Expression and Application of Peptide Materials Containing Functionalizing Sequence as a Metallothionein

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ABSTRACT

The present research focus on the peptides that are rich in cysteine or histidine amino acids, which are potential ligands for metal ions. These peptide sequence were designed from the nine kinds of metal binding fragments of *xenopas laevies* transcription factor III A zinc finger protein. The protein material was prepared by the rolling circle amplification (RCA) which was a useful and simple technique to amplify oligonucleotide for the biomaterials containing tandem repetitive sequence. The purification and characterization of protein were expressed in *E.coli*. This study targeted on the functionalized protein having the repetitive sequence of (YICSFADCGAAYNKNWKLQAHLCKH $)_n$ as the RCA generated protein materials. The procedure for the purification of histidine tagged proteins has been developed using metal chelating affinity chromatography. This purification method can be used for proteins containing histidine tag and yielding high amounts of purified protein.

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Keywords: protein design, biomaterials, rolling circle amplification, metal binding protein

INTRODUCTION

Metallothioneins are low molecular weight proteins that have high cysteine content (up to 30%) and bind group 11 and 12 metals in vivo. Metallothioneins are ubiquitous in nature with highly conserved sequence positions of the cysteine residues over many different organisms. Molecular modeling is becoming widely used as an alternative to , or supplemental to , the customary experimental methods. Molecular mechanics and molecular dynamics are classical techniques that are computationally for less expensive than semi-empirical and thus are most suitable for large systems such as proteins. (1) The cysteine rich peptides that are synthesized for binding a wide range of heavy metals. (2) Expression of metallothioneins in E.coi to improve the bioadsorption of heavy metals is a promising technology for the development of microbial-based biosorbents . (3)

Industrial and agricultural activities have lead to substantial release of toxic heavy metals in the environment, which can constitute a major hazard for ecosystems and human health (4) . Therefore, it is an urgent demand to seek for an efficient and environmentally compatible means able to remove or detoxify heavy metals in an economical and natural way. A lot of biomaterials are reported by the biological techniques and the newly designed peptides have also been developed as useful tools in molecular biology (5). Bioremediation, using bacteria or plants , is often regarded as a relatively inexpensive and efficient way of cleaning up waste, sediments or soils contaminated with toxic heavy metals (6).

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A promising way of improving bioremediation processes is to genetically engineer bacterial strains to confer increased abilities to accumulate toxic heavy metals. Attempts to enhance the metal content of bacterial cells have been made by overexpressing metal- binding peptides or proteins such as poly-histidines (7) or metallothioneins (8) .The present work involves quantifying metal binding to selected peptide sequence. An important component of these studies involves the protein material was prepared by the rolling circle amplification (RCA) (9, 10) which was a useful and simple technique to amplify oligonucleotide for the biomaterials containing tandem repetitive sequence. RCA is the
prolonged extension of an oligonucleotide primer annealed to a circular template DNA. A continuous sequence of tandem copies of the circle is synthesized. RCA has the advantage of not requiring a thermal cycling instrument (11). A fundamental requirement for molecular biology is the isolation and amplification of specific DNA sequences. Target sequences are typically inserted into circular vectors, propagated in a biological host, and isolated by physical methods (Sambrook et al. 1989). Utilizing metal-binding proteins or peptides developed from metal-tolerant organisms to promote the heavy metal resistance or metal-accumulation ability of a proper host has caugh the attention of several researchers. Protein with the ability of heavy metal binding, such as metallothioneins and phytochelatins, has been explored and genetically engineered to bacteria or plants. Recently, several metal-binding peptides were expressed in *Escherichia coli* or yeast strains in order to increase their metal resistance or bioaccumulation ability. Therefore, this study was designed to investigate the potential to create a more effective metal biosorbent by using *E.coli* strains that can overexpress the metal binding proteins (12).

Materials and Method

Oligonucleotides

All oligonucleotides were purchased (Hokkaido system .science) as described below.
Primer 1; GGAT CCTACATCTGCTCTTTCGCCGACTGCGGCGCTGCTTATAAC (45 base),
Primer 2; AAGCTTTAGTGTTTGCACAGATGCGCCTGCAGT TTCCAGTTCTT (44 base),
Template 1;
AGAGCAGATGTAGTGTTTGCACAGATGCGCCTGCAGTTTCCAGTTCTTGTTATAAGC
AGCAGCGCCGCAGTCGGCGAA(75 base),
Template 2;
CTGTGCAAACACTACATCTGCTCTTTCGCCGACTGCGGCGCTGCTTATAACAAGAAC
TGGAAACTGCAGGCGCAT(75 base).

Bacterial Strains and Vectors

E.coli strain BL21(DE3) was used as the host to express the recombinant proteins. *E.coli* strains containing plasmid vectors conferring ampicillin resistance were maintained on Luria-Bertani medium supplemented with 50 ug/ml ampicillin. The vector pT7 blue was used as TA vector that contain *Bam HI* and *Hind III* fragments of the plasmid DNA. The vector pET-32a with histidine tags was selected for the protein expression.

pET System

The pET System is most powerful system yet developed for the cloning and expression of recombinant proteins in *E.coli*. Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and translation signals ; expression is induced by providing a source of T7 RNA polymerase in the host cell. T7 RNA polymerase is so selective and active that almost all of the cell's resources are converted to target gene expression ; the desired product can comprise more than 50% of the total cell protein after a few hours of induction.

Another important benefit of this system is its ability to maintain target genes transcriptionally silent in the uninduced state. Target genes are initially cloned using hosts that do not contain the T7 RNA polymerase gene, thus eliminating plasmid instability due to the production of proteins potentially toxic to the host cell. Once established in a non-expression host, plasmid are then transferred into expression hosts containing a chromosomal copy of the T7 RNA polymerase gene under lacUV5 control, and expression is induced by the addition of IPTG. Two types of T7 promoter and several hosts that differ in their stringency of suppressing basal expression levels are available, providing great flexibility and optimizing the expression of a wide variety of targrt genes.

Rolling Circle Amplification

Fire, S.Xu (1995) found the RCA to make the repetitive arrangement of the gene which made circle DNA the uniform by carry out an elongation reaction by polymerization reaction.

RCA technique was performed according to the described procedure (Fire,A.et al, 1995). Firstly, the template oligonucleotides were circularized (Template 1 and 2) using a partially complementary oligonucleotides (Primer 1 and 2), respectively. These template and primer were then reacted with DNA polymerase in the presence of deoxynucleotide triphosphates. In the proceeds of nucleotide synthesis, the polymerase produced duplex DNA. At some points, the circularized DNA became constrained so tightly because polymerization could not proceed without some relief of the restraint. The amplified product can also be used in vito cloning, library construction and molecular biology application.

Expression of protein in *Escherichia coli strains*

The plasmid DNA was assembled using the expression vector pET-32a (Novagen). Standard procedures were used for ligation, transformation, restriction analysis and plasmid preparation (Sambrook et al., 2001). The recombinant strains *E.coli* BL 21 was cultivated at 37 c in Luria-Bertani (LB) broth amended by 100 mg/ml of ampicillin until the optical density (at 600 nm) of the culture reached 0.6.The culture was subsequently induced with 1M isopropyl-B-D-thiogalactopyranoside (IPTG) for 4 hrs. Cell were harvested by centrifugation (16,000 g at 4 C for 10 min). The cell pellets (6 g) were resuspended in 18 ml of 20mM Kpi buffer. Cells were broken by sonication. Protein samples were boiled in sample buffer prior to electrophoresis. The protein was analyzed on a standard 12% polyacrylamide gel and stained with Coomassive Blue (Erni et al., 1982).The control strain of BL 21 was also cultivated and treated in the same way, except that the medium for growth of *E.coli* BL21 did not contain antibiotics. Protein analysis (via SDS PAGE) for the tested and control strains shows that proteins overexpressrd and with molecular weight of approximately 23 kDa(s).

Purification of histidine-tagged proteins

In this research we used his trap kit for purification of proteins. His Trap kit is designed for affinity purification of histidine-tagged proteins. Using his trap HP kit,histidine-tagged proteins can be prepared to high purity in one step.His-tagged protein can be purified directly from pre-treated bacterial lysates and are recovered from the matrix under mild elution conditions that preserve their antigenicity and functionality.

His Trap HP columns are supplied pre charged with Ni $^{2+}$ ions, and will selectively retain proteins if complex-forming amino acid residues, in particular histidine, are exposed on the surface of the protein. Tagging proteins with additional histiding, increases the affinity for Ni $^{2+}$ and generally makes the his-tagged protin the strongest binder among other proteins, in e.g., an *E.coli* extract.

Histidine-tagged proteins can be eluted from his trap HP with buffers containing imidazole. There is, a balance between the concentration of imidazole needed to prevent non specific binding of contaminants and the concentration of imidazole needed to elute the histidine-tagged protein.

RESULTS

Figure 1 shows the RCA products. The resulting smear bands for cir 1, cir 2 was consistent with a tandem array structure derived from rolling circle synthesis. Extension of an oligonucleotide was taken by DNA polymerase in the presence of excess dNTPs for over 12 hours.

The sence change elongated single-stranded (ss) DNA and antisence change elongated single-stranded (ss) DNA were annealed to get double-stranded (ds) DNA by PCR in the present of taq.polymerase and dNTP mixture. (see fig 1 A,B).

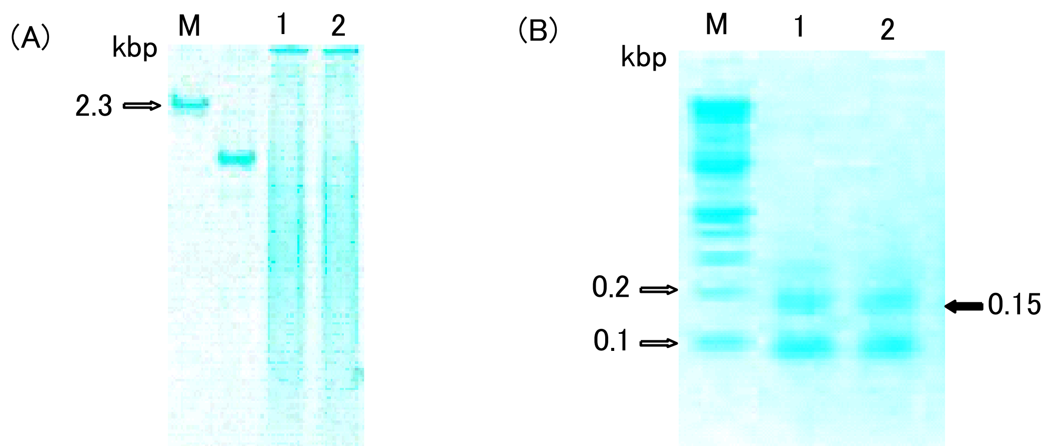


Figure 1.(A) ;The RCA products after the elongation reaction for 12 hours were analyzed by polyacrylamide gel (0.7%) electrophoresis.Both lane 1 and 2 indicate the RCA products. (B); the two time repetitive product of double stranded RCA product after checking by PCR.

After the purification of the purpose DNA that verified gene was sub cloned into pT7 blue within *Bam HI* and *Hind III* restriction sites for restriction enzyme digesting. The repetitive

gene fragment of 600 bp (8 times repeated gene) was collected by transformation in *E.coli* . (see fig 2 .A)

The verified gene fragments were subcloned into pET-32a (Novagen) within *Bam HI* and *Hind III* sites. The resulting figure 2(b) describe the targeted DNA, molecular weight about 0.6 kbp in lane 1,3 and lane 4.

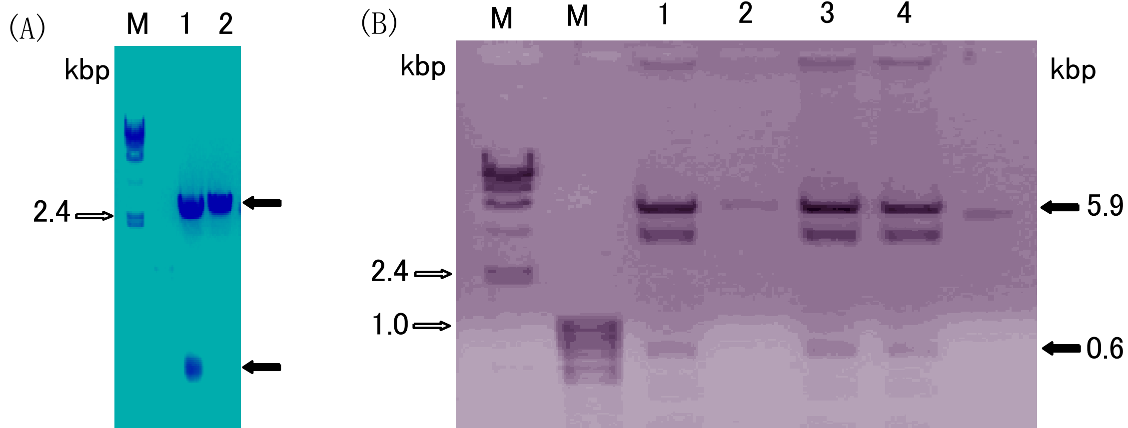


Figure (2) . (A); Agarose electrophoresis analysis after the digestion of restriction enzyme in TA vector. The inserted DNA (600 bp) was observed in the lane 1 and the upper arrows is pT7 blue 2.88 k bp. (B); All lanes indicates the products after the restriction enzyme digestion of the RCA product in the expression vector. The inserted DNA (600 bp) were observed in lane 1,3 and 4. The upper arrow is pET-32a of 5.9 k bp.

E-coli strain BL 21 (DE3) which carried out the transformation was cultivated by the LB-Amp culture medium 250ml, for 5 hours at 37, IPTG was added so that the last concentration might become 1mM in the place where OD $_{600}$ amounted to 0.6, and it cultivated for 12 hours. The new band has been checked to 21 KDa(s) after IPTG addition and 1,2 and 3 hr. (fig 3).

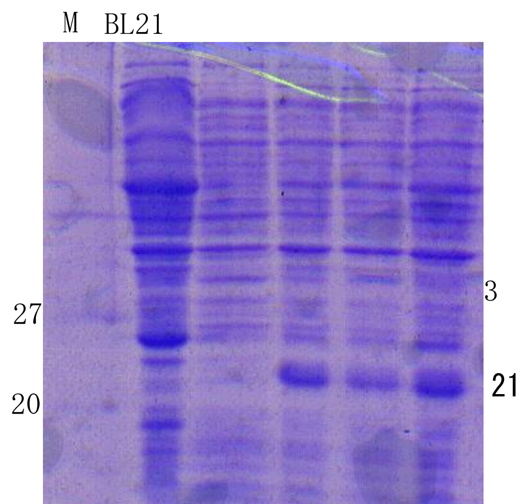


Figure (3), Protein expression was analyzed by SDS-PAGE. From lane 1 to lane 4 were expressed for 1, 2, 3 and 4 hours after induction , respectively. The expressed protein was observed from lane 2 to lane 4 as about 21 kDa in size.

Purification of protein

We used his trap kit for affinity purification of our target protein. The vector pET-32a with histidine tags sequence was used for the protein expression. Histidine tagged protein can purify by using metal chelate affinity. In this research we used his trap kit for purification of proteins. His Trap kit is designed for affinity purification of histidine-tagged proteins.Using his trap kit,histidine-tagged proteins can be prepared to high purity in one step.His-tagged protein can be purified directly from pre-treated bacterial lysates and are recovered from the matrix under mild elution conditions that preserve their antigenicity and functionality. Histidine-tagged proteins can be eluted from his trap HP with buffers containing imidazole. There is a balance between the concentration of imidazole needed to prevent non specific binding of contaminants and the concentration of imidazole needed to elute the histidine-tagged protein.After the purification of protein we got high concentration of protein and we could remove some impurities from protein (see fig.4).

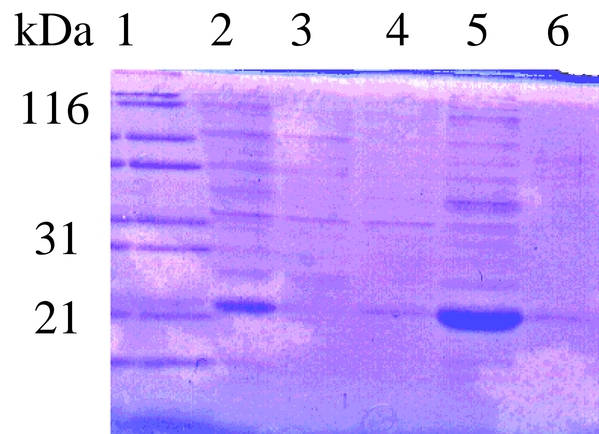
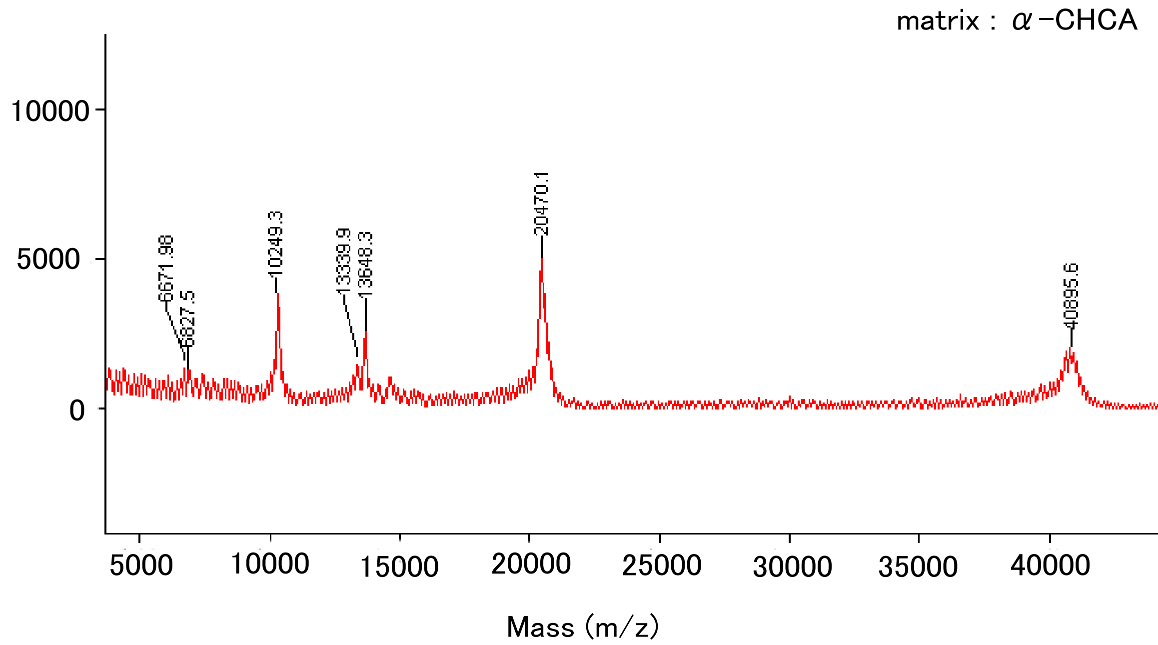


Fig (4). Purified protein by using His Trap column. Lanes:1, protein molecular weight marker; 2,supernatant with protein after sonication; 3,after washing protein with binding buffer; 4, sample after 1st eluted with elution buffer; 5,sample after 2nd eluted with elution buffer; 6,sample after 3rd eluted with elution buffer.

Analysis of Molecular Weight of Protein by Matrix-Assisted Laser Desorption / Ionization (MALDI) Mass Spectrometry



DISCUSSION

RCA has several substantial advantages over other amplification techniques. (13) Most importantly , RCA is an isothermal procedure and , therefore , has no need of special instrumentation to cycle the temperature, which is required with the widely used PCR based - diagnostics. This RCA feature significantly simplifies the automation and miniaturization of RCA -based diagnostics. In addition, RCA can be performed by a large variety of DNA polymerase compare to PCR, which relies on only thermo stable enzymes. Besides, RCA represent an inexpensive, and more sensitive (compared to PCR) analytical technology with a very wide dynamic range and higher multiplexity to serve as a potent alternative to the thermocycling diagnostic methods. In comparison with other isothermal methods of signal, probe, or target amplification, such as transcription- based amplification ,strand -displacement amplification, loop-mediated amplification, the RCA-based assays are less complicated and in many case do not require any substantial preoptimization of an experimental protocol. The RCA -based diagnostics exhibit an exceptional specificity for particular DNA/RNA sequences, as well as for marker molecules other than DNA/RNA ,allowing the multiplex detection of single -based mutations and specific antigens. Besides, RCA is an ultra sensitive method of detection: a variety of RCA format permit essentially single-molecule counting of the DNA, RNA, or protein targets. Furthermore, RCA -based diagnostics are characterized by good reproducibility, with amplification errors being at a lower level compared to PCR.

In this research designed the peptide sequence from first sequence of *xenopas laevis* transcription factor III A zinc finger that sequence has metal binding activity. The ovserved protein has more specific sequences than other polypeptides and proteins because of protein contains 8 times repeated of YICSFADCGAAYNKNWKLQAHLCKH sequence. After the purification and the assignment of protein structure of this product, the metal binding activity will be characterized. And this protein material will be expected for the use of metal recovery system. In this paper, we have demonstrated that the RCA reaction can be useful for the design and preparation of the functionalized protein biomaterials.

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