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pT7MT, a Metallothionein 2A-Tagged Novel Prokaryotic Fusion Expression Vector

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Abstract In the present article, a novel fusion expression vector for *Escherichia coli* was developed based on the pTORG plasmid, a derivative of pET32a. This vector, named pT7MT (GenBank Accession No DQ504436), carries a T7 promoter and it drives the downstream gene encoding Metallothionein 2A (MT2A). There are in-framed multiple cloning sites (MCS) downstream of the MT2A gene. A target gene can be cloned into the MCS and fused to the C-terminal of the MT2A gene in a compatible open reading frame (ORF) to achieve fusion expression. The metal-binding capability of MT2A allows the purification of fusion proteins by metal chelating affinity chromatography, known as Ni^{2+}-affinity chromatography. Using this expression vector, we successfully got the stable and high-yield expression of MT2A-GST and MT2A-Troponin I fusion proteins. These two proteins were easily purified from the supernatant of cell lysates by one-step Ni^{2+}-affinity chromatography. The final yields of MT2A-GST and MT2A-Troponin I were 30 mg/l and 28 mg/l in LB culture, respectively. Taken together, our data suggest that pT7MT can be applied as a useful expression vector for stable and high-yield production of fusion proteins.

Keywords: MT2A, pT7MT, fusion protein, metal chelate affinity chromatography

Several fusion protein expression vectors have been developed for the expression of recombinant proteins [14] in *Escherichia coli* (*E. coli*). It has been known that fusion expression vectors enhance the productivity, solubility, and uniformity of the target proteins compared with non-fusion proteins, and an N-terminal fusion tag often stabilizes eukaryotic proteins in a bacterial cell [1, 15]. Fusion proteins can be easily purified by affinity chromatography because the binding specificity of affinity tags towards their corresponding immobilized ligands [7]. Two of the most widely used protein fusion tags are GST [13] and the small His_6 or His_{10} tags [5, 13]. A lot of vectors are designed to produce target proteins fused with a GST-tag or His-tag and therefore facilitate the purification of target proteins. For example, GST fusion proteins can be purified from cell lysates by substrate-affinity chromatography [3, 4, 8, 13] and His-tag can be used for the purification by metal-affinity chromatography [2, 16].

Metallothioneins (MTs) are small intracellular proteins that contain 30% cysteine residue content and may act as an antioxidant by scavenging hydroxyl radicals and by binding metals [10]. MTs are unusually rich in cysteine residues that coordinate multiple zinc and copper atoms under physiological conditions [11]. In single-cell eukaryotes, MTs bind copper predominantly [6]. In mammals, MTs bind zinc predominantly, but zinc can be readily displaced by copper or cadmium [12]. Researchers have tried to produce MTs via recombinant DNA technology in *E. coli.* Unfortunately, the majority of expressed mammalian MT2A fusion protein formed inclusion bodies and constituted about 20% of the total cellular proteins in transformed *E. coli* [18]. Fusion expression of MT3 with N-terminal tag (GST-MT3) has also been reported to be unstable and inclined to degradation, although GST can highly improve the solubility of MT3 [20]. Therefore, it is extremely difficult to get the soluble and stable expression of MT with high yield in either non-fusion or fusion forms.

In the present paper, we first fulfilled the stable and highly soluble expression of MT2A in an N-terminal fusion form with GST. Then, MT2A-Troponin I was also expressed *via* the same strategy. Both fusion proteins were easily purified through Ni^{2+}-affinity chromatography by virtue of MT2A's physiological property of metal binding, indicating the possibility that MT2A could be used as an applicable fusion tag. Based on these data, pT7MT vector, a novel

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fusion expression vector with an N-terminal MT2A tag developed in the article, displays promising application potential for gene expression.

MATRIALS AND METHODS

Bacterial Strains and Materials

E. coli strain BL21 (DE3) was used as the host strain for the expression of foreign genes. Restriction enzymes were from Takara (Shuzo, Japan), Ni-NTA chromatographic resin was from Pharmacia Biotech (Piscataway, NJ, U.S.A.), Glutathione-Sepharose 4B was from Sigma (St. Louis, MO, U.S.A.), and PCR Supermix was obtained from Life Technologies (Boston, MA, U.S.A.). Plasmid preparation, enzymatic manipulation of DNA, bacterial transformation, and PCR amplification were performed according to standard protocols.

Construction of Novel Vector pT7MT

pT7MT is a derivative of the pTORG expression vector (Hua, Z.C. and H.X. Zhang. 2003. A novel method for enhancing solubility of recombinant protein products in *E. coli*. China Patent ZL01127171.X), in which the GST was replaced with the MT2A gene. Initially, the pTORG vector was double digested with NcoI and BamHI to remove GST from the plasmid. PCR amplification was performed from pACT-MT2A [9], which contains the MT2A gene, using an upstream primer, 5'CAT GCC ATG GAC TGC TCC TGC GCC GCC 3', in which the underlined NcoI site contains the translation initiation codon ATG, and a downstream primer, 5'CGG GAT CCC ACG ACC TTC GAT GGC GCA GCA G 3', where the BamHI site is also underlined. PCR cycles were as follows: 95°C, 50 s; 58°C, 50 s; 72°C, 50 s; 25 cycles. The PCR product was then isolated from agarose gel electrophoresis using a Gel-Out kit (Bocai, Shanghai, China) and digested with BamHI and NcoI, and then ligated to the same digested pTORG with T4 ligase to construct the new plasmid pT7MT. The sequence of pT7MT has been deposited in GenBank under the accession number DQ504436.

Construction of Fusion Protein

N-Terminal MT2A fusion expression plasmids were constructed by inserting Troponin I (Tn I) or the GST gene into the pT7MT vector, respectively. The Tn I gene was obtained by PCR after 25 cycles of amplification (PCR cycles: 95°C, 1 min; 55°C, 1 min; 72°C, 1 min) from pET28a-Tn I [19], with the upstream primer 5' CGCGGATCCAAA TGGGAGATGAGGA 3', and downstream primer 5' GCCCTCGAGCTATTG TGAGGTCGGAGA 3'. The GST gene was obtained after 25 cycles of amplification (PCR cycles: 95°C, 1 min; 56°C, 1 min; 72°C, 1 min) with the upstream primer 5' CGCGGATCCATGGCCAGCTACCGAC 3', and downstream primer 5' TCCCTCGAGTTATACGA-CAAAGCGGG 3', respectively. The amplified DNA fragments were digested with BamHI and XhoI, and cloned into pT7MT digested with the same restriction enzymes to create expression plasmids pT7MT-GST and pT7MT-Tn I. Plasmid pTORG-MT2A was also constructed to express recombinant GST-MT2A fusion protein as a control. MT2A cDNA was obtained by PCR after 25 cycles of amplification (PCR cycles: 95°C, 50 s; 58°C, 50 s; 72°C, 50 s) by using the upstream primer 5' CCGG-GATCCTCATATGGCCATGGA 3', and downstream primer 5' CGGCTCGAGTCACATTATTTCATAGA 3'. The MT2A gene was then inserted into pTORG between the BamHI and XhoI sites [17, 18].

Expression and Purification of Target Proteins

The transformed cells were grown overnight in 100 ml Luria-Bertani (LB) medium containing 100 μg/ml ampicillin. For large-scale preparation of MT2A-GST fusion protein, a 20-ml aliquot of the overnight culture was added to 1 l of fresh medium in a 2-l Erlenmeyer flask and grown at 37°C, 225 rpm. After a 3-h culture, when the bacterial density at 600 nm (OD_{600}) reached 1.0, fusion protein expression was induced by the addition of IPTG (0.5 mM final concentration) and the culture was grown for 3 more hours. Cells were harvested by centrifugation at 8,000 ×*g* for 5 min, and then resuspended in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and sonicated with an ultrasound (Glenmills, Clifton, U.S.A.) (1 min, power 4, 50% duty cycle). Supernatant was collected after 5-min centrifugation at 14,000 ×*g* and loaded onto a Ni-NTA column, which was preequilibrated with Buffer A (50 mM sodium phosphate, 0.3 M sodium chloride, 10 mM imidazole, pH 8.0). After washing the column by gradually increasing the imidazole concentration, proteins were eluted with buffer A containing 250 mM imidazole. Eluted fraction was loaded onto a Sephadex G-15 filtration column to remove imidazole from the protein. MT2A-Tn I was expressed and purified by the same method as described above.

For GST-MT2A fusion protein, the induced cells were resuspended in PBS and sonicated with an ultrasound (Glenmills, Clifton, U.S.A.) (1 min, power 4, 50% duty cycle). Supernatant was collected after a 5-min centrifugation at 14,000 ×*g*, and the supernatant of the cell lysate was loaded onto a glutathione-Sepharose column, which was preequilibrated with PBS. After washing the column with PBS, proteins were eluted with 10 mM glutathione in 50 mM Tris-HCl, pH 8.0. The elution was collected in 1-ml fractions, and eluted fractions were subjected to a 12% SDS- PAGE for further analysis.

The protein concentration of each step during the purification process for MT2A-GST, MT2A-Tn I, and GST-MT2A was determined by a BCA assay kit (Pierce, IL, U.S.A.) using BSA as the reference.

To compare the solubility of MT2A and Tn I fusion proteins under different strategies, cells from 3 ml of LB culture were harvested and resuspended in pre-cold PBS (50 mM sodium phosphate, 0.3 M NaCl, pH 8.0) to adjust to the same optical density at 600 nm. Three-hundred μl of resuspended cells were collected and then lysed by ultrasonication. The separated total, soluble, or insoluble fractions of crude bacterial lysates and purified fusion proteins were analyzed by 12% SDS-PAGE. Gel staining, scanning, and protein band quantification were performed by scanning densitometry using software Grab-it 2.5 and Gelwork (UVP, CA, U.S.A.). The percentage of fusion protein expression was determined as induced target protein/total bacterial proteins×100%. The solubility was determined as soluble target protein/total expressed target protein×100%. The purity was determined as purified target protein/total proteins in elution fraction×100%.

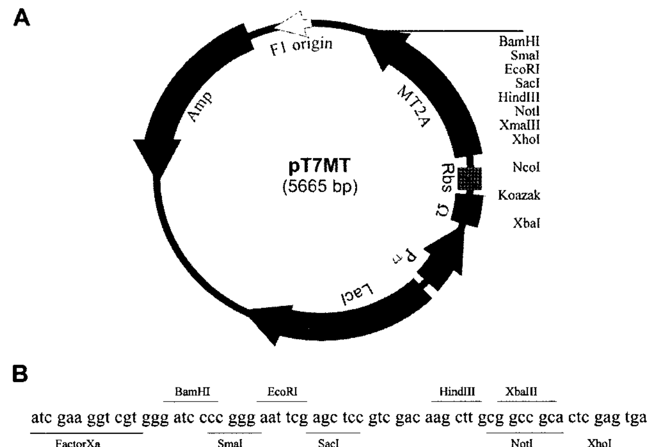

Fig. 1. Map of pT7MT and its MCS.
A. Diagram of the expression vector pT7MT with relevant unique restriction sites. B. pT7MT MCS. The relevant restriction sites and the recognition sequence of factor Xa are indicated.

RESULTS

In this study, we explored to employ the metal binding property of MT2A to construct a novel fusion expression vector. We amplified the MT2A-encoding region by PCR and cloned the PCR product into the pTORG plasmid to construct a new expression vector named as pT7MT (Fig. 1A). This pT7MT vector carries a T7 promoter driving the gene encoding Metallothionein 2A (MT2A) and the downstream in-framed multiple cloning sites (MCS) (Fig. 1B). The target gene could be cloned into the MCS and fused in a compatible open reading frame with MT2A and expressed as MT2A fusion protein. The expression efficiency of this vector was tested by expressing plasmids pT7MT-GST and pT7MT-Tn I.

E. coli BL21 (DE3) cells transformed with plasmid pT7MT-GST (Fig. 2A) was induced by IPTG, which resulted in the production of a fusion protein with molecular mass of 34 kDa, revealed by SDS-PAGE analysis. The fusion protein was purified by Ni-NTA affinity chromatography, and the purified MT2A-GST was stable and exhibited no degradation. The final yield of the purified MT2A-GST was 30 mg/l (Table 1). As a control, MT2A was also expressed in the conventional GST fusion form of GST-MT2A and purified by a glutathione-Sepharose 4B affinity column. However, the purified protein was less than 1 mg/l LB culture, which was extremely low compared with MT2A-GST (Fig. 2B). Fig. 2B shows the reason for low production of the GST-MT2A target product. When MT2A was expressed in the GST-MT2A form, the GST-MT2A product was easily degraded after cell lysis. After glutathione-Sepharose 4B affinity chromatography, the GST-MT2A

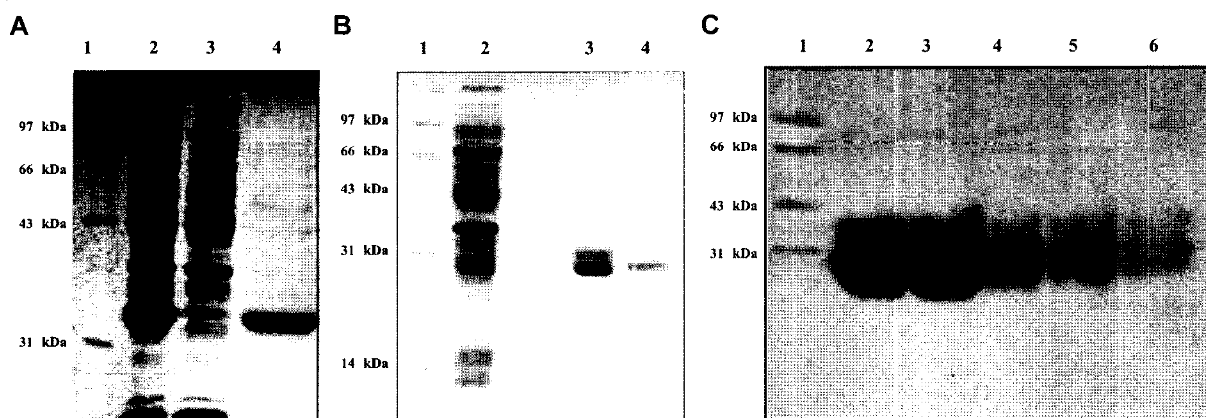

Fig. 2. Twelve % SDS-PAGE analyses of recombinant MT- or GST-tagged proteins from *E. coli.*
A. Expression of MT2A-GST fusion protein. Lane 1, molecular mass marker (Promega); lane 2, soluble fraction after cell lysis; lane 3, wash fraction (non-binding fraction); lane 4, eluted fraction. B. Expression of GST-MT2A fusion protein. Lane 1, molecular mass marker (Promega); lane 2, soluble fraction after cell lysis; lane 3, eluted fraction with 10 mM GSH; lane 4, purified GST-MT2A through Sephadex G-15 column. C. Degradation of GST-MT2A protein after purification. Lane 1, molecular mass marker (Promega); lane 2, 10 min; lane 3, 20 min; lane 4, 30 min; lane 5, 40 min; lane 6, 50 min incubation.

Table 1. Purification of MT-tagged GST and Tn I expressed in *E. coli* by one-step metal affinity chromatography.

Fusion protein	Fraction	Protein amount (mg)[a]	Fusion protein content (%)[b]	Purification fold
MT-GST	Total lysate	285	15	1.0
	Ni^{2+}-affinity chromatography	30	87	5.8
MT-TnI	Total lysate	257	18	1.0
	Ni^{2+}-affinity chromatography	28	80	4.4

[a]Total protein was determined by the Pierce BCA assay using BSA as a standard.

[b]Purity of protein at each step of isolation and purification was estimated by analyzing images of the SDS-PAGE gel stained with Coomassie blue using Grab-it 2.5 and Gelwork (UVP). Target protein content was calculated by the following formula: expressed target protein amount/total protein amount.

fusion protein, whose original molecular mass was around 33 kDa, was eluted with the main molecular mass around 27 kDa, indicating that the C-terminal MT2A was completely degraded within 1 h (Fig. 2C). When MT2A was shifted to the N-terminal of the fusion protein in MT2A-GST form, the degradation completely diminished (Fig. 2A). Moreover, in addition to the product stability, the MT2A-GST fusion protein showed a higher expression level and better solubility than the GST-MT2A fusion form.

To validate the applicability of the pT7MT vector to other target protein, we further expressed Troponin I (Tn I) in pT7MT. According to our previous study [17] and other reports [20], the Tn I protein tends to aggregate in insoluble inclusion body form when expressed in *E. coli*. Previous study from our laboratory demonstrated that soluble Tn I-His_6 was less than 10% of expression product (Fig. 3A). A large amount of biologically active Tn I was finally obtained *via in vitro* renaturation process [19]. When Tn I was fused to the C-terminal of MT2A, more than 60% of the expressed MT2A-Tn I protein was soluble (Fig. 3B), and purified MT2A-Tn I was easily obtained by one-step Ni^{2+}-affinity chromatography with a yield of 28 mg/l (Table 1). These results strongly suggest that MT2A provides an alternative fusion partner that facilitates high expression of soluble recombinant protein and makes protein purification simple and easy.

DISCUSSION

In this study, we constructed a novel fusion expression plasmid, pT7MT, by using MT2A as a fusion partner and affinity tag. The initial aim of the present study was to obtain large amounts of soluble MT2A for function study. MT2A was first expressed in GST-MT2A fusion protein form, and although soluble expression could be achieved, the obtained soluble fusion protein tended highly to degrade during both the expression and purification process. This made it impossible to get intact MT2A fusion protein *via* this route. Hence, we tried another way to produce MT2A in an N-terminal fusion form with GST by constructing fusion expression plasmid pT7MT-GST. The following results demonstrated that MT2A-GST was stably expressed in a soluble form with high yield. Based on the physiological

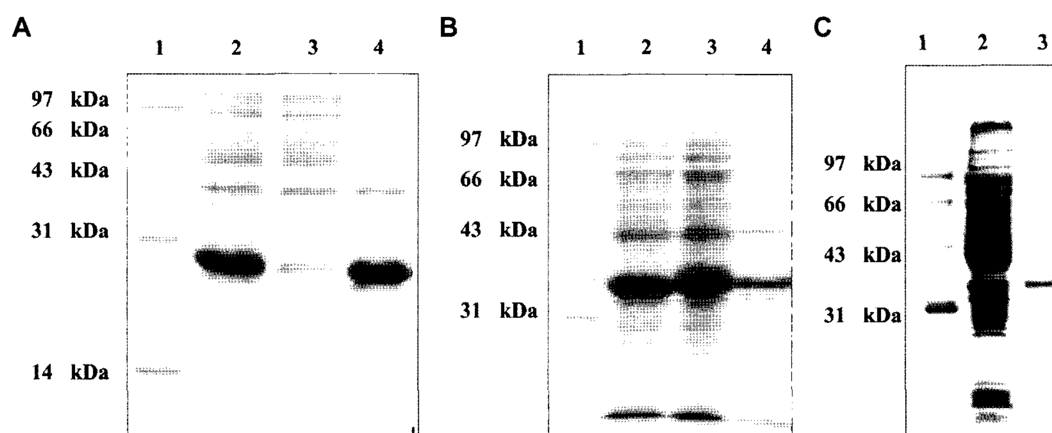

Fig. 3. Comparison of protein expression and solubility of Tn I in cells containing pET28a-Tn I and pT7MT-Tn I.
A. Expression of Tn I-His_6 from pET28a-Tn I-containing cells. Lane 1, molecular mass marker (Promega); lane 2, total cellular proteins of Tn I-His_6 expressing cells after 3-h IPTG induction; lane 3, cell lysate after sonication; lane 4, insoluble precipitates fraction. **B.** Expression of MT2A-Troponin I. Lane 1, molecular mass marker (Promega); lane 2, total cellular protein from pT7MT-Tn I expressing cells after 3-h IPTG induction; lane 3, cell lysate after sonication; lane 4, insoluble precipitates fraction. C. Purification of MT2A-Tn I. Lane 1, molecular mass marker (Promega); lane 2, cell lysate after sonication; lane 3, eluted fraction from 250 mM imidazole Ni-NTA column.

property of MT2A to bind metal ion, we tried to purify MT2A-GST by Ni^{2+}-affinity chromatography and got good purification efficiency. Therefore, we could conclude that the N-terminal MT2A form is stable and can be used as an affinity tag to facilitate purification of target protein. Because GST itself is a well-known fusion partner to improve the solubility and yield of target protein, we could not attribute the improvement to the N-terminal MT2A alone. To test the universality of MT2A as a fusion tag to improve the solubility of target protein solely, we constructed another MT2A fusion expression plasmid, pT7MT-Tn I. The solubility of MT2A-Tn I was highly improved (increased from 10% to 60%) compared with that of Tn I fused with His tag. Although the purity of MT2A-GST and MT2A-Tn I obtained by Ni^{2+}-affinity chromatography was not high, only 87% and 80%, respectively (Table 1), further modification of MT2A to improve its binding capacity to Ni^{2+} chromatography might significantly increase the purity.

In summary, we constructed a novel *E. coli* fusion protein expression vector. We selected MT2A as a fusion tag because MT2A is being recognized for its intrinsic high metal-binding capacity and this property can be used for affinity chromatography. Moreover, the MT2A fusion protein showed high and soluble expression. The wide applications of pT7MT in protein expression will be investigated and modification of pT7MT will also be made to further increase its expression level and improve its binding capacity to Ni^{2+} chromatography.

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