Trends in Peptide and Protein Sciences
Volume 8 (2023): e5

DOI: https://doi.org/10.22037/tpps.v8i1.42962

Original Article

Soluble Expression of Recombinant Human Bone Morphogenetic Protein-7 (rhBMP-7) in *Escherichia coli* Using SUMO Fusion System

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Article history:
Received: 9 August 2023
Accepted: 8 October 2023

Keywords:
BMP-7
Escherichia coli
Recombinant protein
Soluble expression
SUMO fusion system

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HIGHLIGHTS

- SUMO is a well characterized family of ubiquitin-like molecules.
- SUMO fusion led to increased expression and solubility of BMP-7.
- BMP-7 is involved in the process of bone formation.

ABSTRACT

BMPs belong to transforming growth factor β superfamilies, which their principal role is inducing bone and cartilage formation at heterotopic and orthotopic sites. Since the formation of inclusion bodies is the main limitation of producing these proteins in *Escherichia coli*, in this study, the small ubiquitin-like modifiers (SUMO) fusion system was employed to improve solubility and expression of recombinant human BMP-7 (rhBMP-7) in *E. coli*. The SUMO fusion system has the ability to enhance protein expression, reduce target protein proteolytic degradation, and increase protein folding and solubility. In the current study, the SUMO protein gene was fused to the N-terminus of the BMP-7 gene, and cloned in the pET-28a vector. After purification of the expressed SUMO-BMP-7 protein by Ni-NTA chromatography, SUMO was removed from the BMP-7 protein using SUMO protease. In the second step of purification using Ni-NTA chromatography, the cleaved BMP-7 protein was purified and then identified by Western blot analysis. The results of the current study demonstrated that the SUMO fusion system is able to increase the soluble form of rhBMP-7. Furthermore, rhBMP-7 can be purified by a two-step purification strategy including: 1) purification of SUMO-BMP-7 and 2) purification of rBMP-7 after cleavage using Ni-NTA chromatography. Altogether, this research has provided a feasible approach for large-scale production of soluble rhBMP-7, to facilitate its further medical development.

Cite this article as: Sadipour, G., Dugmehchi, A., Talebkhan, Y., Barkhordari, F., Mohit, E. and L. Nematollahi, (2023). Soluble expression of recombinant human bone morphogenetic protein-7 (rhBMP-7) in *Escherichia coli* using SUMO fusion system. *Trends Pept. Protein Sci.*, **8**: e5.

Introduction

Bone morphogenetic proteins (BMPs) are a group of cytokines that belong to the transforming growth factor-β (TGF-β) superfamily and were described by Dr. Marshall Urist in 1965 (Chen et al., 2013; Wang et al., 2014; Chen et al., 2016; Aslani et al., 2019). Their hallmark ability is induction of bone and cartilage tissue differentiation. These proteins play important roles during embryonic patterning and early skeletal growth (Wang et al., 2014; Deng et al., 2018) .To date, More than 20 different BMPs have been identified with varying degrees of bone induction (Rao et al., 2013; Wang et al., 2014; Sheikh et al., 2015; Ye and Jiang 2016; Khorsand et al., 2017). BMP-7 gained approval for local treatment of distinct bone-related conditions, e.g., posterolateral spinal lumbar fusion and complicated permanent tibial pseudarthrosis (Neuerburg et al., 2019; Zang et al., 2019) BMP-7 also induced cementogenesis and stimulated periodontal ligament and alveolar bone formation in surgically induced mandibular molar class II and class III furcation defects in baboons and beagles, demonstrating its beneficial effect in periodontal wound healing/regeneration (Zang et al., 2019). Recombinant forms of BMP-2 and BMP-7 are the only BMPs approved by the Food and Drug Administration (FDA) for clinical use (Haversath et al., 2012; Epstein, 2013; Begam et al., 2017; Gillman and Jayasuriya, 2021). Preclinical and clinical studies have shown that rhBMP-7 has been used in various therapeutic applications including spinal fusion, non-union fractures, and long bone defects (Blokhuis et al., 2013; Sampath and Reddi, 2020). Hence Expression of rhBMP-7 in heterologous systems seems to be necessary. *Escherichia coli* (*E. coli*) is still one of the most used hosts for the expression of recombinant proteins because of its simplicity, rapid growth, and low cost. Although *E. coli* is a preferred host for the expression of recombinant proteins, most heterologous proteins such as BMPs tend to form inclusion bodies in *E. coli* (Sun et al., 2012; Hou et al., 2013; Jeong et al., 2014; Nasrabadi et al., 2018). Various strategies, including changing of promoters, co-expression of chaperones and foldases (Sun et al., 2012; Gupta and Shukla, 2016; Bhatwa et al., 2021), secretion of proteins to the *E. coli* periplasmic space (Sun et al., 2012; Malik, 2016) and using fusions partners (Sun et al., 2012; Bhatwa et al., 2021; Ko et al., 2021), have been applied to improve soluble expression of recombinant protein in *E. coli* (Wang et al., 2010; Gupta and Shukla, 2016; Bhatwa et al., 2021). Fusion systems can not only enhance protein expression and folding, but also reduce proteolytic degradation and simplify purification and detection of the recombinant protein (Wang et al., 2010; Yadav et al., 2016; Cheng et al., 2017). Available some common structures have been used as fusion tags, including Thioredoxin (Trx) (Xiao et al., 2018; Ki and Pack, 2020; Saffari et al., 2020), the small ubiquitin-like modifiers (SUMO) (Wang et al., 2010; Guerrero et al., 2015; Yang et al., 2017; Hanif et al., 2018), maltose binding protein (MBP) (Wang et al., 2010; Vu et al., 2014; Lee, 2022), N-utilization substance A (NusA) (Wang et al., 2010; Raran-Kurussi and Waugh, 2014), alkaline phosphatase (Kuo et al., 2011), glutathione S-transferase (GST) (Wang et al., 2010; Williams et al., 2020), ubiquitin (Wang et al., 2010) and the DsbA protein (Liu et al., 2015; Hajihassan et al., 2016) are some of the available commonly structures have been used as fusion tags. In this study, the effect of the SUMO fusion system on improving the solubility and expression of rhBMP-7 in *E. coli* was evaluated.

SUMO is a well characterized family of ubiquitin-like molecules, which becomes covalently and reversibly conjugated to specific protein substrates on lysine residues in eukaryotic cells. Sumoylation is a post-translational modification process and is involved in many cellular processes, such as transcriptional regulation, nuclear-cytosolic transport, chromosome organization, apoptosis, protein activation and stability, response to stress, progression through the cell cycle, DNA repair, and signal transduction (Wang et al., 2010; Kuo et al., 2011; Hou et al., 2013; Wang et al., 2013; Yu et al., 2013; Liew et al., 2014; Sriramachandran and Dohmen, 2014; Hasan and Saluja, 2015; Sang et al., 2019; Acuña, 2022). SUMO has an external hydrophilic surface and an inner hydrophobic core that exert a detergent-like effect on insoluble proteins (Hou et al., 2013; Sang et al., 2019; Zia et al., 2022), SUMO and its associated enzymes and pathways are present in all eukaryotes and are highly conserved from yeast to human (Linova et al., 2020). It was found that fusion of recombinant proteins with SUMO protein has many advantages including improved protein folding, enhanced expression level, and enhanced protection of the protein from degradation via its chaperoning properties. Moreover, recombinant proteins with SUMO tags can be effectively cleaved by robust and efficient SUMO

protease, which recognizes the tertiary sequence of SUMO protein, and native-like target protein can be generated after removing SUMO tags (Wang et al., 2010; Wang et al., 2012; Hou et al., 2013; Truong et al., 2013; Wang et al., 2013; Yu et al., 2013; Zhang et al., 2014; Li et al., 2018; Linova et al., 2020). In previous studies, several difficult to-express proteins including the *Staphylococcus aureus* PyrC enzyme (SaPyrC) (Truong et al., 2013), active single-chain antibody fragment (scFv) (Ye et al., 2008), antimicrobial peptide scolopin 1 (AMP-scolopin 1) (Hou et al., 2013), human PYY (hPYY) (Fazen et al., 2012), recombinant hepcidin 25 (Sadr et al., 2017), mature α-luffin (Namvar et al., 2018), melittin (Chen et al., 2021), antimicrobial peptide AP2 (Mo et al., 2018) and allium sativum leaf agglutinin (ASAL) (Upadhyay et al., 2010) have been successfully expressed and purified in *E. coli* using SUMO as fusion tag (Hou et al., 2013).

In the present study, the expression of rhBMP-7 using the SUMO fusion system in *E. coli* was evaluated for the first time. Furthermore, the effect of SUMO fusion on rhBMP-7 solubility and purification by Ni–NTA chromatography was also examined.

Materials and Methods

Strains, plasmids and culture media

E. coli strain Top 10F' and *E. coli* strain BL21 (DE3), which were used for cloning experiments and as hosts for protein expression, were obtained from Thermo Fischer Scientific (Massachusetts, USA). The pET-28a expression vector was obtained from Novagen, (Madison, WI). The gene was synthesized by GeneRayBiotech© (Shanghai, China) and cloned in PGH vector, Prestained Protein Ladder, pageruler unstained protein ladder, restriction enzymes, and buffers for gene construction and molecular cloning were from Thermo Fisher Scientific (Massachusetts, USA). Anti-His6-Peroxidase and T4 DNA ligase were purchased from Roche Diagnostics (Germany). Imidazole, $Na_2H_2PO_4$, NaCl, isopropyl b-D-thiogalactopyranoside (IPTG), luria–bertani (LB) medium, and Coomassie brilliant blue G-250 were obtained from Sigma (USA). A plasmid DNA purification kit was obtained from iNtRON Biotechnology (USA). The SUMO protease was obtained from LifeSensors (USA). The Ni- NTA Agarose was ordered from QIAGEN (Germany). 3, 3'-Diaminobenzidine (DAB) conjugate substrate kit was obtained from Bio-Rad (USA). Goat anti-rabbit antibody peroxidase and rabbit polyclonal antibody to BMP-7 were purchased from Razi Biotech (Tehran, Iran) and Abcam (United Kingdom), respectively. Other reagents were obtained from standard commercial sources.

Construction of BMP-7 expression cassettes with and without SUMO fusion partner

The coding region of the human BMP-7 mature domain (139 amino acids, GenBank with accession number NM_001719.3) with either His6SUMO or His sequence tag at the N-terminal region was synthesized and cloned in the PGH vector. Codon optimization for expression in *E. coli* was done by GenBank and confirmed using the codon adaption index calculator. Then, the genes were cloned in *Nco*I/*Eco*RI sites of pET-28a (+), and the transformants were selected in Luria Bertani medium (LB) containing kanamycin (30 µg/mL). To confirm the recombinant clone, colony PCR screening using T7 primers was performed. To further confirm the recombinant clone, the plasmid of one positive clone was sequenced. Finally, pET-28a-SUMO-BMP7and pET-28a-BMP7 plasmids were transformed into the expression strains *E. coli* BL21 (DE3) competent cells.

Expression of SUMO-BMP-7 and BMP-7 fusion proteins

In this study, a single colony of transformed *E. coli* strain BL21 (DE3) was inoculated into 5 ml of LB-broth (containing 30 µg/mL kanamycin) to grow overnight under a constant temperature 37°C in a shaker-incubator. Then, overnight culture was sub-cultured 1:100 into 50 ml LB-broth (30 µg/mL kanamycin) until its optical density (OD_{600}) reached 0.4-0.6. In order to induce recombinant protein expression, 1 mM IPTG (isopropyl b-D-1-thiogalactopyranoside) was added at 37 °C while shaking at 170 rpm. Four hours after induction, the cells were harvested by centrifugation at 9000 rpm for 3 min.

Soluble proteins analysis

In order to assess the solubility, the bacterial pellets were resuspended in 15 mL lysis buffer (50 mM Na_2HPO_4/NaH_2PO_4, 300 mM NaCl, 40 mM imidazole, pH 8.0). A mild sonication (Hielscher, Germany) for 15 cycles (20 s pulses and 20 s intervals) on ice was employed in order to lyse the cells Then, the resulting lysate cells were centrifuged at 13000 rpm for 20 min at 4°C. The supernatant and the pellet which contained the

soluble proteins and cell debris as well as inclusion bodies (IB) were separated by 15% SDS–PAGE, and proteins were visualized by Coomassie staining. Densitometry analysis of SDS–PAGE gels was carried out using Quantity One® software (ver. 4.6.3; Bio-Rad Laboratories, Hercules, CA) to determine the relative soluble expression level of SUMO-BMP7 and BMP7.

Purification and western blotting of SUMO-BMP-7 fusion protein

In order to purify SUMO-BMP7 and BMP7, the soluble protein fraction from 200 mL LB-broth culture medium, was applied to a Ni-NTA superflow column, that was packed with 2 mL resin and pre-equilibrated with 10 mL binding buffer (50 mM Na_2HPO_4/NaH_2PO_4, 300 mM NaCl, 40 mM imidazole at pH 8.0) at a flow rate of 1 mL/min. Then, to wash unbound proteins wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 50 mM Imidazole, pH 8.0) was used. Eventually, the fusion protein was eluted with 10 mL elution buffer (50 mM Na_2HPO_4/NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, pH 8.0). 15% SDS–PAGE was used to detect the purified SUMO-BMP-7 protein.

Anti-His6 tag antibody was used in Western blot analysis to confirm the His-tagged SUMO-BMP-7 expression in soluble form and His-tagged BMP-7 expression. A semi-dry transfer cell apparatus (Bio-Rad, USA) was applied to transfer the recombinant proteins to a nitrocellulose membrane (Amersham Company, UK). Then, the membrane was blocked with 3% skimmed milk in PBS buffer for 60 min. Then, the blot was incubated at room temperature with an anti-His6 tag antibody at 1:1000 dilution (diluted in PBS) for 120 min. Finally, positive bands were visualized by a DAB staining kit (Thermo Fisher Scientific, Waltham, MA, USA).

Enzymatic cleavage of SUMO-BMP7 fusion protein and purification of recombinant BMP-7

In order to remove imidazole, the elutes consisting of purified SUMO-BMP-7 were dialyzed with molecular weight cut-offs 12-15 kDa (Sigma, USA) against 20 mM Tris-HCl, 150 mM NaCl, and 10% glycerol (pH 8.0). Then, digestion of SUMO-BMP-7 fusion was done by adding 2 mM dithiothreitol (DTT) and incubating with SUMO Protease (Lucigen©, Middleton, WI) at 1:100 enzyme to substrate molar ratio at 4 °C, pH 8.0 for overnight. The resulting cleaved product was analyzed with 15% SDS–PAGE. To further purify BMP-7 and to remove the SUMO tag and SUMO Express Protease, as well as any residual of uncleaved fusion protein, purification using the Ni-NTA column was performed as described earlier.

Western blot analysis of the purified BMP-7 protein

As His6 tags were removed after purification of hBMP-7 protein, rabbit polyclonal antibody (primary antibody) and goat anti-rabbit antibody conjugated to horse radish peroxidase (secondary antibody) was used in western blot analysis to confirm purified hBMP-7. Accordingly, the blot was incubated at room temperature with rabbit polyclonal antibody against BMP-7 (primary antibody) at 1:5000 dilution (diluted in PBS) for 90 min. Finally, the blot was incubated at room temperature for 60 min with goat anti rabbit antibody conjugated to horse radish peroxidase (secondary antibody) at 1:20000 dilution (diluted in PBS). Positive bands were visualized by a DAB staining kit (Thermo Fisher Scientific, Waltham, MA, USA).

Results

PET28-SUMO-BMP-7 and PET28-BMP-7 vector construction

BMP-7 has been reported to express as inclusion bodies in *E. coli*, thus, for the soluble and high yield expression of mature BMP-7 in *E. coli*, we employed the SUMO-fusion system. Therefore, two expression constructs (pET-28a-BMP-7 and pET-28a-SUMO-BMP-7) were prepared (Fig. 1) to compare the solubility of SUMO-free and SUMO-tagged expressed proteins. The synthesized genes segments with the size 452 bp and 752 bp were cut from PGH-BMP-7 and PGH-SUMO-BMP-7, respectively, and cloned into *Nco*I/*Eco*RI linearized pET-28a (+) vector. Digestion of pET-28a-SUMO-BMP-7 and pET-28a-BMP-7 using *Nco*I/*Eco*RI enzymes resulted in expected bands sizes of 5265 and 752 bp as well as 5265 and 452 bp, respectively (Fig. 2). Additionally, DNA sequencing analysis and colony PCR screening using T7 primers confirmed the sequence of positive clones (data not shown).

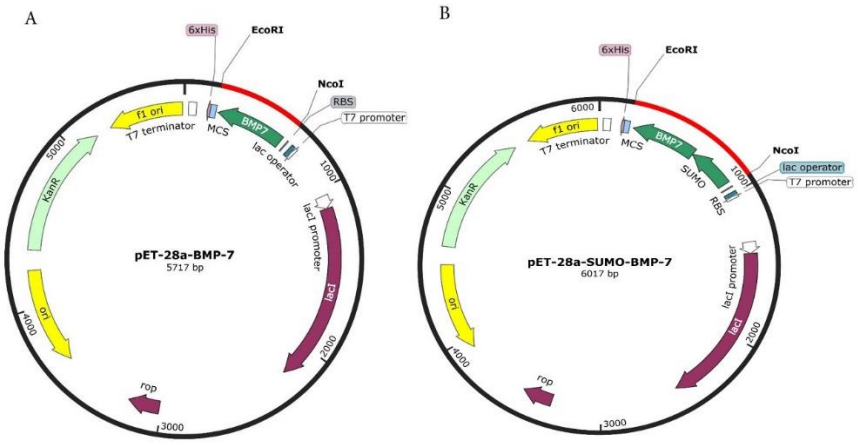

Figure 1. Schematic representation of recombinant pET-28a-BMP-7 and pET-28a-SUMO-BMP-7 construct map. A) The human BMP-7 and B) the SUMO-BMP-7 genes were cloned into *Nco*I/*Eco*RI sites of pET-28a(+).

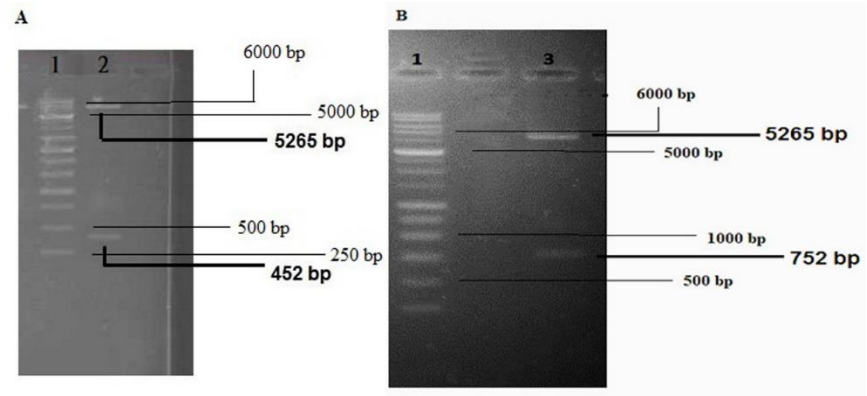

Figure 2. A) Restriction Analysis of A) pET-28a- BMP-7 and B) ET-28a- SUMO-BMP-7. A) Lane 1: Size Marker, Lane 2: Digestion of pET-28a- BMP-7 using *Nco*I/*Eco*RI created two fragments with expected size at 5265 bp and 452 bp. B) Lane 1: Size Marker, Lane 3: Digestion of pET-28a- BMP-7 with *Nco*I/*Eco*RI created two fragments with expected size at 5265 and 752 bp.

Expression analysis of SUMO-free BMP-7 and SUMO-fused BMP-7 in E. coli

To compare the soluble expression, both pET-28a-BMP-7 and pET-28a-SUMO-BMP-7 were transformed into *E. coli* BL21 (DE3). The expression was induced with 1 mM IPTG at different combinations of time and temperature (25, 30, and 37 °C for 4, 8, 10, 12, and 24 h). The results showed that the highest amount of SUMO-BMP-7 protein was expressed when pET-28a-SUMO-BMP-7 was induced at 37 °C for 4 h (data not shown). A predominant band at approximately 30 kDa for SUMO-BMP-7 and 16 kDa for BMP-7 protein was observed in induced samples. Western blotting using anti-His6 tag antibody confirmed the expression of the BMP-7 and SUMO-BMP-7 proteins in *E. coli* (Fig. 3).

Quantification of 15% SDS-PAGE gel using Quantity One® software revealed that the expression level of the BMP-7 accounts for about 18.79% of total proteins. Although BMP-7 protein was found in both the soluble and insoluble fractions, it was mainly localized in inclusion bodies (11.11%). The fusion of SUMO to BMP-7 (SUMO-BMP-7) protein led to a considerable increase in expression level to 38.5% of total proteins. Furthermore, a substantial proportion of the expressed fusion protein (SUMO-BMP-7) was present in soluble form (36.4%), which was higher than that of BMP-7 (7.68%) (Fig. 4).

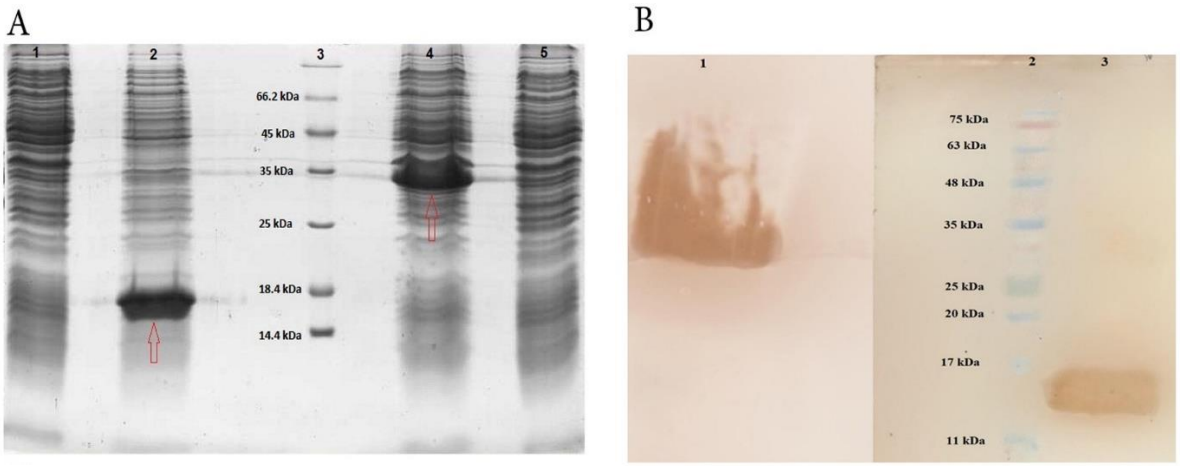

Figure 3. A) SDS-PAGE and B) Western blot analysis of BMP-7 and SUMO-BMP-7 expression. A) Lane 1 and 2: before and after induction samples of BMP-7, respectively, Lane 3: Protein Marker (Thermo Scientific, Cat. No. 26610), Lane 4 and 5: after and before induction samples of SUMO-BMP-7 protein, respectively. B) Lane 1 and 3: SUMO-BMP-7 and BMP-7 protein samples after induction. Lane 2: prestained protein ladder (SinaClon, Cat. No. PR901641).

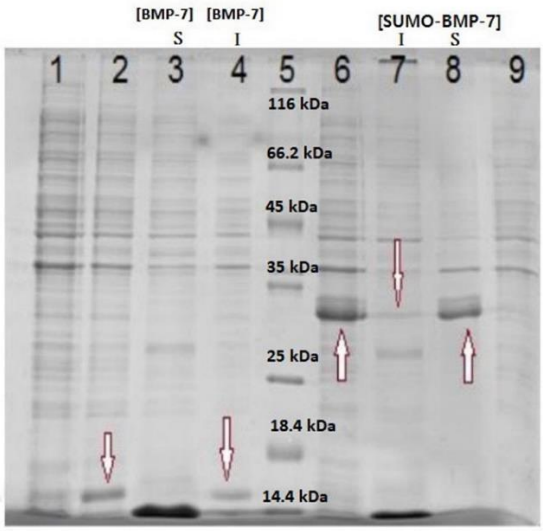

Figure 4. SDS- PAGE analysis of soluble and insoluble expression of BMP- 7 and SUMO-BMP-7 recombinant proteins. BMP-7 protein sample Lane 1: before, Lane 2: after induction, Lane 3: soluble and Lane 4: insoluble fraction of BMP-7 protein sample. Lane 5: Protein Marker (Thermo Scientific), Lane 6: SUMO-BMP-7 protein sample after induction. Lane 7: insoluble and Lane 8: soluble fractions of SUMO-BMP-7 protein, Lane 9: SUMO-BMP-7 protein sample before induction.

Purification of SUMO–BMP-7 fusion protein

Ni–NTA resin was used for fusion protein purification. Proteins without His6 tags were removed from the Ni–NTA resin using washing buffer containing 40 mM imidazole, and the His6-tagged SUMO-BMP-7 was eluted using elution buffer containing 250 mM imidazole (Fig. 5A, lane 2, 3). Observing an approximate 30 kDa band in Western blot analysis (Fig. 5B) confirmed the purification of SUMO-BMP-7.

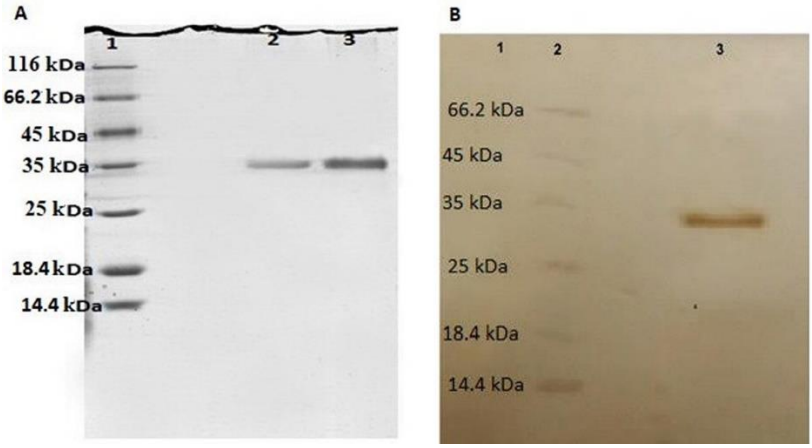

Figure 5. A) SDS-PAGE and B) Western blot analysis of SUMO-BMP-7 purification. A) Lane 1: protein marker (Thermo Scientific), Lane 2-3: elution 1-2 of SUMO-BMP-7. B) Lane 2: Protein Marker (Thermo Scientific), Lane 3: purified SUMO-BMP-7 protein.

Enzymatic cleavage and Purification of BMP-7

The SUMO-BMP-7 protein (50 µg) was almost entirely cleaved after incubation with SUMO protease (1 U) at 37 °C overnight. As demonstrated in Fig. 6, BMP-7 was released from the fusion protein after cleavage with protease. Then, to remove His6-tagged SUMO and SUMO protease, the mixture of cleavage reaction was re-loaded to the Ni–NTA column. After purification of the cleavage mixture, the His6-tagged SUMO was eluted using elution buffer containing 250 mM imidazole, which was observed as ~18 kDa band corresponding to the SUMO tag (Fig. 6, Lane 5). BMP-7 without His6 tags was removed from the Ni–NTA resin using a washing buffer containing 40 mM imidazole and an approximately 16 kDa band corresponding to recombinant BMP-7 was observed (Fig. 6, Lane 6). Observing a ~ 16 kDa band in Western blot analysis confirmed purification of BMP-7 (Fig. 7).

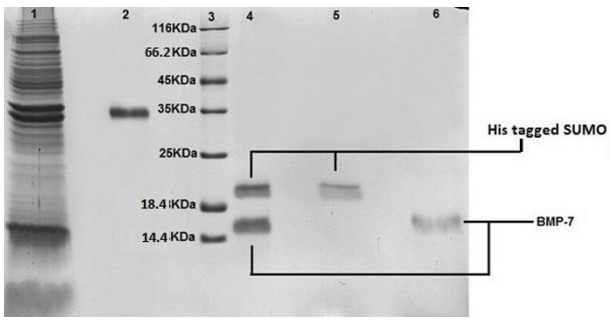

Figure 6: SDS-PAGE analysis of SUMO-cleaved after Ni-NTA purification. Lane 1: soluble fraction of SUMO-BMP-7 protein sample, Lane 2: purified SUMO-BMP protein, Lane 3: protein marker (Thermo Scientific), Lane 4: cleavage mixture of SUMO-BMP-7 protein with SUMO protease, Lane 5: SUMO fusion tag, Lane 6: purified recombinant BMP-7 protein.

Figure 7: Western blot analysis of purified recombinant BMP-7 protein using anti-BMP-7 antibody. Lane 1: prestained protein ladder (SinaClon), Lane 2: Purified BMP-7 protein.

Discussion

Clinical trials have provided supportive evidence for the use of BMP-7 in the treatment of open tibial fractures, distal tibial fractures, tibial nonunions, scaphoid nonunions, and atrophic long bone nonunions (Moghaddam et al., 2010; Blokhuis et al., 2013; Sampath and Reddi, 2020; Sandler et al., 2023). Given the current medical applications of BMPs, applying different strategies to improve the quality and yield of their expression is of great importance (Kübler et al., 2000). Producing recombinant rhBMP-7 has become the

principal method for high yield production. Reducing production costs and increasing the yields of rhBMP-7 production can enhance the clinical use of BMP-7 (Zhang et al., 2010). The clinical efficacy of rBMP-7 has been demonstrated in the treatment of orthopedic injuries through topical application. However, the pharmaceutical development of rBMP-7 for systemic delivery has presented many challenges. Specifically, the expression level of recombinant mature BMP-7 protein in mammalian cells is very low, the molecule has poor solubility at neutral pH, and intracellular proteolytic processing events result in a secreted BMP-7 having multiple amino-termini, creating a heterogeneous mixture of proteins (Gazzerro and Minetti, 2007).

Researchers have used the BL21 (DE3) strain and the expression vector of pET-25 for the cytoplasmic expression of BMP-4, BMP-9, BMP-10, BMP-11, and BMP-14 (Bessa et al., 2009). Herein, we also selected an *E. coli* host for rBMP-7 production. *E. coli* is the most commonly used heterologous expression host and can provide a simple and inexpensive way to produce recombinant proteins. The disadvantages of protein expression in *E. coli* cytoplasm are the accumulation of inclusion bodies resulting from incorrect protein folding (Wang et al., 2013; Yu et al., 2013). Promoting the secretion of recombinant protein into *E. coli* periplasm, in which the oxidizing conditions can favor the formation of the disulfide bonds, can circumvent the problem of inclusion body formation. However, the presence of incomplete chaperone machinery that can impair correct folding at a high expression rate and limited volume of the compartment which can lead to overcrowding are the major drawbacks of the accumulation of recombinant proteins in periplasmic space (de Marco, 2020). Totally, guiding recombinant proteins across the cytoplasmic membrane can cause protein aggregation and result in low yields of protein production (Karyolaimos et al., 2020). An alternative method is a soluble cytoplasmic expression by fusing the protein encoding gene to the solubilizing protein tags. Several protein fusion technologies have been developed to enhanc the expression and purification of heterologous proteins. In a systematic comparison of various fusion tags, SUMO fusion was reported to be superior for increasing the solubility of recombinant proteins (Ceylan and Erdoğan, 2017; Rezaie et al., 2017; Pratheesh et al., 2019). This effect was attributed to the chaperonin-like properties of SUMO which help to stabilize the folded structure of the attached protein and to prevent protein aggregation. It was also reported that a His6-tag at the N-terminus of SUMO resulted in rapid purification using nickel affinity chromatography (Satakarni and Curtis, 2011; Tileva et al., 2016; Ceylan and Erdoğan, 2017). Although the fusion tags are often highly expressed in the host cells, the presence of the fusion tags may prevent or interfere with the function of the target protein/peptide. Therefore, the peptide/protein of interest should be released from the fusion protein by enzymatic or chemical cleavage. SUMO protease cleaves SUMO peptide with remarkable fidelity and efficiency and gives the desired N-terminus (Wang et al., 2013; Li et al., 2018; Linova et al., 2020). The small size of SUMO resulted in a higher stoichiometric ratio of the target polypeptide in fusion protein and increased yield (Wang et al., 2011). It was found that when the target protein is fused directly to the C terminus of SUMO, cleavage by SUMO protease will result in the release of the target protein with the desired N-terminal amino acid sequence (Wang et al., 2013). SUMO fusion technology has been applied for the expression of different recombinant proteins and antibody fragments such as Hepcidin25 in *E. coli* (Sadr et al., 2017). For instance, Lee et al. (2009) have reported the successful expression of foot-and-mouth disease virus capsid protein as a stable heterodimer complex using SUMO fusion technology.

In this study, we use two different constructs having (pET-28a-SUMO-BMP-7) and lacking (pET-28a-BMP-7) SUMO partner for expression in the *E. coli* BL21 strain. In addition, the effects of duration and temperature of induction on the total yield of SUMO-BMP-7 were investigated to obtain optimal expression conditions. The most SUMO-BMP-7 was expressed 4 h after induction at 37°C. In order to lyse the cells and improve the yield and solubility of the recombinants, sonication with different pulses and in different solution concentrations was evaluated. Then, various buffer conditions such as lysis buffer (Na_2HPO_4/NaH_2PO_4, NaCl, different concentrations of imidazole), lysis buffer (Na_2HPO_4/NaH_2PO_4, Tris-HCL, urea, pH 8.0/ pH 6.3/ pH 5.9/ pH 4.5), TE buffer (Tris and EDTA), lysis buffer+ protease inhibitor mini+ Lysozyme (data not shown) were screened. It was found that lysis buffer with 40 mM imidazole and mild sonication for 15 cycles (20 s pulses and 20 s intervals) are the best conditions for soluble expression and purification.

Our results demonstrated that BMP-7 expressed in the BL21 strain was mostly insoluble and accumulated as inclusion bodies and it was present in soluble fractions of cell lysates to a lesser extent. As indicated in Fig. 4, the SUMO-BMP-7 fusion protein was expressed in both soluble and insoluble fraction of cell lysates, which resulted in higher production yield compared to BMP-7

expression. Similarly, Sun et al. (2008) reported that SUMO-urodilatin fusion protein is expressed in both soluble and insoluble fractions of the cell lysate. In the current study, fusion of SUMO to BMP-7 resulted in increased recombinant BMP-7 expression from 18.79% to 38.5%. Taking advantage of the SUMO technology, 36.4% of the soluble fraction was accounted for SUMO-BMP-7. However, SUMO-free BMP-7 with an N-terminal His tag was considerably less soluble (7.68%) than SUMO-BMP-7. In this study, it was demonstrated that expression of rhBMP-7, without SUMO fusion partner, causes inclusion bodies production. However, when SUMO was fused to BMP-7, the solubility of SUMO-BMP-7 was improved. The soluble expression of aggregation-prone recombinant proteins using different fusion tags and *E. coli* strains has been associated with different outcomes. For example, Rezaie et al. (2017) demonstrated that the combination of SUMO fusion technology, EnBase® cultivation system, and recruiting a redox mutant of *E. coli* can efficiently enhance the solubility and productivity of recombinant Fab fragments. In our previous study, we showed that the expression of rhBMP-7 in the SHuffle® T7 Express strain causes enhanced solubility compared to its expression in the BL21 strain. It emphasized the application of SHuffle® T7 Express strain for the efficient production of therapeutic proteins containing disulfide bonds (Dugmehchi et al., 2022). Sadr et al. (2017) revealed that thioredoxin and SUMO dual fusion system is an efficient production system for the synthesis of functional human hepcidin25 with a final yield of 3.9 mg/L of media. Peciak et al. (2014) have reported the successful expression of interferon consensus protein (IFN-con) using a combination of SUMO-tagged construct and SHuffle strain. Based on their results, 86– 88% of total expressed protein was recovered in soluble fraction. Wang et al. (2013) successfully developed a procedure to express soluble and functional recombinant HPS using the SUMO fusion system combined with Tf chaperone in *E. coli*. Zhang et al. (2014) demonstrated that ELP and SUMO tags can be combined to produce soluble proteins. Fazen et al. (2012) reported that hPYY (3–36) is successfully expressed as a fusion with the SUMO protein and expressed in a yield of 30 ± 7 mg/L of induced culture in *E. coli*. The presence of the SUMO tag was necessary since rapid degradation of unmodified hPYY (3–36) was observed (Fazen et al., 2012). Hou et al. (2013) constructed an efficient system for the expression and purification of scolopin 1 in *E. coli* by the pSUMO expression vector. Herein, two steps of Ni-NTA chromatography were used to purify the soluble rhBMP-7. First, the soluble fraction of SUMO-BMP-7 was purified with high purity. Then, the purified SUMO-BMP-7 was dialyzed to reduce the concentration of imidazole because of the inhibitory effect of imidazole on the SUMO protease. In the next step, the rhBMP-7 protein was separated and released from the fusion protein by SUMO protease. In the second step of purification, the cleavage mixture was loaded onto the Ni–NTA column. Highly efficient cleavage of the fusion protein between the C-terminus of SUMO and the N-terminus was confirmed by observing the BMP-7 band at the expected position of 16 kDa in SDS-PAGE and Western blot analysis. The theoretical molecular weight of the SUMO proteins is approximately 11 kDa. However, as reported by Hilgarth and Sarge (2005) each added SUMO typically causes an increase in the range of 15–17 kDa on SDS-PAGE. In contrast to our study, the SUMO protease failed to cleave the SUMO peptide from ASAL in the study of Santosh Kumar. This may be due to steric hindrance caused by the homodimer structure of the Chimeric ASAL (Upadhyay et al., 2010).

Here, we have reported for the first time that rhBMP-7 can be expressed more soluble in *E. coli* using SUMO fusion technology. Furthermore, rhBMP-7 can be successfully released from SUMO-BMP-7 after cleavage by SUMO protease. Additionally, rhBMP-7 can be purified by a two-step purification strategy including 1) purification of SUMO-BMP-7 and 2) purification of rBMP-7 after cleavage using Ni-NTA chromatography. Altogether, this research has provided a feasible approach for large-scale production of soluble rhBMP-7, to facilitate its further medical development. It is highly required to examine the effect of the SUMO fusion system on the biological activity of hBMP-7 in our future studies.

Conclusion

In conclusion, it was found that the fusion of SUMO to hBMP-7 and its expression in *E. coli* increases expression and solubility compared to the expression of SUMO-free BMP-7, Further studies are highly required to investigate the effect of the SUMO fusion system on the biological activity of hBMP-7. Additionally, to apply this system for industrial application, it is required to compare the level of active purified soluble rhBMP-7 expressed after SUMO protease cleavage with those of active rhBMP-7, which is expressed with no fusion tags and achieved after the refolding process.

Ethical Statement

This article does not contain any studies with human and animal subjects performed by any of the authors.

Competing Interests

The authors declare no conflict of interest.

Funding

This study was supported financially by Pasteur Institute of Iran (Grant#736).

Authors Contribution

Ghazal Sadipour performed literature searches, experimental studies, and manuscript preparation. Alireza Dugmehchi assisted in laboratory experiments. Yeganeh Talebkhan provided critical feedback and assisted in the revision of the manuscript. Elham Mohit and Leila Nematollahi were chief investigators and contributed to revise the main manuscript.

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