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Expression and purification of recombinant proteins in *Escherichia coli* tagged with the metal-binding protein CusF

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ABSTRACT

Production of recombinant proteins in *Escherichia coli* has been improved considerably through the use of fusion proteins, because they increase protein solubility and facilitate purification via affinity chromatography. In this article, we propose the use of CusF as a new fusion partner for expression and purification of recombinant proteins in *E. coli*. Using a cell-free protein expression system, based on the *E. coli* S30 extract, Green Fluorescent Protein (GFP) was expressed with a series of different N-terminal tags, immobilized on self-assembled protein microarrays, and its fluorescence quantified. GFP tagged with CusF showed the highest fluorescence intensity, and this was greater than the intensities from corresponding GFP constructs that contained MBP or GST tags. Analysis of protein production *in vivo* showed that CusF produces large amounts of soluble protein with low levels of inclusion bodies. Furthermore, fusion proteins can be exported to the cellular periplasm, if CusF contains the signal sequence. Taking advantage of its ability to bind copper ions, recombinant proteins can be purified with readily available IMAC resins charged with this metal ion, producing pure proteins after purification and tag removal. We therefore recommend the use of CusF as a viable alternative to MBP or GST as a fusion protein/affinity tag for the production of soluble recombinant proteins in *E. coli*.

1. Introduction

Fusion proteins have played an important role in the expression and purification of recombinant proteins within the bacterium *Escherichia coli* [1]. Furthermore, it has been demonstrated that the use of fusion proteins also improves levels of protein synthesis when employing *in vitro* cell-free expression systems based on the *E. coli* S30 extract [2]. The most common fusion protein-affinity tags are maltose-binding protein (MBP) [3], glutathione S-transferase (GST) [4], and thioredoxin [5]. Nevertheless, since these fusion proteins are not universally applicable, it is important to continue searching for alternatives that may serve to optimize the production of target proteins.

CusF, a periplasmic protein that is part of the CusCBFA efflux complex, plays a role in the resistance of *E. coli* to elevated levels of copper and silver. The mature polypeptide is small (just 9.9 kDa), and forms a beta-barrel structure [6]. In this work, we propose CusF as a new fusion protein-affinity tag for protein expression and purification in *E. coli*. Several globular proteins tagged with CusF showed high levels of soluble protein with low formation of inclusion bodies, as compared to those tagged with MBP or GST. Expression of Red Fluorescent Protein confirmed that CusF 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 Cu(II) ions. Since CusF is a small protein, high yields of the target proteins are obtained after removal of the CusF tag. Finally, results show that CusF is an exceptional fusion protein for *in vitro* protein synthesis using

Abbreviations: CusF, Cu sensitive free protein; GFP, Green Fluorescent Protein; MBP, maltose-binding protein; GST, glutathione S-transferase; IMAC, immobilized-metal affinity chromatography; AXR3, Auxin-responsive protein IAA17; dNTP, deoxynucleotide triphosphate; LB, Luria–Bertani broth; IPTG, isopropyl β-D-1-thiogalactopyranoside; OD_{600}, optical density at 600 nm; SHY2, Short hypocotyl 2; LovR, response regulator of LovK; RFP, Red Fluorescent Protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tris, Tris(hydroxymethyl)-aminomethane.

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the *E. coli* S30 cell-free expression system.

2. Materials and methods

2.1. DNA constructs

The constructs for cell-free expression on protein microarrays of untagged GFP and GFP tagged with MBP, AXR3 (auxin-responsive protein IAA17 from *Arabidopsis thaliana*), CusF, and GST were those described previously [7–9], using the pIVEX2.3d vector, which contains the T7 promoter. For protein expression *in vivo*, full-length CusF (which includes the signal peptide MKKALQVAMFSLFTVIGF-NAQA for periplasmic expression) was amplified with primers 5′ - AGTCAGTCA**CATATG**AAAAAAGCACTGCAAGTCG - 3′ (NdeI, forward) and 5′ - ATGCATGCA**GGTACC**CTGGCTGACTTTAATATCCTGTAA - 3′ (KpnI, reverse). The 50 μL reaction comprised 10 ng of template DNA, 60 pmol of each primer, 1.5 μL of 10 mM dNTPs 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, 59 °C–1 min, 72 °C–1 min; and a final extension at 72 °C for 10 min. Amplification of CusF lacking the signal sequence (for cytoplasmic expression) was done with forward primer 5′ - AGTCAGTCA**CATATG**GCTAACGAA-CATCATCATGAAAC - 3′ (NdeI) and the same reverse primer and thermocycler conditions as before. pET30a vector (EMD Millipore) was linearized with NdeI and KpnI, and the CusF insert was ligated into it following digestion with the same enzymes. The gene for GST was cloned with the same restriction sites to compare soluble protein production. Target proteins were cloned using NcoI and XhoI restriction sites, in order to place the enterokinase recognition sequence between CusF and the target protein for tag removal. The reporter proteins involved in this study were the S65T mutant of Green Fluorescent Protein (GFP) [10]; Red Fluorescent Protein (RFP) [11]; LovR, the response regulator of LovK from *Caulobacter crescentus* [12], and the *A. thaliana* protein Short Hypocotyl 2 (SHY2) [13].

2.2. Protein expression in vitro

CusF-GFP expression *in vitro* was compared to MBP-GFP, GST-GFP, AXR3-GFP, and GFP alone (lacking an N-terminal tag) by measuring the fluorescence intensities on self-assembled protein microarrays. These microarrays were produced by applying a cell-free expression system based on the S30 *E. coli* extract on anti-GFP antibody arrays as previously described [7]. Fluorescence intensities were detected using the GenePix 4200AL microarray scanner, with excitation at 488 nm and emission detected at 511 nm. The fluorescence median values from each array element were extracted and averaged for each sub-array.

2.3. Protein expression in vivo

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) was inoculated with a single colony and incubated at 37 °C and 220 rpm until an OD_{600} of 0.5 was attained. IPTG was added 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 by centrifugation and resuspended in 100 μL 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 residual pellet was resuspended in 100 μL 8 M urea buffer and boiled for 10 min to solubilize the inclusion bodies, then centrifuged at the same speed; the supernatant was used for the analysis of insoluble protein content. For large-scale protein expression, cells were grown in baffled flasks until an OD_{600} of 0.5 was reached. Expression was then induced by adding IPTG to 0.1 mM, and the cells were incubated overnight at room temperature (25 °C) and 220 rpm.

2.4. Protein purification

For purification of CusF-GFP, cells from 2.5 L of culture were harvested by centrifugation at 4 °C and then resuspended in 40 mL of 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 (BioSpec Products, Bartlesville, OK). The lysate was clarified by centrifugation at 4 °C and 15,000 rpm for 20 min. The purification was carried out using the ÄKTA Primer Plus FPLC system (GE Healthcare). The clear lysate was loaded into a HisTrap FF 5-mL column charged with Cu(II) previously equilibrated with lysis buffer. After loading, the column was washed with 5 column-volumes of washing buffer (50 mM Tris–HCl, 500 mM NaCl, 2.5 mM imidazole, pH 8.0). After no more protein absorption was detected, CusF-GFP 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. Protein quantification was performed using the Bradford reagent (BIO-RAD, Hercules, CA).

For purification of CusF-RFP, using the construct that contained the signal sequence for transport to the periplasm, an osmotic shock procedure was performed to obtain the periplasmic fraction. 14 g of wet weight cells from a 2.5 L culture were resuspended in 70 mL of sucrose buffer (20 mM Tris–HCl, 30% sucrose, 2.5 mM EDTA, pH 8.0; 5 mL per gram of cell pellet), and were 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 50 mL of ice-cold 5 mM $MgSO_4$ 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, also considered as periplasmic fraction, was pooled with the first supernatant, and applied to a HisTrap HP 1-mL column. Purification was done as described above.

2.5. CusF-tag removal

1 mg of CusF-RFP in 125 μL was mixed with 25 units (2 μL) of enterokinase light chain (New England Biolabs, Ipswitch, MA) for 16 h at room temperature. CusF was removed by incubating the reaction mixture with 150 μL of Profinity IMAC resin (BIO-RAD) charged with Cu(II) for 1 h at 4 °C, then centrifuged at 5000 rpm for 1 min, and the supernatant was analyzed by SDS-PAGE. Since free RFP bound to the Cu(II) resin, it was necessary to elute it using small concentrations of imidazole (5 and10 mM); fractions were analyzed by SDS-PAGE.

3. Results and discussion

Fig. 1 shows a microarray image illustrating the transcription and translation of different GFP constructs using a cell-free system based on the *E. coli* S30 extract. GFP was used as the epitope for protein immobilization and provided direct measurement of protein expression levels in terms of fluorescence intensity. GFP tagged with CusF provided the highest fluorescence intensity signals. The fluorescence values of more than 35 proteins tagged with GFP at the C-terminus were determined in a previous study, where different cell-free expression systems were tested [7]. Although not mentioned in that report, CusF consistently displayed the highest fluorescent values when using the S30 extract or the hybrid system.