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Original Article

Cytoplasmic Expression of Human Bone Morphogenetic Protein-7 by a Genetically Engineered Strain of *Escherichia coli*, SHuffle® Strain

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HIGHLIGHTS

- *E. coli* SHuffle® T7 Express strain is an effective host to express disulfide-bonded proteins.
- BMP-7 is involved in the process of bone formation.
- Expression of human BMP-7 in SHuffle® strain increased its solubility.

ABSTRACT

Homodimeric bone morphogenetic protein-7 (BMP-7) plays a key role in bone metabolism. The functionality of human BMP-7 protein is dependent on its disulfide bond formation and proper folding. Therefore, the expression of soluble recombinant BMP-7 using *Escherichia coli* cells as the host remains a challenge. Given the need for these disulfide-bonded proteins for stabilized native conformation, the cytoplasm of SHuffle® T7 Express as an *E. coli* engineered strain can effectively fold disulfide-bonded proteins with a need for proper oxidative folding. These cellular features turn the SHuffle® expression system into an efficient host for the recombinant production of human BMP-7 protein. A soluble dimeric form of recombinant human BMP-7 (rhBMP-7) which has a wide range of applications in medicine and can be used in the treatment of bone defects was produced using the SHuffle® strain as the expression system. This study demonstrated the production of rhBMP-7 using *E. coli* SHuffle® T7 Express strain. Also, an effective protocol was proposed for the expression and purification of soluble human BMP-7. In addition, it was found that the genetically engineered SHuffle® strain can efficiently enhance the solubility of recombinant human BMP-7 as a therapeutic target.

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Introduction

In the 1960s, proteins known as bone morphogenetic proteins (BMPs) belonging to the transforming growth

factor-beta (TGF-β) superfamily of cytokines were isolated from the extract of bone matrix. Further studies demonstrated that they have a key role in bone formation and skeletal development (Urist and Strates, 1971). The C-terminal of a large majority of the BMP family members contains seven cysteine residues. Six out of these seven residues are involved in the formation of inter-polypeptide disulfide bonds, known as cysteine knot, which forms the core of the monomer. While the seventh cysteine residue is critical for the dimerization process and forms a disulfide bond that links the two monomers. The dimeric structure is an absolute requirement for biological activity and interaction with receptors. BMP-7 (also known as osteogenic protein-1), is a member of bone morphogenetic proteins. The mature BMP-7 is a disulfide-linked homodimer (Kirker-Head, 2000). *In vitro* and *in vivo* studies have shown that BMP-7 plays a major role in the differentiation of osteoblast cells from mesenchymal stem cells (MSCs), a process mediated in part by the BMP-7 signaling pathway. The osteoinductive property of BMP-7 has made it clinically valuable (Westerhuis *et al*., 2005). Recombinant human BMP-7 (rhBMP-7) has been approved by the United States Food and Drug Administration (FDA) in 2002 (Bessa *et al*., 2008) for the treatment of defects and fractures associated with long bone non-unions (Westerhuis *et al*., 2005). Producing disulfide-bonded proteins such as BMPs is generally challenging and unpredictable. Disulfide bonds play a major role in the folding, activity, and structural stability of these proteins (Cheng *et al*., 2003). Prokaryotic systems remain the most appropriate expression system for disulfide-bonded proteins given their high expression level, relatively low cost, ease of genetic engineering, high speed, and high growth rate and yield (Zhang *et al*., 2010). The cytoplasm of wild-type *E. coli* contains two destructive reducing pathways including glutaredoxin and thioredoxin pathways, which cause cysteine residues to be maintained in a reduced state (Ritz *et al*., 2001). In the case of cytoplasmic expression, to restore the native BMP-7 folding, refolding procedures, which is costly and time-consuming are required (Collet and Messens, 2010). However, disulfide bonds can be formed in the periplasm of gram-negative bacteria such as *E. coli*. Although, given the very low periplasmic expression of disulfide-bonded proteins, their production in the periplasm is not economical (Devi and Mittl, 2011). To genetically engineer SHuffle® expression strain (New England Biolabs, USA), glutaredoxin reductase (*gor*) and thioredoxin reductase (*trxB*) were deleted. Thus, the expression of disulfide-bonded proteins has become possible in the oxidative cytoplasmic environment of SHuffle® strain (Jalomo-Khayrova *et al*., 2018). Furthermore, in this novel engineered *E. coli* strain, cytoplasmic disulfide bond isomerase (DsbC), which is essential for the formation of correct disulfide bridges is over-expressed (Kurokawa *et al*., 2001). Accordingly, this genetically-engineered *E. coli* strain not only offers an expression solution to disulfide-bonded proteins but also corrects misfolded protein substrates and promotes proper folding and activity (Lobstein *et al*., 2012; Jalomo-Khayrova *et al*., 2018). In recent years, several disulfide-bonded proteins have been expressed in the cytoplasm of SHuffle® T7 Express strain and this strain is found to produce soluble forms of proteins and to correct the mis-oxidized bonds (Lobstein *et al*., 2012). In the present study, we aimed to investigate the expression of rhBMP-7 in *E. coli* SHuffle® strain. To the best of the authors' knowledge, this research pioneered the study of rhBMP-7 expression in the engineered SHuffle® strain to develop an easy and economical process for the production of soluble rhBMP-7.

Materials and Methods

Strains, culture media and reagents

The *E. coli* TOP10F′ strain (Thermo Fisher Scientific, MA, USA) was used to propagate recombinant plasmids. *E.coli* BL21 (DE3) (Novagen, Madison, WI, USA) and *E. coli* SHuffle® T7 Express (New England BioLabs, Ipswich, MA, USA) cells were used as expression hosts. The pET-28a (+) expression vector (Novagen, Madison, Wisconsin, USA) was used for high-level expression of recombinant BMP-7 protein. Bacterial strains were cultured in Luria-Bertani (LB) broth or on LB agar plates. The restriction enzymes such as *Nco*I and *Eco*RI were procured from Thermo Fisher Scientific (MA, USA). Rabbit polyclonal primary antibody to hBMP-7 and IgG-HRP conjugated goat anti-rabbit secondary antibody were purchased from Abcam (Cambridge, UK) and Razi BioTech (Tehran, Iran) respectively. Moreover, vectors and DNA fragments were purified using Miniprep kits (QIAGEN, USA) and gel extraction kits (QIAGEN, USA), respectively. Other reagents were obtained from standard commercial sources.

Construction of hBMP-7 expression cassette

After obtaining the sequence of the hBMP-7 gene from GenBank with accession number NM_001719.3 and codon optimization according to the codon usage of SHuffle®, human cDNA encoding the mature domain of BMP-7 (139 amino acids) was synthesized by GeneRayBiotech© company (Shanghai, China). *Nco*I and *Eco*RI restriction sites were inserted upstream and downstream of the gene, respectively. In this construct, the 6×His tag was fused to the N-terminal of the hBMP-7 sequence immediately after the start codon.

Cloning of hBMP-7 gene and screening for recombinants

The synthetic gene was digested from the intermediate vector (pGH) using *Nco*I/*Eco*RI and subsequently was cloned into the corresponding sites of pET-28a (+). The recombinant plasmid, pET-28a-BMP-7, was transferred to the *E. coli* TOP10F′ strain (Thermo Fisher Scientific, MA, USA) using the heat shock method. Plasmids were extracted using Miniprep kits (Qiagen, USA) according to the manufacturer's instructions. The presence of the desired recombinant plasmid in *E. coli* TOP10F′ colonies was studied by PCR and double digestion. In PCR, T7_f (5′-TAATACGACTCACTATAGGG-3′) as the T7 promoter and T7 Terminal_r (5′-GCTAGTTATTGCTCAGCGG-3′) as the T7 terminator primers were used. The thermal cycles of PCR were as follows: pre-incubation at 95 °C for 5 minutes followed by 30 cycles of 94 °C for 45 s, 50 °C for 1 min, 72 °C for 1 min and a final elongation (72 °C for 5min). Recombinant pET-28a-BMP-7 plasmid was also confirmed through a restriction analysis using *Nco*I/*Eco*RI enzymes. Finally, the sequence of the recombinant plasmid, which was confirmed by PCR and double digestions was analyzed.

Expression of rhBMP7 in the SHuffle® T7 Express strain

The heat shock method was used to transform the recombinant pET-28a-BMP-7 vector into the SHuffle® expression strain. The cells were placed onto LB agar containing 50 µg.mL^{-1} of kanamycin (Sigma-Aldrich, USA) and 30 µg.mL^{-1} spectinomycin (Fisiopharma, Salerno, Italy), and incubated overnight at 30 °C. Single colonies on the plates were transferred to 5 mL of the LB medium containing 50 µg.mL^{-1} of kanamycin and 30 µg.mL^{-1} spectinomycin and incubated overnight at 30 °C and 180 rpm. This culture was diluted at 1:100 using 500 mL of LB broth supplemented with kanamycin and spectinomycin. The cells were cultured at 30 °C and 180 rpm until reaching an OD_{600} of 0.6-0.8. The rhBMP-7 expression was induced by adding 1 mM IPTG. Subsequently, the cells were incubated for 4 h at 30 °C and 180 rpm and then the cellular debris was removed by centrifugation at 8000 × g for 20 min at 4 °C and the pellets were stored at -20 °C. The expression of rhBMP-7 in *E. coli* BL21 (DE3) was performed under the same conditions as that in SHuffle® T7 Express strain. However, BL21 (DE3) cells containing pET-28a-BMP-7 were incubated at 37 °C.

Determining the solubility of rhBMP7 expressed in SHuffle® T7 Express and E. coli BL21 (DE3) strains

Pellets were resuspended in 5 mL of lysis buffer (NaH_2PO_4 50 mM, NaCl 300 mM, Imidazole 10 mM, pH 8.0; supplemented with 0.2 mg.mL^{-1} lysozyme) and sonicated on an ice bath for 10 cycles of 30 s pulses and 30 s intervals. The disrupted cells were ultimately centrifuged at 8000 × g for 30 min at 4 °C and finally, the clear supernatant was collected. The samples related to soluble and insoluble fractions were evaluated using SDS-PAGE analysis. The density of the bands related to soluble human BMP-7 was determined in both hosts by performing densitometry analysis on the SDS-PAGE gel using Quantity One™ software (Bio-Rad Laboratories, Hercules, CA).

Purification of rhBMP7

The recombinant His-tagged hBMP-7 was purified from the supernatant by affinity chromatography using a HisTrap™ High Performance 1 mL prepacked column (GE Healthcare, USA). The column was equilibrated using binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4). After washing the weakly-bound proteins with washing buffer (20 mM sodium phosphate, 0.5 M NaCl, 30 mM imidazole, pH 7.4), the column was eluted with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4) according to the manufacturer's instructions. The samples were then analyzed using SDS-PAGE under reducing (in the presence of 2-mercaptoethanol) and non-reducing conditions.

Western blot analysis

The purified sample was analyzed using 15% SDS-PAGE gel and visualized by coomassie brilliant blue stain. Then, western blot analysis was performed to confirm the expression of rhBMP-7. Accordingly, the protein bands were transferred from SDS-PAGE gel onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Sweden) using an electroblotter in the wet transfer method (100 mA/2.5 h). The membrane was blocked with 5% non-fat dry milk in phosphate-buffered saline (PBS) with 0.1% Tween 20 at 4 °C with gentle shaking, overnight. Then, the blots were incubated with 1:1000 dilution of polyclonal rabbit anti-human BMP-7 (Abcam, Cambridge, UK) for 90 min at room temperature followed by incubation with 1:1000 dilution of goat anti-rabbit conjugated with horseradish peroxidase (Razi BioTech, Iran) for 45 min at room temperature. The antigen-antibody complex was then visualized using 3,3′-Diaminobenzidine (DAB) substrate kits (Bio-Rad, USA).

Results

Construction of hBMP-7-expressing vector

The synthesized codon-optimized hBMP-7 gene was digested from pGH-BMP-7 plasmid and cloned into the *Nco*I/*Eco*RI site of the pET-28a (+) vector (Fig. 1).

Colony PCR analysis using T7 promoter and T7 terminator primers confirmed the presence of the hBMP-7 coding region inside the pET-28a (+) vector with the predicted size of 648 bp (Fig. 2A). As demonstrated in Figure 2B, *Nco*I/*Eco*RI double digestion of the pET-28a-BMP-7 plasmid resulted in the expected fragments with sizes of 5265 and 452 bp (Fig. 2B). Furthermore, sequence analysis of pET-28a-BMP-7 plasmid confirmed the cloning procedures (data not shown).

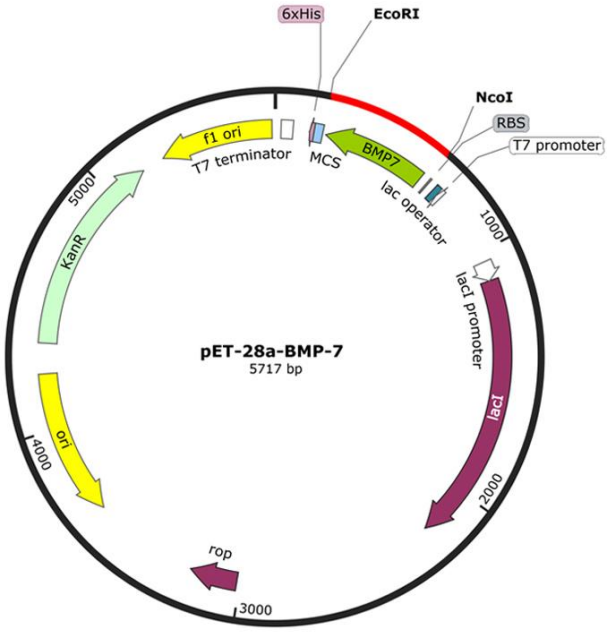

Figure 1: Schematic representation of recombinant pET-28a-BMP-7 construct map. The human BMP-7 gene was cloned into NcoI/EcoRI sites of pET-28a(+).

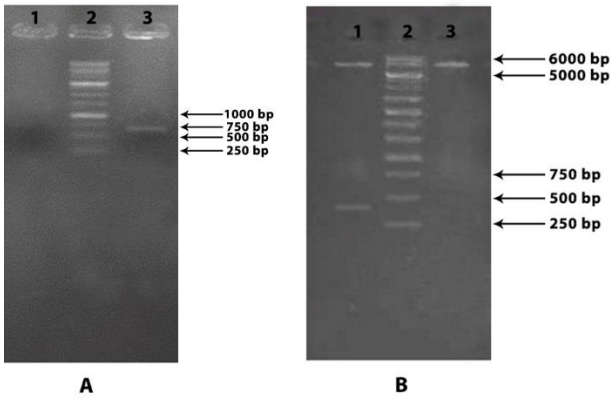

Figure 2: Colony PCR of *E. coli* TOP10F′ transformants and restriction analysis of pET-28a-BMP-7 construct. (A) PCR products were electrophoresed on a 1% agarose gel. Lane 1: negative control, Lane 2: 1 kb DNA ladder, Lane 3: amlification of BMP-7 gene. (B) Restriction analysis of pET-28a-BMP-7 plasmid. Lane 1: fragments created by *Nco*I/*Eco*RI digestion of pET-28a-BMP-7 construct with sizes of 5265 and 452 bp, Lane 2: 1 kb DNA ladder, Lane 3: *Nco*I-linearized pET-28a-BMP-7 with size of 5717 bp.

Expression and purification of rhBMP-7

According to Fig. 3, SDS-PAGE analysis revealed the expression of hBMP-7 in the *E. coli* SHuffle® T7 Express strain. In SDS-PAGE analysis, the obtained molecular weights of both monomeric and dimeric forms of rhBMP-7 were consistent and active dimeric forms of hBMP-7 (~16 and ~32 kDa respectively). Since recombinant rhBMP-7 protein contained an N-terminal His-tag, immobilized metal affinity chromatography (IMAC) was performed to purify it. Given that two polypeptide chains of BMP-7 homodimer are joined together by a disulfide bond, it can be expected that in non-reducing SDS-PAGE analysis, the BMP-7 product shows the correct homodimer molecular weight, while in reducing condition only the monomer form is detectable. Therefore, the purified protein samples were electrophoresed under reducing and non-reducing conditions (Fig. 4A).

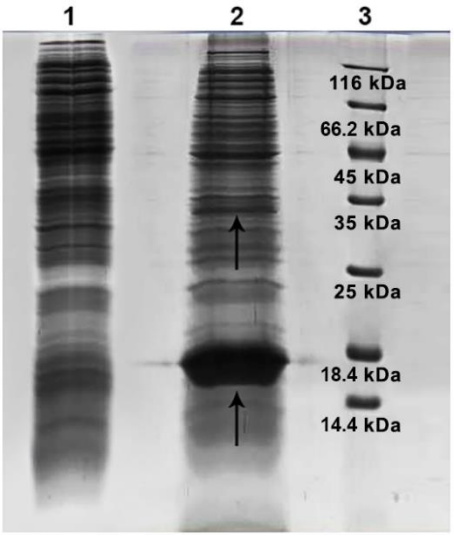

Figure 3: SDS-PAGE analysis of the expressed hBMP-7 in SHuffle® T7 Express strain, Lane 1: before induction, Lane 2: protein sample after induction, Lane 3: protein marker (Thermo Scientific, Cat. No. 26610). The arrows show the bands related to the monomeric and dimeric forms of rhBMP-7.

Western blot analysis of rhBMP-7

Western blot analysis was performed on the purified rhBMP-7. Consequently, two major protein bands (~32 kDa and ~16 kDa) were evident when the recombinant hBMP-7 was resolved under non-reducing condition by SDS-PAGE (Fig. 4B). Upon reduction of the protein sample with 2-ME, only the ~16 kDa protein band was seen (Fig. 4B). These data indicate the formation of disulfide-linked homodimers. The rhBMP-7 dimers are converted to monomers only by reducing reagents.

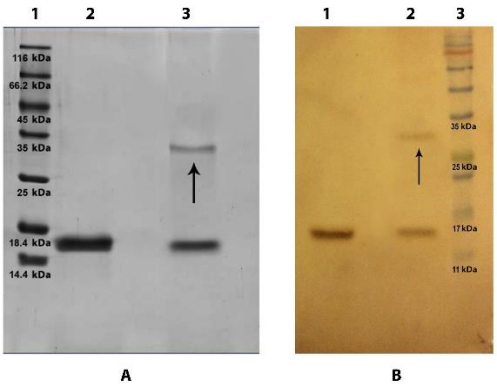

Figure 4: (A) SDS-PAGE analysis of the purified hBMP-7 using affinity chromatography. Lane 1: protein marker (Thermo Scientific, Cat. No. 26610), Lane 2: purified rhBMP-7 under reducing condition and Lane 3: under non-reducing condition. The arrow indicates the dimeric form of rhBMP-7. (B) Western blot analysis of the purified rhBMP-7. Lane 1: under reducing condition, Lane 2: under non-reducing condition and Lane 3: protein marker (prestained protein ladder, SinaClon, Cat. No. PR901641). The arrow indicates the dimeric form of rhBMP-7.

The effects of expression strains on the expression and solubility of hBMP-7

In this study, the soluble expression of hBMP-7 in SHuffle® and BL21 (DE3) strains, which were cultured under the same conditions was compared. SDS-PAGE analysis showed that the hBMP-7 monomer, with a predicted molecular weight of ~16 kDa accumulated as inclusion bodies in BL21 (DE3) strain (Fig. 5A), while rhBMP-7 was expressed in soluble form in SHuffle® strain (Fig. 5B). The correct weight of homodimer rhBMP-7 (~32 kDa) was demonstrated only in *E. coli* SHuffle® expression system. These results demonstrated that the application of SHuffle® T7 Express strain has an effect on the solubility and folding of the rhBMP-7 protein.

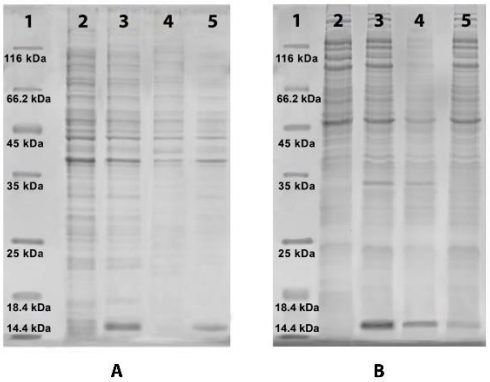

Figure 5: SDS-PAGE analysis of the hBMP-7 expression in the BL21 (DE3) and *E. coli* SHuffle® T7 Express strains. (A) BL21 (DE3) strain. Lane 1: protein marker (Thermo Scientific, Cat. No. 26610), Lane 2: before, Lane 3: after induction, Lane 4: soluble and Lane 5: insolube fractions of the expressed rhBMP-7 in the BL21 strain. (B) *E. coli* SHuffle® T7 Express strain. Lane 1: protein marker (Thermo Scientific, Cat. No. 26610), Lane 2: before, Lane 3: after induction, Lane 4: soluble and Lane 5: insolube fractions of the expressed rhBMP-7 in the SHuffle® strain.

Discussion

Given the current medical applications of BMPs, applying different strategies to improve the quality and yield of their expression are of great importance (Kübler *et al*., 2000). Despite the advantages of protein expression in *E. coli* including high growth rate, cost-effectiveness, simple genetic manipulation and high cell density as well as the possibility of producing proteins at industrial scales, many disadvantages such as improper disulfide bonds formation in the cytoplasm, which may cause protein misfolding and low level of soluble expression exist (Derman *et al*., 1993; Bessette *et al*., 1999). Since the volume of the periplasm is about 20% of the cytoplasm, the expression of proteins containing disulfide bonds in the cytoplasm of genetically engineered strains is a very effective strategy (Levy *et al*., 2001). So far, researchers have used 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). Given that the cytoplasm of this bacterium cannot properly fold disulfide-bonded proteins, the expression of these proteins generally results in misfolded inclusion bodies (Klösch *et al*., 2005). In addition to being time-consuming and expensive, refolding of inclusion bodies reduces the production efficiency of proteins (Soares *et al*., 2003, Robbens *et al*., 2006; Hernández *et al*., 2008). Despite the problems in producing complex proteins in the *E. coli* cytoplasm, different strategies such as reducing the induction temperature, and simultaneous expression of chaperones, regulating the concentration of inducer (IPTG) (Larentis *et al*., 2014), selecting appropriate promoters (Francis and Page, 2010) and using protein fusion (Gopal and Kumar, 2013) have been applied to increase the solubility of the expressed proteins and improve folding in *E. coli* (Martínez-Alonso *et al*., 2010). Nevertheless, the soluble expression of disulfide-bonded proteins with proper folding is possible in the engineered *E. coli* SHuffle® T7 Express cytoplasm. The oxidative cytoplasmic environment of this strain accelerates the formation of disulfide bonds. Moreover, over-expression of DsbC in the cytoplasmic environment of SHuffle® strain effectively enhances the amounts of correctly folded disulfide-bonded proteins (Lobstein *et al*., 2012). In previous studies, many proteins such as luciferase, cellulase Cel9A and alkaline phosphatase PhoA, which require disulfide bridges to achieve their native folded state, were expressed in the oxidizing cytoplasm of *E. coli* SHuffle® T7 Express strain. It was found that the expression of the mentioned proteins in the engineered SHuffle® expression strain significantly increased the solubility, folding and activity of these proteins (Lobstein *et al*., 2012). In the present study, SDS-PAGE and

western blot analysis revealed that both the monomeric and dimeric forms of rhBMP-7 have been expressed in the cytoplasm of SHuffle® strain. The expression of the dimeric form of this recombinant protein is important, given that only its dimeric form has biological activities, pharmaceutical properties and potential for industrial production. Subsequently, the synthesized rhBMP-7 was purified using immobilized metal affinity chromatography. Western blot analysis of the purified rhBMP-7 confirmed the expression of the monomeric with an approximate size of 16 kDa and dimeric with an approximate size of 32 kDa forms of rhBMP-7. So far, human BMP-7 protein has been expressed in different strains of *E. coli* such as BL21 (DE3), but the reducing cytoplasm of *E. coli* is a serious obstacle to the formation of disulfide bonds. Accordingly, insoluble inclusion bodies are formed in the cytoplasm. Also, the refolding process may not be able to fully reform the native protein fold and may significantly reduce the functionality of the protein. Moreover, the active dimeric form of human BMP-7 is generally not formed in *E. coli* strains such as BL21 (DE3) and the protein is only seen in monomeric form (Tóth *et al*., 2021). In addition, the results revealed that the soluble rhBMP-7 form was expressed only in the oxidative cytoplasm of SHuffle® T7 Express strain, while the recombinant protein accumulated as insoluble inclusion bodies in the cytoplasm of BL21 (DE3). However, to further enhance the solubility of the expressed rhBMP-7, it is required to optimize the cultivation and induction conditions (Fathi-Roudsari *et al*., 2016; Kasekarn *et al*., 2020). Our results are consistent with the study of Ritthisan *et al.* in which EhCP1 enzyme was expressed in SHuffle® strain. They found that the solubility and activity of EhCP1 are increased when it is expressed in *E. coli* SHuffle® strain (Ritthisan *et al*., 2018). In another study, the expression of TNF-α in SHuffle® expression system resulted in a significant increase in disulfide bond formation compared to its expression in BL21 (DE3) strain (Safarpour *et al*., 2017). Furthermore, recently anti-HER2 scFv was expressed in the soluble and active form in the oxidative cytoplasm of *E. coli* SHuffle® T7 Express cell (Ahmadzadeh *et al*., 2020).

Conclusion

In conclusion, it was shown that the expression of rhBMP-7 in SHuffle® T7 Express strain enhanced its solubility compared to its expression in BL21 strain. Further studies are highly required to investigate the effect of the expression of rhBMP-7 in SHuffle® on its biological activity. Furthermore, to compare these two strains for industrial application, it is required to compare the level of active soluble rhBMP-7 expressed in SHuffle® with those of active rhBMP-7, which is expressed in BL21 and achieved after refolding process. The findings of the present study emphasized the application of SHuffle® T7 Express strain for efficient production of therapeutic proteins containing disulfide bonds.

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Ethical Statement

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

Authors' Contributions

A. Dugmehchi performed the literature search, experimental studies and manuscript preparation. G. Sadipour assisted in laboratory experiments. Y. Talebkhan, H. Jahandar and F. Nemati provided critical feedback and assisted in the revision of the manuscript. E. Mohit and L. Nematollahi were chief investigators and contributed to revising the main manuscript.

Competing Interests

The authors declare no conflict of interest.

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