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Abstract: Metallothioneins (MTs) are a family of low molecular weight, cysteine-rich, metal-binding proteins, which play important roles in metal homeostasis and heavy metal detoxification. In our previous study, a novel full length MT cDNA was successfully cloned from the freshwater crab (Sinopotamon henanense). In the present study, tandem repeats of two and three copies of the crab MT gene were integrated by overlap extension PCR (SOE-PCR) and expressed in Escherichia coli. The SUMO fusion expression system was adopted to increase the stability and solubility of the recombinant MT proteins. Furthermore, the metal tolerance and bioaccumulation of E. coli cells expressing oligomeric MTs were determined. Results showed that the recombinant plasmids pET28a-SUMO-2MT and pET28a-SUMO-3MT were successfully constructed with high-fidelity. Oligomeric MTs expression significantly enhanced Cu, Cd and Zn tolerance and accumulation in E.coli. in the order: SUMO-3MT > SUMO-2MT > SUMO-MT > control. Cells harboring pET28a-SUMO -3MT exhibited the highest Cu, Cd and Zn bioaccumulation at 5.8-fold, 3.1-fold and 6.7-fold respectively higher than that of the control cells. Therefore, oligomeric MTs have potential as candidates for heavy metal bioremediation. Our research lays the foundation for the development and application of MTs in the biological treatment of heavy metals.

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***Cover Letter**

Dear editor,

We would like to submit a manuscript entitled "Tandem oligomeric expression of metallothionein from freshwater crab (*Sinopotamon henanense*) in *Escherichia coli* enhances heavy metal tolerance and bioaccumulation" by Wenli Ma *et al.* for publication in Ecotoxicology and Environmental Safety.

In the present study, tandem repeats of *S. henanense* MT genes were integrated in series with gene splicing by overlap extension and the souble expressions of oligomeric MTs were achieved with the adoption of SUMO fusion system. Results showed that oligomeric MTs expression significantly enhanced Cu, Cd and Zn tolerance and accumulation in *E.coli*. Our research lays the foundation for the development and application of MTs in the biological treatment of heavy metals.

Thank you very much for your kind consideration.

Best wishes,

Yours sincerely,
Wenlima

Statement of novelty

1. For the first time, tandem repeats of *S. henanense* MT genes were integrated in series with gene splicing by overlap extension.
2. For the first time, the souble expressions of oligomeric MTs were achieved with the adoption of SUMO fusion system.
3. Oligomeric MTs expression significantly enhanced Cu, Cd and Zn tolerance and accumulation in *E.coli*.

Tandem oligomeric expression of metallothionein from freshwater crab (*Sinopotamon henanense*) in *Escherichia coli* enhances heavy metal tolerance and bioaccumulation

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Abstract

Metallothioneins (MTs) are a family of low molecular weight, cysteine-rich, metal-binding proteins, which play important roles in metal homeostasis and heavy metal detoxification. In our previous study, a novel full length MT cDNA was successfully cloned from the freshwater crab (*Sinopotamon henanense*). In the present study, tandem repeats of two and three copies of the crab MT gene were integrated by overlap extension PCR (SOE-PCR) and expressed in *Escherichia coli*. The SUMO fusion expression system was adopted to increase the stability and solubility of the recombinant MT proteins. Furthermore, the metal tolerance and bioaccumulation of *E. coli* cells expressing oligomeric MTs were determined. Results showed that the recombinant plasmids pET28a-SUMO-2MT and pET28a-SUMO-3MT were successfully constructed with high-fidelity. Oligomeric MTs expression significantly enhanced Cu, Cd and Zn tolerance and accumulation in *E.coli*. in the order: SUMO-3MT > SUMO-2MT > SUMO-MT > control. Cells harboring pET28a-SUMO -3MT exhibited the highest Cu, Cd and Zn bioaccumulation at 5.8-fold, 3.1-fold and 6.7-fold respectively higher than that of the control cells. Therefore, oligomeric MTs have potential as candidates for heavy metal bioremediation. Our research lays the foundation for the development and application of MTs in the biological treatment of heavy metals.

Keywords

Metallothionein

Gene splicing by overlap extension

SUMO fusion expression

Metal tolerance

Metal bioaccumulation

1. Introduction

In recent decades heavy metal pollution has become an increasingly serious environmental problem for humans and other organisms due to industrialization, urbanization and population growth (Cheng, 2003). Unlike other pollutants, heavy metals are difficult to remove from the environment and cannot be chemically or biologically degraded (Mejáre et al., 2001). They can enter into food chains and do serious harm to both animal and human health (Chen et al., 1999).

The conventional technologies used in removing heavy metals are mainly physico-chemical, such as chemical precipitation, filtration, ion exchange, electrochemical treatment, membrane technologies, and adsorption on activated carbon (Wang et al., 2009). However, these treatments are usually expensive and their metal binding capacities are not high (Hamidi et al., 2004; Gecol et al., 2004; Litter et al., 2010). In recent years, bioremediation, for example microbial flocculation and biosorption, has become an emerging tool for removal of heavy metals (Singh et al., 2011). Bioremediation has the advantages of low cost, high binding affinity, large available quantities and relatively little damage to the environment compared to physico-chemical methods (Malik 2004; Shukla et al., 2010). Among them, the use of genetically engineered bacteria over-expressing metal-binding proteins such as metallothioneins (MTs) has drawn increasing attention (Deng et al., 2007; Su et al., 2009; Sauge-Merle et al., 2012).

Metallothioneins (MTs) are a family of low molecular weight, cysteine rich, metal-binding proteins, which exist in many organisms (Kagi 1988). MTs are involved in essential-metal homeostasis, heavy metal detoxification and cytoprotection (Margoshes et al., 1957; Roesijadi 1992). The freshwater crab *Sinopotamon henanense* is widely distributed in Shanxi Province, China, an area that has endured serious environmental pollution. The crabs live in sediment and face heavy metals directly both via their integument and via their food. In our previous study, *S. henanense* showed a strong capability to accumulate heavy metals (Ma et al., 2008). A novel MT from *S. henanense* has been purified and its full length cDNA has

been cloned. Sequence analyses indicated that the isolated cDNA encoded a protein with 59 amino acids containing 18 cysteine residues, implying that the *S. henanense* MT protein has a high metal-binding capacity and unique properties of metal affinity (Ma et al., 2009).

The recombinant *E. coli* expressing MT gene showed enhanced metal bioaccumulation, indicating that both MT and the engineered bacteria have great potential as biomaterials for bioremediation (Kim et al., 2005; Deng et al., 2007; Singh et al., 2008; Sekhar et al., 2011). However, the expression efficiency of functional MT proteins in *E.coli* have so far been limited, because MTs are unstable due to their low molecular weight, as well as the oxidation of their cycteine thiol groups, resulting in proteolysis during expression in the host cells (Suleman et al., 2012; Morris et al., 1999; He et al., 2014).

To improve the expression efficiency of functional MT proteins in *E.coli*, a useful strategy is constructing an expression vector with tandem repeated multiple MT genes (Hou et al., 2009; Rao et al., 2005). But this leads to a new problem of an increase in inclusion bodies due to the increase of the molecular weight of the recombinant protein. In recent years, small ubiquitin-related modifier (SUMO) has become an effective biotechnological tool as a fusion system (Zhang et al., 2018; Liu et al., 2012). Advantages of the SUMO fusion system include promoting correct protein folding, increasing protein solubility, reducing proteolytic degradation of the target protein and simplification of purification and detection compared with traditional fusion systems (Marblestone et al., 2010).

In this study, tandem repeats of *S. henanense* MT genes were integrated in series and expressed in *E. coli* in order to enhance heavy metal binding capacity. Furthermore the SUMO fusion system was adopted to improve protein folding, solubility and yield of recombinant oligomeric MTs. These oligomeric MTs can bind larger amounts of heavy metals than monomeric MTs, and thus both oligomeric MTs and the genetically modified *E. coli* have great potential for use in bioremediation of heavy metal pollution.

2. Materials and Methods

2.1 Bacterial Strains, Vectors, Primers and Enzymes

E. coli competent cells DH5α and BL21 (DE3) were purchased from TransGen Biotech (Beijing, China). Expression vector pET28a-SUMO was purchased from Miaoling Biotech (Wuhan, China). Cloning vector pMD18-T vector was purchased from TaKaRa (Dalian, China). The restriction enzymes BamH Ⅰ and Hind Ⅲ, T4 DNA ligase and the high-fidelity thermostable DNA polymerase were from TaKaRa. Primers were synthesized by Shanghai Sangon (China). Immobilized metal ion affinity chromatography Ni-NTA (Pre-Packed Gravity Column) was from Shanghai Sangon (China).

2.2 Construction of expression plasmids by SOE-PCR

The dimer of the crab MT gene was integrated by overlap extension PCR (SOE-PCR) (Horton et al., 1993; Heckman et al., 2007); see Fig.1 for primer design and PCR strategy. SOE-PCR involved three separate PCRs: the two DNA fragments produced in the first stage reactions were mixed to form the template for the second stage. The recombinant plasmid pET28a- SUMO-MT which we previously constructed served as the PCR template in the two first stage reactions. The first and second PCR amplifications were performed using one cycle at 98℃ for 3 min; 30 cycles at 98℃ for 30s, 60℃ for 30s, and 72℃ for 30s; followed by one cycle at 72℃ for 7 min, and hold at 4℃. The first and second PCR products were mixed as templates for the third PCR. The third PCR amplification was performed using one cycle at 98℃ for 3 min; 25 cycles at 98℃ for 30s, 64℃ for 45s, and 72℃ for 30s; followed by one cycle at 72 ℃ for 7 min, and hold at 4℃. High-fidelity thermostable DNA polymerase (TaKaRa) was employed. The purified fragment MT-MT was digested with BamHI and HindIII, ligated to the pET-28a-SUMO expression vector, and transformed into *E.coli* DH5α competent cells. The trimer of the crab MT gene was integrated by the same strategy. The recombinant plasmid pET28a-SUMO-2MT and pET28a-SUMO-MT served as PCR templates in the two first stage reactions. The constructed plasmids were sequenced by Shanghai

Sangon Biotech Co., Ltd.

2.3 Optimization of expression conditions and purification of recombinant dimeric and trimeric MT

After sequencing, the recombinant vectors pET-28a-SUMO-2MT and pET-28a-SUMO-3MT were transformed into *E. coli* BL21 (DE3) strain for protein expression. Selection of transformed colonies was performed on LB agar plates containing 50 μg/mL kanamycin. Successfully transformed *E. coli* cells were picked from a single colony and grown overnight at 37℃ 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℃ under continuous shaking (180rpm), until the OD600 reached 0.6, and expression of the recombinant protein was induced by different concentrations (0.1mM, 0.3mM, 0.7mM and 1.0mM) of isopropyl-beta-D-thiogalactopyranoside (IPTG), at different induction times (1hr, 3hr, 5hr, 7hr and 9hr) and different temperatures (20℃, 30℃ and 37℃). The cells were harvested by centrifugation at 6000 g for 20 min at 4℃ and lysed by sonication with 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). Cell debris was removed by centrifugation at 12500 g for 20 min at 4℃ and the supernatants analyzed by SDS-PAGE to detect the optimal IPTG concentration, the optimal induction time and temperature. Then the recombinant proteins were purified with Ni-NTA (Pre-Packed Gravity Column) and eluted with 50, 100, 250 and 500 mM imidazole buffers. The purified fractions were further treated using an ultrafiltration centrifugal tube (Millipore, 3kD) to remove the imidazole.

2.4 Characteristics of oligomeric MTs

The purified apo-MT, apo-2MT and apo-3MT were incubated overnight with the addition of 300mM $ZnCl_2$, $CdCl_2$ and $CuSO_4$ in the presence of 100mM DTT, pH7.8, 10mM Tris-HCl buffer. Then, the ultraviolet absorption spectra of metal-incubated and apo-forms of MTs were determined between 200 and 300nm using a spectrophotometer (UNICO UV-2102PC).

2.5 Metal tolerance of E. coli cells expressing MTs

Metal tolerance assays were carried out according to Zhang *et al.* (2014) with slight modification. *E. coli* cells harboring pET28a-SUMO, pET28a-SUMO-MT, pET28a-SUMO-2MT and pET28a-SUMO-3MT were cultured in liquid LB medium until the OD600 reached 0.6, 0.7mM IPTG was added and cells were grown for an additional 4hr at 30℃. After serial dilutions (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, respectively), each dilution was spotted either on solid LB plates or on plates with $CuSO_4$ (140, 280, 420μM), $CdCl_2$ (50, 100, 150μM) or $ZnSO_4$ (160, 320, 480μM). Plates were photographed after incubation at 37℃ for 12h.

2.6 Growth kinetics of E. coli cells harboring oligomeric MTs

Growth kinetics of *E. coli* cells were used to analyze the tolerance of *E. coli* expressing oligomeric MTs to Cu^{2+}, $Cd^{2+}$and Zn^{2+} according to the method of Niu *et al*. (2018). *E. coli* cells (harboring pET28a-SUMO-MT, pET28a-SUMO-2MT and pET28a-SUMO-3MT) were grown at 37ºC to mid-logarithmic phase (OD600 reached 0.5-0.6). Then IPTG was added at a final concentration of 0.7mM. After 0.5 h, $CuSO_4$, $CdCl_2$ or $ZnSO_4$ were added at a final concentration of 200, 50 and 100μM, respectively. *E. coli* cells (harboring pET28a-SUMO) were treated identically as control. OD600 measurements were conducted to analyze the effects of Cu^{2+}, Cd^{2+} and Zn^{2+} on bacterial growth at 1 h intervals for 8 h at 30ºC.

2.7 Metal bioaccumulations in E. coli cells expressing MTs

The metal bioaccumulations in *E. coli* cells expressing MTs were determined according to the method of Sauge-Merle *et al.* (2012). Cultures of the control cells (harboring pET-28a-SUMO) and the three recombinant bacteria (harboring pET28a-SUMO-MT, pET28a-SUMO-2MT and pET28a-SUMO-3MT) were diluted 1:100 in 50 mL LB medium with 50 μg/mL kanamycin. IPTG (0.7mM) and metal ions with the same concentration (300 μM $ZnSO_4$, $CdCl_2$, and $CuSO_4$) were added and cells were cultured for 6h at 37℃. Cells were collected by centrifugation at 6000g for 20 min at 4 ℃. Cell pellets were washed twice in fresh LB medium and dry weight of cells measured after dehydration at 80℃ for 48h. Metal ion accumulations were

determined by flame atomic spectrometry (SHIMADZU AA-6300, Japan), measuring Cu at 324.8nm, Cd at 228.8nm and Zn at 213.9nm. Metal ion accumulation was expressed as μmol/g dry weight bacterial cells.

2.8 Statistical Analysis

All experiments were repeated three times, and measurements expressed as mean±standard deviation(SD). Statistical analysis was performed using SPSS 19.0 software. One-way analysis of variance (ANOVA) was used to test the differences between the control and the bacterial cells harboring pET28a-SUMO-2MT and pET28a-SUMO-3MT. $p<0.05$ was regarded as significant, $p<0.01$ was regarded as very significant.

3. Results and discussion

3.1 Construction of oligomeric MTs

DNA sequencing confirmed that the pET-28a-SUMO-2MT and pET-28a-SUMO-3MT contained no nucleotide substitutions, indicating the recombinant expression vectors were constructed successfully by SOE-PCR. SOE-PCR has proved to be a rapid and efficient technique for gene splicing and site-directed mutagenesis (Heckman et al., 2007). Initial PCRs generate overlapping gene segments that are then used as template DNA for another PCR to create a full-length product. Primer design is critical for SOE-PCR; if the overlapping portion of the primers is too short, it would be difficult to generate full-length amplification. If the overlapping portion of the primers was too long, it would more likely to produce diffuse or non-specific amplification. Considering that the crab MT gene in this study has high GC content (55%) and there is no linker at the junction of the two MT genes, about 25bp of the overlapping portion was adopted in the primer design and results showed the full-length dimeric and trimeric MTs were created successfully with our designed primers.

3.2 Expression of recombinant dimeric and tetrameric MTs

SDS-PAGE analysis showed that the maximal levels of the SUMO-2MT and SUMO-3MT expression were achieved at 8h after 0.7 mM IPTG induction at 30℃

and expressed mainly in the soluble form(Fig.2A). Compared with the uninduced control, recombinant *E. coli* harboring pET28a-SUMO-MT, pET28a-SUMO-2MT and pET28a-SUMO-3MT showed an additional smear and broad band of approximate molecular weight 28kDa, 36kDa and 42kDa, respectively(Fig.2A), which were slightly higher than the predicted molecular weights of the MTs (SUMO 20KD, MT 7KD, SUMO-MT 27KD, SUMO-2MT 34KD, SUMO-3MT 41KD). These results were consistent with the study of oligomeric human MTs expressed in *E.coli* (Hong et al., 2000). This phenomenon may due to the rich cysteine residues in oligomeric MTs, because when the SH groups were blocked with carboxymethylation, sharp bands on SDS electrophoresis were obtained (Kimura et al., 1991).

Expression of MTs in *E.coli* has been a major problem due to their high cysteine content and low molecular weight (Sekhar et al., 2011; Suleman et al., 2012; He et al., 2019). In addition, because of their strong metal-binding characteristics, they can be toxic to host cells (Berka et al., 1988). When we adopted the common expression vectors pET-28a and pGEX-6p-1 for *S. henanense* MT expression in *E. coli*, although we obtained recombinant 6×His-MT and GST-MT, the soluble expression was limited and mainly expressed as inclusion bodies (data not shown).

The most common strategy for soluble expression of recombinant proteins in *E. coli* is to reduce the protein synthesis rate by decreasing the incubation temperature and the concentration of the inducer. However, this method is not effective with many heterologous proteins and often results in lower protein productivity (Makrides et al., 1996).

There are only two related studies where oligomeric human MTs have been integrated and expressed in *E. coli* (Hong et al., 2000; Ma et al., 2011). However, both of them were mostly expressed in inclusion bodies, and soluble expressions were very low. In the present study, we solved this obstacle by employing the SUMO fusion system. Recently, SUMO has emerged as an effective biotechnological tool that enhances the soluble expression of recombinant proteins (Marblestone et al., 2010). It has the great advantage of decreasing proteolytic degradation and simplifying

purification owing to its inherent chaperone properties (Butt et al., 2005). In the present study, the soluble expression of the recombinant dimeric and trimeric MTs were considerably increased by the adoption of the SUMO fusion expression system. Further research should focus on the metal binding characteristics of purified dimeric MTs and trimeric MTs after cleaving the SUMO tag by SUMO protease.

3.3 Purification and characteristics of oligomeric MTs

The recombinant proteins were purified with Ni-NTA (Pre-Packed Gravity Column) and the target proteins were eluted with 100mM imidazole buffers(Fig.2B). The purified fractions were further treated with an ultrafiltration centrifugal tube (Millipore, 3kD) to remove the imidazole and incubated overnight with 300μM $ZnSO_4$, $CdCl_2$ or $CuSO_4$. Both the ultraviolet absorption spectra of purified apo-MTs and metal-incubated oligomeric MTs showed low absorbance at 280nm; this is an ultraviolet absorption characteristic of metallothionein because of the lack of aromatic amino acids. The ultraviolet absorption spectra of metal-incubated oligomeric MTs showed a significant absorption shoulder at about 225nm, 250nm and 270nm respectively(Fig.3). These spectral profiles were typical of Zn-thiolate, Cd-thiolate and Cu-thiolate ligand absorbance (Duan et al., 2018), indicating that the recombinant oligomeric MTs have the abilities of binding Zn, Cd and Cu.

3.3 Metal tolerance of E. coli cells expressing MTs

E. coli cells harboring pET28a-SUMO (as control), pET28a-SUMO-MT, pET28a-SUMO-2MT and pET28a-SUMO-3MT were spotted either on LB solid plates or on plates with different concentrations of $ZnSO_4$, $CdCl_2$ or $CuSO_4$ to test the Cu, Cd and Zn tolerance abilities (Fig.4). When grown in the control medium, all of the *E.coli* cells could grow well. When grown in a medium containing $CuSO_4$, $CdCl_2$ or $ZnSO_4$, the growth of *E.coli* cells harboring different recombinant vectors showed distinctly different growth patterns. Growth of *E. coli* cells harboring pET28a-SUMO (as control) and pET28a-SUMO-MT were inhibited; however, *E. coli* cells harboring pET28a-SUMO-2MT and pET28a-SUMO-3MT reduced this growth defect, indicating that *E. coli* cells expressing dimeric and trimeric MTs increased Cu, Cd and

Zn tolerance compared to the cells harboring pET28a-SUMO and pET28a-SUMO-MT.

3.4 Growth kinetics of E. coli cells harboring oligomeric MTs

To further test the metal tolerance abilities of *E. coli* harboring pET28a-SUMO (as control), pET28a-SUMO-MT, pET28a-SUMO-2MT and pET28a-SUMO-3MT, the control strain and the three recombinant strains were subjected to CuSO4 (200μM), $CdCl_2$ (50μM) or ZnSO4 (100μM). When grown in the control medium, all of the *E. coli* cells harboring pET28a-SUMO, pET28a-SUMO-MT, pET28a-SUMO-2MT and pET28a- SUMO-3MT grew well (Fig.5a). When grown in medium containing Cu, Cd or Zn, the growth of control cells was inhibited; however the expression of oligomeric MTs markedly mitigated this growth defect. The control cells stopped growth after treatment with 200μM Cu^{2+} for 4h when the OD600 reached 0.85, whereas cells expressing SUMO-3MT stopped growth with Cu^{2+} treatment for 7h when the OD600 reached 1.80 (Fig.5b). The control cells ceased growth after treatment with 50μM Cd^{2+} for 2h when the OD600 reached 0.58, whereas cells expressing SUMO-3MT stopped growth after Cd treatment for 5h when the OD600 reached 1.49 (Fig.5c). The control cells stopped growth after treatment with 100μM Zn^{2+} for 3h when the OD600 reached 0.70, whereas cells expressing SUMO-3MT stopped growth with Zn^{2+} treatment for 7h when the OD600 reached 1.48 (Fig.5d).

E. coli cells expressing SUMO-MT, SUMO-2MT and SUMO-3MT showed a significant tolerance for Zn^{2+}, Cu^{2+} and Cd^{2+} compared to the control cells. The metal tolerance in various strains followed the following order: SUMO-3MT> SUMO-2MT >SUMO-MT>control. Our results showed that oligomeric MTs expression in *E. coli* cells leads to an increase in metal tolerance.

3.5 Bioaccumulation Capacity of E. coli Expressing MTs

Similar trends were found for Cu, Cd and Zn bioaccumulation in *E.coli* cells (Fig.6). Expression of SUMO-MT, SUMO-2MT and SUMO-3MT significantly increased the Cu, Cd and Zn concentration in *E.coli* compared to control cells harboring pET28a-SUMO. The cells containing pET28a-SUMO-3MT exhibited the

highest Cu, Cd and Zn bioaccumulation at 1.80, 1.94 and 1.32μmol/g dry cells, respectively. They were 5.8-fold, 3.1-fold and 6.7-fold higher than that of the control cells, respectively. Compared to that of the cells harboring pET28a-SUMO-MT, they were 2.25-fold, 1.41-fold, and 1.67-fold higher, respectively.

In our previous study, we showed that *S.henanense* MT cDNA contains 18 cysteine residues arranged in 5 CysXaa-Cys-, 2 Cys-Cys- and 3 Cys- Xaa- Yaa-Cys-motifs, implying one *S.henanense* MT molecule can bind 6 equivalents of bivalent metal ions (Ma et al., 2009). In the present study, although the expression of dimeric MT and trimeric MT significantly increased the Cu, Cd and Zn bioaccumulation in *E.coli* compared to the cells expression monomeric MT, it did not reach the expected increase of 2-fold or 3-fold higher than the cells expression monomeric MT. Similar results were observed by Ma *et al.* (2011) when oligomeric human metallothioneins were expressed. This may be because the folding of the dimeric and trimeric MTs could have concealed some of the metal-binding sites, even though the SUMO fusion system was adopted to promote protein folding. Further research should focus on site-specific mutagenesis and adding a linker between two MT monomers to further improve the metal tolerance and bioaccumulation.

4.Conclusions

In this study, tandem repeats of two and three copies of the crab MT gene were successfully integrated by overlap extension PCR (SOE-PCR) and expressed in *E. coli*. Furthermore, the soluble expression of the recombinant oligomeric MTs was increased by the adoption of the SUMO fusion expression system. The oligomeric expression of the MT gene not only significantly enhanced metal tolerance but also increased metal ion accumulation in *E. coli* cells. These results suggest that both the *S. henanense* MTs and the *E. coli* cells harboring oligomeric MTs could serve as candidates for bioremediation applications in heavy metal pollution.

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Table1. Sequences of primers used in the present study

Primer	Length	Sequence (5' to 3')
P1	31	CGGGATCC**ATG**CCTGATCCTTGCTGCACAGA
P2	53	CCTTCTGTGCAGCAAGGATCAGGCATGGGGCAGCAGGAGCAAGGCTTCGTGCA
P3	54	TGCACGAAGCCTTGCTCCTGCTGCCCC**ATG**CCTGATCCTTGCTGCACAGAAGGA
P4	31	CCAAGCTT**TTATCA**GGGGCAGCAGGAGCAAG

The bold nucleotides indicate start and stop codons. The underlined nucleotides indicate restriction enzyme cleavage sites. The shadowed nucleotides indicate the overlap sections of the primers.

Figure

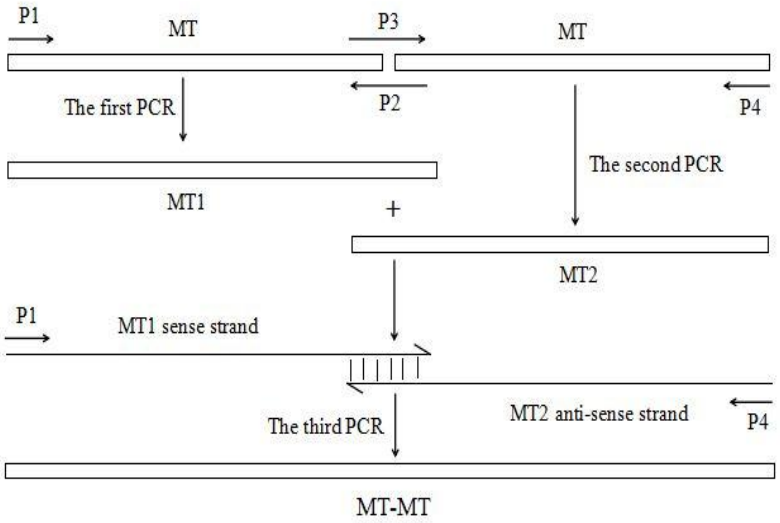

Fig.1. SOE-PCR strategy for integrating the *S. henanense* MT gene to produce dimeric MT.

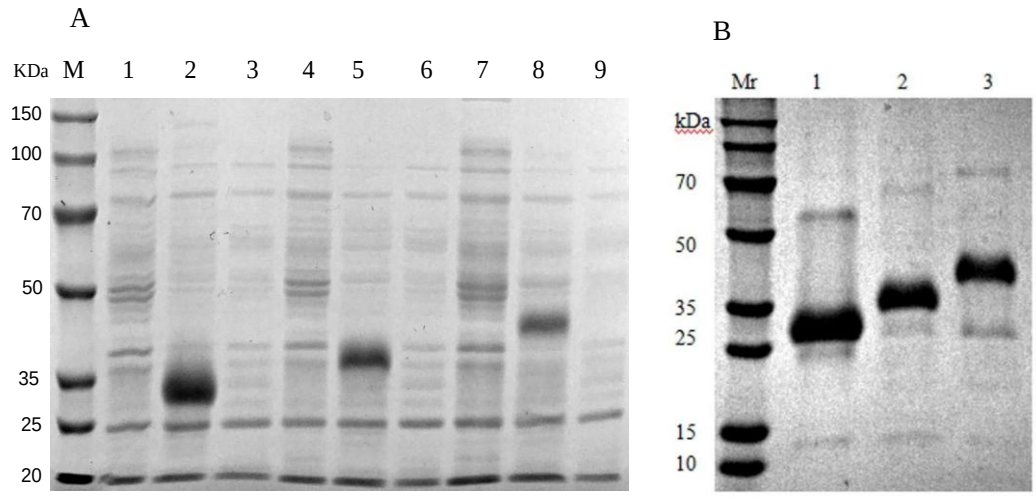

Fig.2. (A) SDS-PAGE analysis of oligomeric *Sinopotamon henanense* MT expression,
Lane1, 4,7, non-induced cell extract supernatant (pET28a-SUMO-MT, pET28a-SUMO-2MT ,ET28a-SUMO-3MT);
Lane2,5,8, induced cell extract supernatant (pET28a-SUMO-MT, pET28a-SUMO-2MT ,ET28a-SUMO-3MT);
Lane3,6,9, induced cell extract precipitation (pET28a-SUMO-MT, pET28a-SUMO-2MT ,ET28a-SUMO-3MT)
(B) Purification of oligomeric *Sinopotamon henanense* MTs. The recombinant proteins were purified with Ni-NTA (Pre-Packed Gravity Column) and eluted with 50, 100, 250 and 500 mM imidazole buffers. The purified fractions were further treated with an ultrafiltration centrifugal tube (Millipore, 3kD) to remove the imidazole. Lane1, monomeric MT, Lane2, dimeric MT, Lane3, trimeric MT

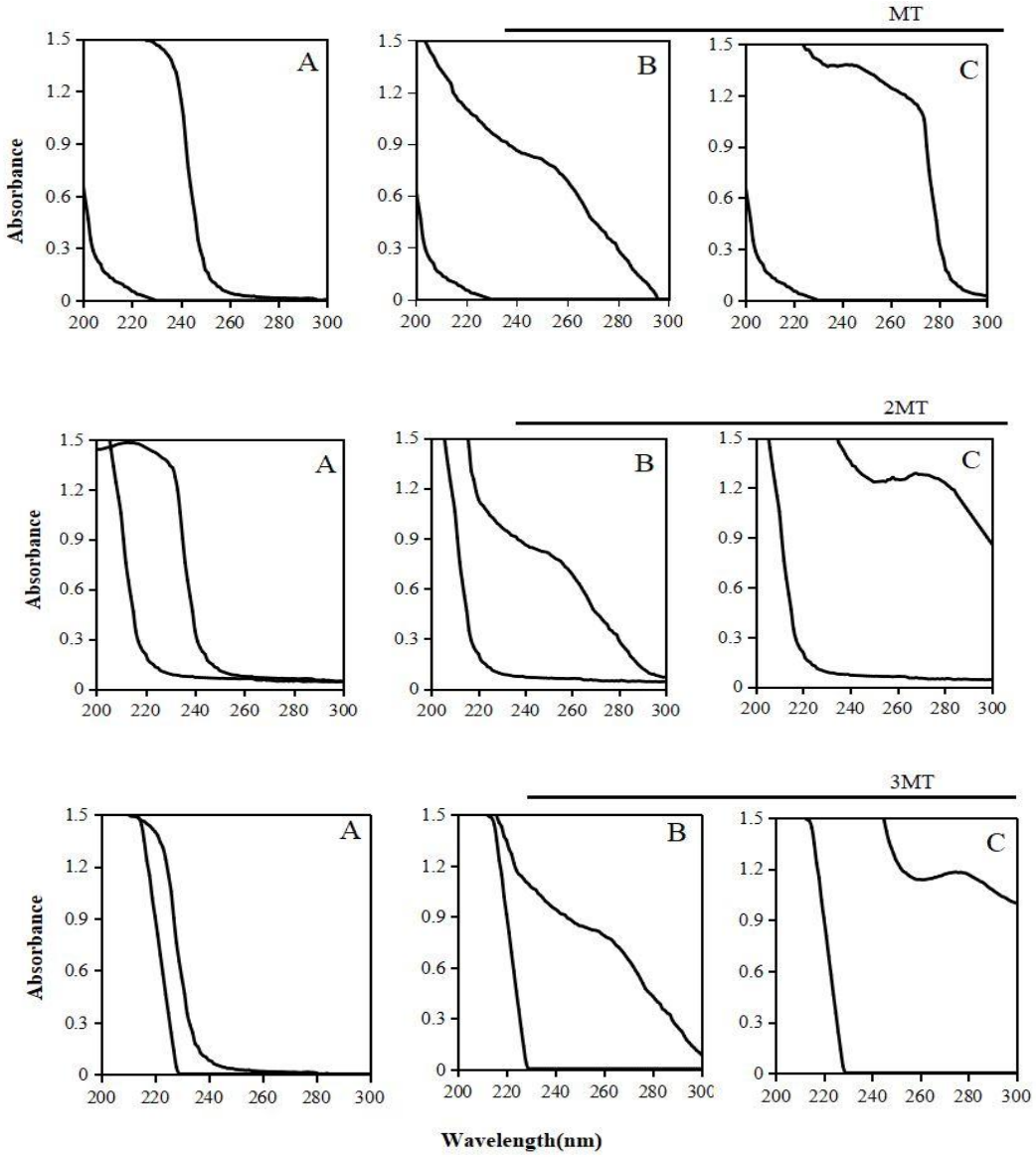

Fig.3 Ultraviolet absorption spectra of purified MT, dimeric MT and trimeric MT proteins with and without $ZnSO_4$ (**A**), $CdCl_2$ (**B**) and CuSO4 (**C**) treatment. The lower spectrum is for the apo form of the MT proteins and the higher spectrum for the metal-treated samples.

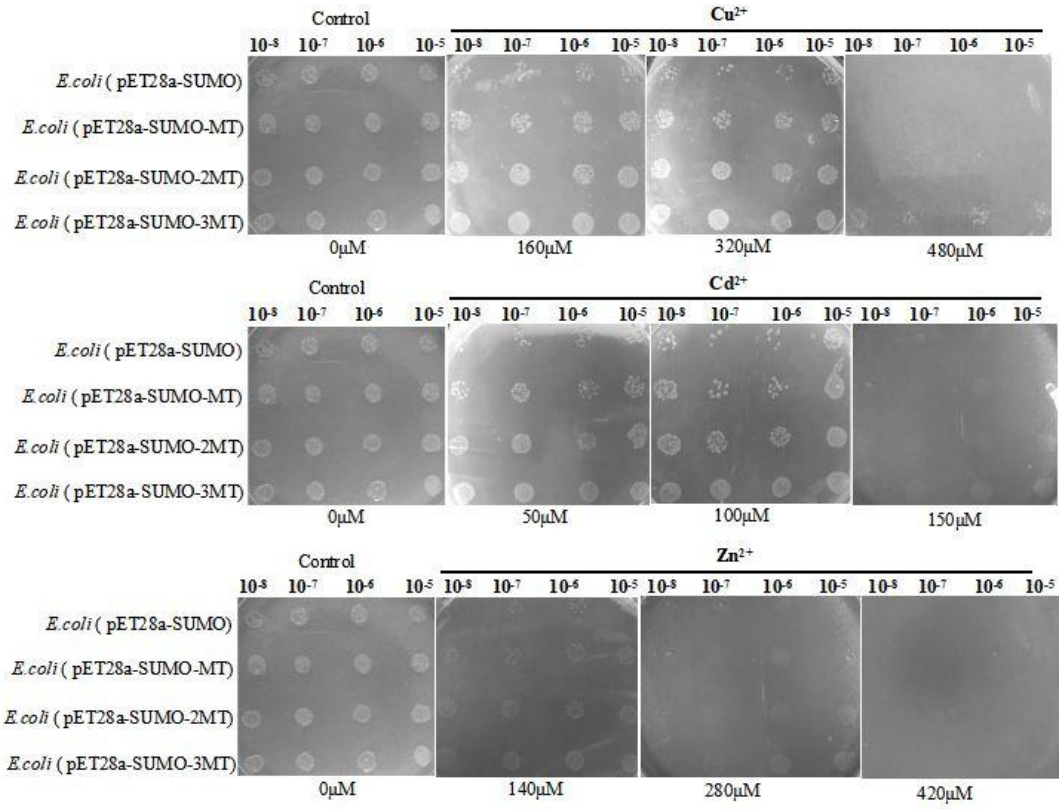

Fig.4. Cu, Cd and Zn tolerance of *E.coli* cells harboring pET28a-SUMO-MT, pET28a-SUMO-2MT and pET28a-SUMO-3MT. *E.coli* cells were cultured in liquid LB medium with 0.7mM IPTG until the OD600 reached 0.6-0.8. After serial dilutions (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, respectively) were prepared, each dilution was spotted either on solid LB plates or on plates with $CuSO_4$ (140, 280, 420μM), $CdCl_2$ (50, 100, 150μM) or $ZnSO_4$ (160, 320, 480μM). Plates were photographed after incubation at 37℃ for 12h.

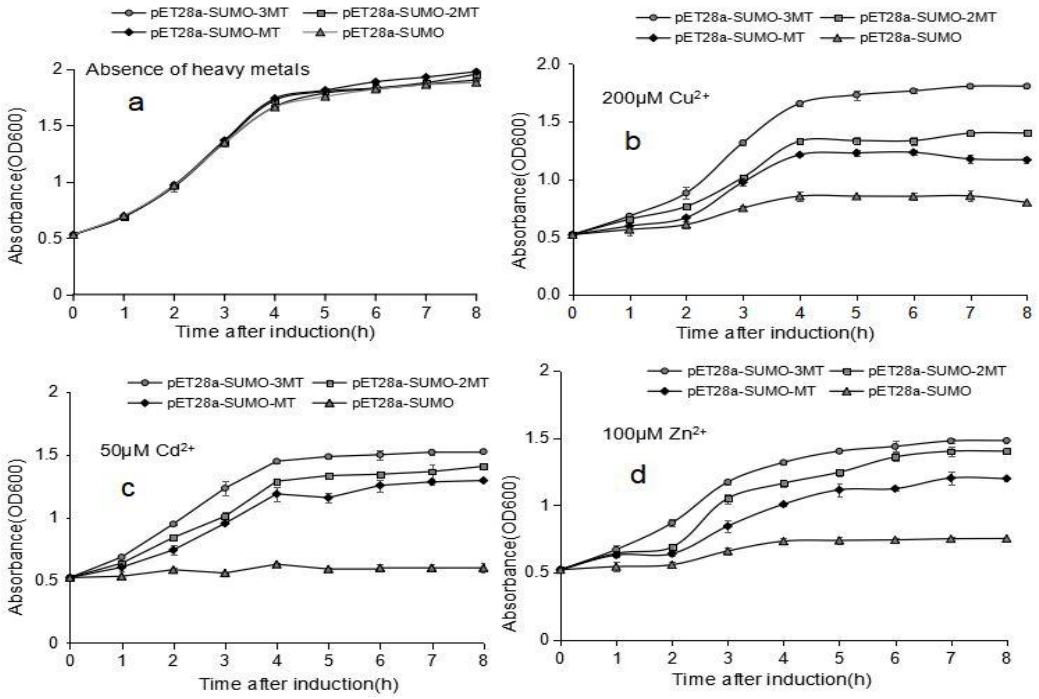

Fig.5. Growth kinetics of *E. coli* cells harboring pET28a-SUMO-MT, pET28a-SUMO-2MT, pET28a-SUMO -3MT in (a) LB, (b) LB containing 200μM Cu^{2+}, (c) 50μM Cd^{2+} and (d) 100μM Zn^{2+}. IPTG was added at a final concentration of 0.7mM when the OD600 reached 0.5. After induction with IPTG for 0.5h, Cu^{2+}, Cd^{2+} or Zn^{2+} was added. OD600 measurements were conducted to analyze the effects of Cu^{2+}, Cd^{2+} and Zn^{2+} on bacterial growth at 1 h intervals for 8 h at 30ºC. The bacterial cells harboring pET28a-SUMO were treated identically to control cells.

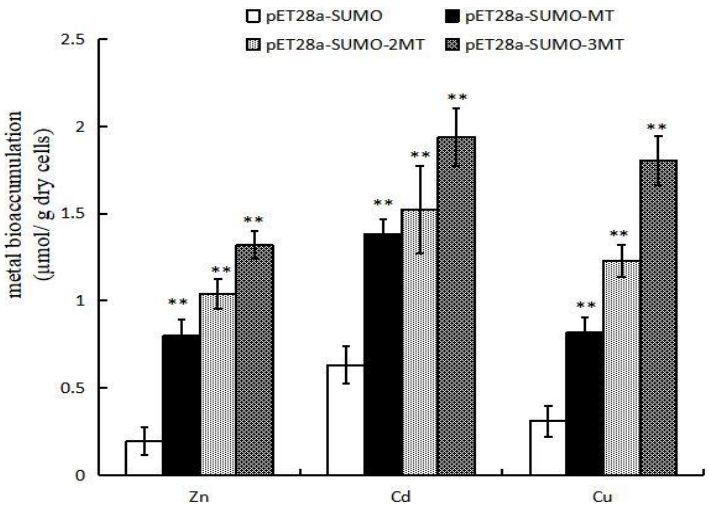

Fig.6. Metal accumulation of *E. coli* cells harboring pET28a-SUMO-MT or pET28a-SUMO-2MT or pET28a-SUMO-3MT in the presence of 300μM $ZnSO_4$, $CdCl_2$ and $CuSO_4$ for 6 h at 37 ℃. Data represent mean±SD of three independent experiments. Statistical significances were analyzed using one-way ANOVA compared with the control (cells harboring pET-28a), *$p<0.05$, **$p<0.01$.