International Journal of Biological Macromolecules 73 (2015) 154–159

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Functional characterization of a type 3 metallolthionein isoform (OsMTI-3a) from rice

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ARTICLE INFO

Article history:
Received 21 August 2014
Received in revised form 29 October 2014
Accepted 30 October 2014
Available online 28 November 2014

Keywords:
Metallothionein
Rice
Heavy metals
Heterologous expression
Functional characterization

ABSTRACT

Metallothioneins (MTs) are low-molecular weight proteins with high Cys content and a high affinity for metals. Plant MTs are classified into four types based on the arrangement of Cys in their amino acid sequences. In the present study, the gene encoding OsMTI-3a, a type 3 MT found in rice, was cloned into pET41a vector. The resulting construct was transformed into the *Escherichia coli* strain Rosetta (DE3). Following the induction with isopropyl β-D-1-thiogalactopyranoside, the OsMTI-3a was expressed as glutathione-S-transferase (GST)-tagged fusion protein. In comparison to control strain, the cells expressing GST-OsMTI-3a accumulated more Cd^{2+}, Ni^{2+} and Zn^{2+} when they were grown in the medium containing $CdCl_2$, $NiCl_2$ or $ZnSO_4$. The recombinant GST-OsMTI-3a was purified using affinity chromatography. The UV absorption spectra recorded after the reconstitution of the apo-protein with different metals confirmed that GST-OsMTI-3a was able to form complexes with Cd^{2+}, Ni^{2+}, and Zn^{2+}. The reaction of the protein–metal complexes with 5-5-dithiobis (2-nitrobenzoic) revealed that the order of affinity of GST-OsMTI-3a toward different metals was $Ni^{2+} \geq Cd^{2+} > Zn^{2+} > Cu^{2+}$.

1. Introduction

Heavy metals are environmental pollutants, many of which are toxic even at very low concentrations. Plants are susceptible to heavy metal toxicity. These toxic effects, including the inhibition of enzyme activities [1], alteration of membrane permeability [2], interference on protein structure and functions [3], oxidative stress [4], and disruption of cellular metabolism lead to reduced photosynthesis [5], chlorosis, and growth inhibition [6]. However, specific processes that occur in plant cells balance the concentration of heavy metals [7,8]. These processes include exclusion of the heavy metals from the symplastic to apoplastic *via* transporter proteins, heavy metal complexation at the cell wall-plasma membrane interface, sequestration of heavy metals from cytoplasm or other cellular compartments into vacuole *via* pumps such as vacuolar proton pyrophosphatase, vacuolar transporters as well as channels, and the chelation of heavy metals in the cytosol by high-affinity ligands such as phytochelatins (PCs), metallothioneins (MTs), and organic acids.

Metallothioneins (MTs) are ubiquitous, low-molecular weight (4–8 kDa), cysteine-rich, metal-binding polypeptides that bind the heavy metals *via* the thiol groups of their cysteine residues [9]. Sequestration of the toxic heavy metals from the cytosol and protection against the intracellular oxidative damage are the main known roles of MTs. The expression of MTs is transcriptionally regulated and is induced by several factors, including oxidative stress and heavy metals such as Hg^{2+}, Cu^{2+}, Ag^{2+}, Co^{2+}, and Ni^{2+} [10]. In plants, a variety of genes encode different MT isoforms, which are classified into four types based on the distribution of their N-terminal Cys residues [11]. The MT types 1–3 are characterized by the presence of two Cys-rich regions that are separated by a central Cys-free spacer of 30–40 amino acid residues. With few exceptions, a typical type 1 MT contains six Cys residues in their N-terminal CXC motifs (where X denotes any amino acid other than Cys). Type 2 MTs contain eight Cys residues at their N-terminus, arranged in the form of CC, CXC, and CXXC motifs. Type 3 MTs are small proteins with four Cys residues arranged in the form of CXXC and CXC motifs in their N-terminal Cys-rich region. In contrast, the distribution of Cys residues within the C-terminus follows the consensus sequence CXCXXXCXCXXCXC, which is highly conserved in all three types of MTs. Type 4 MTs contain three Cys-rich regions, each containing five or six conserved Cys residues. Indicative of their tissue-specific

Abbreviations: MT, metallothionein; Os, *Oryza sativa*; IPTG, isopropyl β-D-1-thiogalactopyranoside; GST, glutathione-S-transferase; PC, phytochelatins; LB, Luria-Bertani; DTNB, 5,50-dithiobis(2-nitrobenzoic); TNB, 2-nitro-5-thiobenzoic acid; MRE, metal-responsive element.

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http://dx.doi.org/10.1016/j.ijbiomac.2014.10.067
0141-8130/

and developmental stage-specific roles, gene expression analysis has shown that the different MT isoforms are differentially regulated in different tissues and at different developmental stages [12].

Previously, we heterologously expressed and characterized two rice (*Oryza staive*) genes encoding OsMTI-1b and OsMTII-1a, which belong to the type 1 and the type 4 MTs, respectively [13,14]. Further, we showed that these isoforms differed in their ability to bind Ni^{2+}, Cd^{2+}, and Zn^{2+}. We hypothesized that these isoforms may have overlapping functions in the protection against oxidative stress. However, to our knowledge there is no information about the specific capacities of other rice MT isoforms for chelating metals. Therefore in this study, we heterologously expressed a type 3 MT isoform, OsMTI-3a from rice, in *Escherichia coli*. The accumulation of metals in cells expressing OsMTI-3a was evaluated by culturing the cells in medium containing $CdCl_2$, $ZnSO_4$, $CuSO_4$, or $NiCl_2$. The purification of recombinant apo-OsMTI-3a in considerable quantities allowed us to study the relative affinity of OsMTI-3a toward Ni^{2+}, Cd^{2+}, Zn^{2+}, and Cu^{2+} *in vitro.*

2. Materials and methods

2.1. Cloning of gene encoding OsMTI-3a

The full-length cDNA of OsMTI-3a (NCBI accession number: AF001396) cloned into the *Xho*I-*Bam*HI sites of pFLCI vector was purchased from National Institute of Agrobiological Sciences Data Bank (NIAS Data Bank, Tsukuba, Japan; http://www.dna.affrc.go.jp). The longest open reading frame of 189 bp was amplified from the cDNA using *Pfu* DNA polymerase (Thermo Scientific) in a reaction mixture containing template plasmid, deoxynucleotides, reaction buffer and the primers 5′-ATATGAATTCATGTCGGACAAGTGCG-3′, which carries an *Eco*RI restriction site at the 5′ end (underlined), and 5′-ATATAAGCTTTCACTTGCCGCACTTGC-3′ with a *Hin*dIII restriction site (underlined) at the 3′ end. An addition of four bases was included at the 5′ end in each oligonucleotide primer. The thermal profile was as follows: 1 cycle at 95 °C for 5 min; 30 cycles at 94 °C for 1 min; 62 °C for 1 min; 72 °C for 1 min and 1 cycle at 72 °C for 10 min. The PCR product was then digested with enzymes *Eco*RI and *Hin*dIII and ligated into pET-41a as expression vector (Novagen) after linearization with *Eco*RI and *Hin*dIII. The resulting plasmid, termed pET41-OsMTI-3a was verified by sequencing and then introduced in *E. coli* protease-deficient strain Rosetta (DE3) for protein expression. The resulted strain was termed R-pET41a-OsMTI-3a. The control strain, R-pET41a was also made by transferring plasmid pET41a without gene.

2.2. Confirmation of heterologous expression and purification of proteins

E. coli cells containing either pET41a (control strain) or pET41a-OsMTI-3a were grown at 37 °C in Luria-Bertani (LB) medium in the volume of 50 ml supplemented with 50 μg/ml kanamycin and 5 μg/ml chloramphenicol to an OD_{600nm} of about 0.6. At this OD cultures were induced by 100 μM isopropyl β-D-1-thiogalactopyranoside (IPTG). The culture medium was supplemented with 0.6 mM $ZnSO_4.7H_2O$ 20 min after addition of IPTG. To confirm heterologous expression of the protein, 1 ml samples of culture medium were harvested by centrifugation 1–4 h after addition of IPTG and frozen at −80 °C until use. The frozen pellets were resuspended in 250 μl pre-cold 10 mM Tris–HCl, pH 8.0, disrupted by mild sonication at 4 °C and centrifugated at 12,000 × *g*, for 20 min. The soluble proteins recovered in supernatant phase were analyzed by 12% SDS-PAGE and stained by Coomasie Brilliant Blue R-250 [15].

For large-scale production of proteins the cells were grown in 500 ml of medium. Induction with IPTG, and supplementation with 0.6 mM $ZnSO_4{\cdot}7H_2O$ were performed as explained above. The cells from whole volume were harvested 4 h after addition of IPTG. The soluble proteins were extracted as explained above. For purification the extracted soluble proteins were applied on to His Trap HP column (GE, Healthcare) preequilibrated with loading buffer (10 mM imidazole, 500 mM NaCl, 30 mM Tris–HCl, pH 8.0) and the bound proteins were eluted by 68.5–283 mM imidazol gradient. Aliquots of the protein fractions were analyzed by 12% SDS-PAGE and stained by Coomasie Brilliant Blue R-250. The pure fractions were pooled and transferred into 12 kDa molecular weight cutoff cellulose tubes (Sigma) and dialyzed against 10 mM Tris–HCl, pH 8.0, at 4 °C overnight to remove imidazole and other salts. Proteins concentrations were determined by Beer-Lambert law with the molar extinction coefficient of 44,537.5 M^{-1} cm^{-1} and 46,277.5 M^{-1} cm^{-1} for GST and GST-OsMTI-3a, respectively.

2.3. Metal ion tolerance of transformed cells

Tolerance of cells harboring plasmids pET41a and pET-OsMTI-3a to metals in the growth medium was examined in concentrations of 0.3 mM $CdCl_2{\cdot}H_2O$, 1 mM $ZnSO_4{\cdot}7H_2O$, 1 mM $CuSO_4{\cdot}5H_2O$, and 2.5 mM $NiCl_2$. For this analysis 5 ml of the overnight cultures of cells were inoculated in 80 ml of LB medium supplemented with desired antibiotics. The cultures were induced at OD_{600} = 0.6 by addition of 0.1 mM of IPTG. After 20 min, the metals were added to the cultures. Bacterial growth was monitored up to 12 h by OD_{600} measurements. Each data represents the mean obtained from two independent experiments with two replicates.

2.4. Analysis of metal ion accumulation in transformed cells

The growth of cells, induction with IPTG, and supplementation with metals were performed as explained in part 2.3. For the analysis of metals, cells from 10 ml of culture at 0 (T0) and 6 h after metal addition (T1) were precipitated by centrifugation at 6000 × *g* for 20 min. The supernatant was analyzed for metals Zn^{2+}, Cu^{2+}, Ni^{2+}, and Cd^{2+} using inductively coupled plasma atomic absorption spectroscopy (PerkinElmer AAnalyst 700). The metal concentration changes in the medium of R-pET41a and R-pET41a-OsMTI-3a between T1 and T0 (C_{T1}–C_{T0}) was calculated. Each data represents the mean ± SD obtained from two independent experiments with two replicates.

2.5. Preparation of apo-protein and reconstitution with different metals

Preparation of apo-protein and reconstitution with metals were performed as described previously with slight modifications [16]. Aliquots of the dialyzed GST-OsMTI-3a recombinant proteins were acidified with HCl to pH 2.0. The samples were then dialyzed against 0.1 N HCl to remove the bound ions. The concentration of protein was determined after dialysis. Reconstitution of apo-protein with metals: Reconstitution with metals was achieved by the addition of 10 mol equivalents of Cd^{2+}, Zn^{2+}, Cu^{2+} or Ni^{2+} ions followed by neutralization of the samples to pH 8.0 with 200 mM Tris. The unbound metals were removed by dialyzing against 10 mM Tris–HCl, pH 8.0 at 4 °C overnight.

2.6. UV absorption spectra

The absorption spectra of metal-incubated and apo-forms of GST-OsMTI-3a proteins were determined between 220 and 350 nm using spectrophotometer (DU 530).