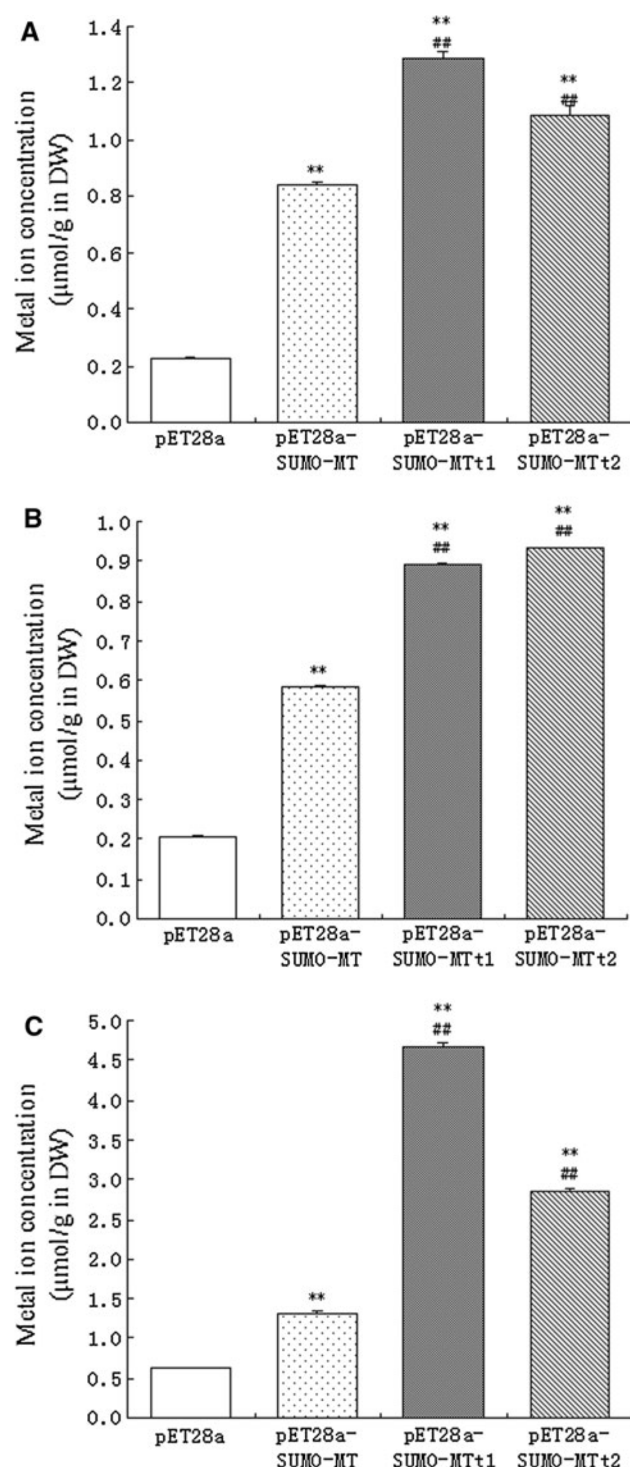


Fig. 6 Metal accumulation of *E. coli* cells expression SUMO-MT or SUMO-MTt1 or SUMO-MTt2 subjected to metal stress following $ZnSO_4$ (**a**), $CuSO_4$ (**b**) and $CdCl_2$ (**c**). Accumulation of metal ions in cells containing PET-28a vector and PET-28a-SUMO-MT or PET-28a-SUMO-MTt1 or PET-28a-SUMO-MTt2 vector in the presence of 300 μM for 6 h at 37 °C. Data represent mean ± SD of five independent experiments. DW represents dry weight. Statistical significances were analyzed using one-way ANOVA compared with the control (PET-28a), $*p < 0.05$, $**p < 0.01$; comparing with the PET-28a-SUMO-MT, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$

may not only increase binding capacity but also alter metal-specificity. Our data demonstrate that the *S. henanense* MT and its mutants can protect bacterial cells from the deleterious effects of heavy metals.

Conclusions

In this study, the *S. henanense* MTs were successfully expressed and purified as fusion protein with SUMO. Overexpression of SUMO-MT gene not only imparted enhanced zinc, copper and cadmium tolerance but also increased metal ion accumulation in *E. coli* cells. The *S. henanense* MTs were supposed to have a functional role in metal tolerance and their expression is highly up-regulated under metal stress. Moreover, the SUMO-MT mutants constructed by site-directed mutagenesis techniques can significantly increase bacterial tolerance and heavy metal accumulation compared with SUMO-MT. These results suggest that we can enhance the efficiency of bioremediation by changing amino acid sequence and adding a new metal-binding site on the basis of transforming the *S. henanense* MTs into bacterial and plant cells. The *S. henanense* MT and mutants can serve as candidates for bioremediation applications of heavy metal pollution.

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Conflict of interest The authors declare that they have no conflict of interest.

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