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Ubiquitin-intein and SUMO2-intein fusion systems for enhanced protein production and purification

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ABSTRACT

Although most commonly used for protein production, expression of soluble and functional recombinant protein in *Escherichia coli* is still a major challenge. The development and application of fusion tags that can facilitate protein expression and solubility partly solve this problem, however, under most circumstance, the fusion tags have to be removed by proteases in order to use the proteins. Because the tag removal using proteases increases cost and introduces extra purification steps, it remains a significant problem that must be resolved before being widely used in industry production. Ubiquitin and SUMO have been successfully used to enhance protein expression and solubility. In the last decades, intein has also been widely used in protein production for its self-cleavage property, which could help to remove the fusion tag without any protease. Here, we take the advantages of ubiquitin, SUMO2 and intein in protein expression. We constructed tandem ubiquitin-intein and SUMO2-intein fusion tags, and chose human MMP13 (amino acid 104–274) and eGFP as the passenger proteins that fused to the C-terminus of the tags. These constructs were expressed in *E. coli* and both MMP13 and eGFP expression and solubility were evaluated. Both tags showed the ability to enhance the solubility of MMP13 and eGFP and improve the expression of eGFP, and the SUMO2-intein having a more significant effect. Both ubiquitin-intein-eGFP and SUMO2-intein-eGFP were purified using Ni–NTA column chromatography and self-cleavaged by changing pH. The recombinant un-tagged eGFP were released and eluted with high homogeneity. In summary, ubiquitin-intein and SUMO2-intein are convenient and useful fusion tags that can enhance the expression, solubility and improve the purification process of the model heterologous protein and these tags may have a good prospect in protein production.

Introduction

In spite of the fast technological development of the protein expression, *Escherichia coli* is still the most attractive host for its rapid and low-cost production of recombinant proteins for scientific research and drug applications [1,2]. However, successful expression of soluble and correctly folded protein is a frequently encountered challenge [3]. These problems have partly been overcome by using various fusion tags, such as maltose-binding protein (MBP)[2] [4], glutathione S-transferase (GST) [5], thioredoxin (TRX) [6], NusA [7], ubiquitin (Ub) [8,9], and SUMO (small ubiquitin-related modifier) [10,11]. Among these tags, Ub and SUMO have recently been successfully used to express several difficult-to-express proteins. In addition, fusion proteins with Ub or SUMO tags can be effectively cleaved by deubiquitinase or SUMO protease with high fidelity, releasing the passenger proteins with natural N-terminal amino acid. However, the removing of Ub or SUMO tags by deubiquitinase or SUMO protease would increase production

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[2] MBP, maltose-binding protein; GST, glutathione S-transferase; TRX, thioredoxin; Ub, ubiquitin; SUMO, small ubiquitin-related modifier; eGFP, enhanced green fluorescent protein; eGFP, elute the cleaved target protein; pHUE, histidine-tagged ubiquitin expression vector; pHS2E, histidine-tagged SUMO2 expression vector; pHUIE, histidine-tagged ubiquitin-intein expression vector; pHSIE, histidine-tagged SUMO2-intein expression vector; DUBs, deubiquitylating enzymes; M, molecular weight marker; UN, un-induced culture; IN, induced culture; S, soluble cellular lysate; IB, inclusion body.

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costs and introduce extra purification steps, which limit their usage in bulk protein production [12].

In the last decade, David W. Wood and his colleagues developed a number of engineered self-cleaving inteins which have broad potential applications [13–16]. The self-cleaving of the intein fusion protein can be induced at the intein's C-terminus or at the intein's N-terminus, and the cleavage leave the protein with natural N-terminal amino acid. The engineered self-cleaving intein exhibits rapid C-terminal cleaving properties in response to mild changes in pH and temperature, while nucleophiles that react with thioesters, such as thiols and hydroxylamine, can effectively inducing the N-terminal cleavage of the intein [13–16]. The low cost and convenient process using intein tag makes it more suitable for industrial application.

In the present study, we tested Ub-intein and human SUMO2-intein as fusion tags for recombinant proteins. Human MMP13 (amino acid 104–274) and enhanced green fluorescent protein (eGFP) were expressed as the fusion proteins with Ub-intein and human SUMO2-intein. We evaluate the expression and solubility of MMP13 and eGFP, and also studied the cleavage and purification of the eGFP fusion protein. We showed that the expression and purification of the eGFP fusion protein using these two tags is easily accomplished by Ni–NTA column chromatography followed by a mild pH shift and elution.

Materials and methods

Construction of the expression plasmid with fusion tags

The *E. coli* strain DH5α (Novagen, Darmstadt, Germany) was chosen for construction and manipulation of the expression plasmids. The pHUE (Histidine-tagged Ubiquitin Expression vector) was generously provided by Dr. Rohan T. Baker. The PET-EI-CAT was kindly provided by Dr. David W. Wood. The pHS2E (Histidine-tagged SUMO2 Expression vector) was constructed by our lab using pHUE as the backbone, and the construction strategy and the primers were shown in our previous article [11]. To generate pHUIE (Histidine-tagged Ubiquitin-Intein Expression vector, Fig. 1A) and pHSIE (Histidine-tagged SUMO2-Intein Expression vector, Fig. 1B), pHUE and pHS2E were both digested by BamHI and EcoRI, and ligated with a DNA insert encoding intein amplified from PET-EI-CAT. The PCR primers used were 5′-TTTTGGATCCGCCC TCGCAGAGGGCACTC-3′ and 5′-TTTTGAATTCGTTGTGTACAACAACC CCTTC-3′ for intein. To generate human MMP13 (amino acid 104–274) and eGFP expression vector, the gene for MMP13 na d eGFP was cloned into pHUIE and pHSIE by inserting the PCR product of MMP13 and eGFP gene using EcoRI and HindIII as the restriction enzyme sites. The PCR primers used were 5′-TTTT GAATTCTACAATGTTTTCCCTCG-3′ and 5′-TTTTAAGCTTTTAGTTGGG GTCTTCATCTC-3′ for MMP13 and 5′-TTTTGAATTCATGGTGAGCAA GGGCGAGGAG-3′ and 5′-TTTTAAGCTTTTACTTGTACAGCTCGTCC ATG-3′ for eGFP.

Expression of recombinant fusion proteins in E. coli

In a typical experiment, the expression vectors were transformed into *E. coli* strain BL21 (DE3) (Novagen, Darmstadt, Germany). For expression, a single colony was inoculated into 4 ml LB-broth supplemented with 30 μg/ml kanamycin (for pET-28b(+) based expression vectors) or 100 μg/ml ampicillin (for pHUE, pHS2E, pHUIE, pHSIE based expression vectors) and grown overnight at 37 °C with shaking. The next day, 2 ml of the overnight cultures were sub-cultured into 200 ml LB supplemented with 30 μg/ml kanamycin or 100 μg/ml ampicillin and grown to OD600 = 0.6 at 37 °C with shaking. Then cultures were cooled on ice for 30 min before induction by 0.4 mM IPTG at 20 °C overnight with shaking. The Cell pellets were harvested by centrifugation at 4000 rpm for 10 min and then resuspended in 20 ml of buffer A (50 mM Tris at pH 8.5, 300 mM NaCl, 20 mM imidazole) containing 1 mM Phenylmethylsulphonyl fluoride. Cells were lysed by mild sonication. The lysates were then partitioned into soluble and insoluble fractions by centrifugation at 12,000 rpm for 30 min at 4 °C, and the supernatant constituting the soluble protein fraction was transferred to a fresh tube and stored at 4 °C (soluble protein sample). The pellet was dissolved in 20 ml of solubilization buffer (20 mM Tris–Cl at pH 8.5, 8 M Urea, 500 mM NaCl) for 1 h at room temperature and then centrifuged at 12,000 rpm for 30 min, and the supernatant was removed and stored at 4 °C (Inclusion body protein sample). The proteins were detected by 12% SDS–PAGE.

Ni–NTA affinity column purification of recombinant eGFP

To avoid the self-cleavage of intein, unless otherwise noted, all of the following purification steps were performed at 4 °C with ice-cold buffers. The supernatant containing Ub-intein-eGFP or SUMO2-intein-eGFP was applied to a Ni–NTA column, which was packed with 1 ml resin and pre-equilibrated with buffer A. After binding, the column was washed with 10 column volumes of buffer A, and then followed by an additional wash with 10 column volumes of the cleaving buffer (PBS buffer supplemented with 40 mM Bis–Tris and 20 mM imidazole, pH 6.0). After the second wash, the column was sealed and incubated at room temperature to allow the self-cleavage of intein. After intein cleavage, 5 column volumes of the cleaving buffer were used to elute the cleaved target protein (eGFP) from the resin. The column was then eluted with 10 column volumes of cleaving buffer supplemented with 500 mM imidazole, and the eluted fraction was also collected. All the fractions were analyzed by 12% SDS–PAGE.

Results

Comparison of ubiquitin-intein and SUMO2-intein fusion systems with His_6, ubiquitin and SUMO2 fusion systems

To evaluate the expression and solubility of the ubiquitin-intein and SUMO2-intein fusion systems, MMP13 and eGFP was chosen as the passenger proteins. We compared ubiquitin-intein and SUMO2-intein fusion systems with His_6, ubiquitin and SUMO2 fusion systems. All the expression vectors were transformed into *E. coli* BL21 (DE3). After culturing the cells, lysates were collected from un-induced (UN) and induced (IN) cultures. The un-induced lysates, the soluble (S) and insoluble (IB) fractions from induced cultures were analyzed by 12% SDS–PAGE (Fig. 2 and Fig. 3).

MMP13 (amino acid residues 103–274) expression and solubility was studied (Fig. 2). As expected, when human MMP13 (amino acid 104–274) fused with His_6, the protein could be expressed in bulk but almost insoluble. However, when expressed with either ubiquitin, ubiquitin-intein, SUMO2, SUMO2-intein, the solubility was improved. Adding intein had negligible influence on protein yields compared to the Ub-MMP13 and SUMO2-MMP13 fusion proteins lacking the intein tag. In agree with our previous study [11], SUMO2 fusion was better than ubiquitin in MMP13 solubility, regardless of whether it coupled with intein or not (Fig. 2).

The eGFP-His_6 construct produced low level protein even with IPTG induction. In contrast, when expressed as fusion proteins with either ubiquitin, SUMO2, ubiquitin-intein or SUMO2-intein, the expression and solubility of eGFP was greatly improved (Fig. 3). Compared with ubiquitin fusion, SUMO2 fusion produced more protein in the soluble fraction, regardless of whether ubiquitin or SUMO2 linked to intein or not. Intein had no effect on