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Expression and purification of recombinant proteins in *Escherichia coli* tagged with a small metal-binding protein from *Nitrosomonas europaea*

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ABSTRACT

Escherichia coli is still the preferred organism for large-scale production of recombinant proteins. The use of fusion proteins has helped considerably in enhancing the solubility of heterologous proteins and their purification with affinity chromatography. Here, the use of a small metal-binding protein (SmbP) from *Nitrosomonas europaea* is described as a new fusion protein for protein expression and purification in *E. coli*. Fluorescent proteins tagged at the N-terminal with SmbP showed high levels of solubility, compared with those of maltose-binding protein and glutathione S-transferase, and low formation of inclusion bodies. Using commercially available IMAC resins charged with Ni(II), highly pure recombinant proteins were obtained after just one chromatography step. Proteins may be purified from the periplasm of *E. coli* if SmbP contains the signal sequence at the N-terminal. After removal of the SmbP tag from the protein of interest, high-yields are obtained since SmbP is a protein of just 9.9 kDa. The results here obtained suggest that SmbP is a good alternative as a fusion protein/affinity tag for the production of soluble recombinant proteins in *E. coli*.

1. Introduction

Currently there are several protein expression and purification methods. Scientists around the world have been focusing on the development of new expression and purification methods that could benefit the research community and the biotechnology industry. The use of fusion proteins for those purposes is very common because they may increase the solubility of heterologous proteins in the bacteria *Escherichia coli* [1]. These fusion proteins usually become affinity tags given that they allow the rapid purification of the recombinant proteins with affinity chromatography, one of the simplest purification methods, sometimes yielding highly pure proteins, depending on the affinity system used. The most common fusion proteins/affinity tags are maltose-binding protein (MBP) [2], glutathione S-transferase (GST) [3], and thioredoxin [4]. Nevertheless, the effectiveness of a fusion protein is not always a given for every protein to be expressed and purified. Some of the factors to be considered when choosing an affinity tag are its molecular weight, the probable improved solubility, the affinity properties it confers in order to purify with affinity chromatography, and the protocol needed to separate it from the protein of interest. Even if a specific fusion protein may seem suitable, based on these characteristics, the grade of success will depend on the nature of the protein of interest. Therefore, it is important to keep searching for different fusion proteins that may perform as good alternatives for specific target proteins.

A small metal-binding protein (SmbP) of 9.9 KDa was isolated from the periplasm of *Nitrosomonas europaea*. The mature protein of just 93 amino acids is a monomer and contains 10 sequential repeats of a seven amino acid motif characterized by a conserved

Abbreviations: SmbP, Small metal-binding protein; IMAC, Immobilized-metal affinity chromatography; MBP, Maltose-binding protein; GST, Glutathione S-transferase; EDTA, Ethylenediaminetetraacetic acid; dNTP, Deoxynucleotide triphosphate; GFP, Green Fluorescent Protein; RFP, Red Fluorescent Protein; LovR, response regulator of LovK; SHY2, Short hypocotyl 2; NDPK2, nucleoside diphosphate kinase 2; IPTG, Isopropyl β-D-1-thiogalactopyranoside; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TRIS, Tris(hydroxymethyl)aminomethane; LB, Luria–Bertani broth.

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histidine residue in the fourth position. SmbP has the capacity to bind different divalent metal ions like Cu, Ni, and Zn; therefore it has been proposed to be a metal scavenging protein in *N. europaea* to deal with high concentrations of copper [5].

In this work, we propose to use SmbP as a new fusion protein-affinity tag for protein expression and purification in *E. coli*. Several globular proteins tagged with SmbP showed high levels of soluble protein production with low formation of inclusion bodies, compared to those of MBP and GST. Expression of active red fluorescent protein showed that SmbP transports proteins to the periplasm if the construct includes the signal peptide. The recombinant proteins can be purified with immobilized metal affinity chromatography (IMAC), using Ni(II) ions. Finally, since SmbP is a small protein, high yields of the target proteins are obtained after removal of the SmbP tag.

2. Materials and methods

2.1. DNA constructs

N. europaea (ATCC 25978) was grown in optimal media: 23.1 mM $(NH_4)_2SO_4$, 2.8 mM $NaH_2PO_4 \cdot 2H_2O$, 40 mM KH_2PO_4, 3.5 mM Na_2CO_3, 222.5 μM $CaCl_2$, 676.2 μM $MgSO_4 \cdot 7H_2O$, 494.4 nM $CuSO_4 \cdot 5H_2O$, 30 mM FeSO4, 50 mM EDTA, the pH was adjusted to 7.5 with Na_2CO_3. Cells were incubated at 26 °C and 150 rpm for 72 h. Once the culture reached an OD_{600} of 1.91 the cells were harvested. The genomic DNA was purified using the Dneasy blood & tissue DNA extraction kit (Qiagen).

Full-length SmbP (SmbPp, for periplasmic expression), which contains a 24-amino acid signal peptide (MKTTLIKVIAASVTALFLSMQVYA) was amplified with primers 5′ – ATGCATGCA**CATATG**AAAACAACCCTGATAAAAGTG – 3′(NdeI, forward) and 5′ – ATGCATGCA**GGTACC**GTGCGATTTATGTTCGGATGC – 3′ (KpnI, reverse). The 50 μL reaction consisted of 100 ng of template DNA (genomic DNA extracted from *N. europaea*), 60 pmoles of each primer, 1.5 μL of 10 mM dNTP mix, and 2 units of *Vent* DNA polymerase (New England Biolabs) in 1X ThermoPol reaction buffer. The thermocycler conditions were 95 °C for 2 min; 30 cycles of 95 °C – 1 min, 56 °C – 1 min, 72 °C – 1 min; and a final extension at 72 °C for 10 min. Amplification of SmbP without the signal sequence (SmbPc, for cytoplasmic expression), was done with a different forward primer 5′ – ATGCATGCA**CATATG**AGCGGACATACTGCTCACGT – 3′ (NdeI) and the same thermocycler conditions. pET30a vector (EMD Millipore) was linearized with NdeI and KpnI, SmbPp and SmbPc were ligated into it after digestion with the same enzymes; the constructs were confirmed by sequencing. The gene for GST was cloned with the same restriction sites to compare soluble and insoluble protein production. Target proteins were cloned using NcoI and XhoI restriction sites in order to have the enterokinase recognition sequence between SmbP and the target protein for tag removal. The target proteins involved in this study were the S65T mutant of green fluorescent protein (GFP) [6], red fluorescent protein (RFP) [7], LovR, the response regulator of LovK from *Caulobacter crescentus* [8], and the *Arabidopsis thaliana* proteins short hypocotyl 2 (SHY2) [9], and nucleoside diphosphate kinase 2 (NDPK2) [10]. The constructs for GFP and RFP tagged with MBP at the N-terminal were the same from a previous study, using the pIVEX2.3d vector, which also contains the T7 promoter [11]. For comparative studies, GFP was cloned into pET30a in frame with a N-terminal His-tag already present in the vector using the restriction sites NotI and XhoI.

2.2. Protein expression

DNA constructs were transformed into *E. coli* BL21(DE3). For small-scale expression experiments, 2 mL of Luria–Bertani broth (with 30 μg/mL kanamycin) were inoculated with a single colony and incubated at 37 °C and 200 rpm until OD_{600} reached 0.5. IPTG was added up to 0.1 mM to induce expression; cells were incubated overnight at room temperature (25 °C) and 220 rpm. The next day cells were harvested and resuspended in 1X SDS-PAGE sample buffer, incubated in boiling water for 10 min, and then centrifuged for 10 min at 13,000 rpm. The supernatant was used for soluble protein content analysis. The pellet was resuspended in an 8 M urea buffer and boiled for 10 min, and then centrifuged at the same speed; the supernatant was used for the analysis of insoluble protein content (inclusion bodies). Both contents were analyzed by SDS-PAGE electrophoresis and soluble protein yields were semi-quantitated using the ImageJ software (version 1.49) from the National Institute of Health [12]. For large-scale protein expression, cells were grown in baffled flasks until OD_{600} reached 0.5, expression was induced by adding IPTG up to 0.1 mM, cells were incubated overnight at room temperature (25 °C) and 220 rpm.

2.3. Protein purification

Cells were harvested by centrifugation at 4 °C and then resuspended in ice-cold lysis buffer (50 mM TRIS–HCl, 500 mM NaCl, pH 8.0). Cells were lysed using a bead-beater and 0.1 mm glass beads (both from BioSpec Products); the lysate was clarified by centrifugation at 4 °C, 15,000 rpm during 20 min. The purification was carried out using the ÄKTA Primer Plus FPLC (GE Healthcare). The supernatant was loaded into a HisTrap FF 5 mL-column charged with Ni(II) previously equilibrated with lysis buffer. Once the lysate was loaded, the column was washed with 5 column-volumes of washing buffer (50 mM TRIS–HCl, 500 mM NaCl, 5 mM imidazole, pH 8.0). After no more protein absorption was detected, the SmbP tagged protein was eluted with 2 column-volumes of elution buffer (50 mM TRIS–HCl, 500 mM NaCl, 250 mM imidazole). Elution fractions of 1 mL were collected and analyzed by SDS-PAGE.

For purification of SmbPp-RFP, an osmotic shock procedure was performed just to obtain the periplasmic fraction. Cells from a 2 L culture were resuspended in 20 mM TRIS–HCl, 30% sucrose, 2.5 mM EDTA, pH 8.0 (5 mL per gram of cell pellet) and incubated on ice for 1 h using a plate shaker at 150 rpm. Cells were centrifuged at 10,000 rpm for 15 min at 4 °C, the supernatant was recovered and saved, and the sediment was resuspended in ice-cold 5 mM $MgSO_4$ buffer for 1 h (on the plate shaker at 150 rpm). Cells were centrifuged again at 10,000 rpm for 15 min at 4 °C, and the supernatant was also considered as periplasmic fraction. The two supernatants were pooled and applied to the HisTrap column and purified the same way as described above. All protein quantification was performed using the Bradford reagent from BIO-RAD.

2.4. SmbP-tag removal

1 mg of SmbPc-GFP in 100 μL was mixed with 25 units (2 μL) of enterokinase light chain (New England Biolabs) for 16 h at room temperature. SmbP was removed by incubating the reaction mixture with 150 μL of Profinity IMAC resin charged with Ni(II) (BIO-RAD) for one hour at 4 °C, then centrifuged at 5000 rpm for 1 min, and the supernatant was analyzed by SDS-PAGE.

2.5. Fluorescence spectroscopy for GFP and RFP

Purified fluorescent proteins were diluted in 20 mM TRIS–HCl pH 8.0 buffer and the fluorescence emission spectrum was obtained with a Luminescence Spectrometer LS 55 from Perkin Elmer. Excitation wavelengths were set at 460 and 530 nm for GFP and RFP respectively.