Protein Expression and Purification 115 (2015) 61–68

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Expression and purification of active recombinant human bone morphogenetic 7-2 dimer fusion protein

Jianli Dang, Lei Jing, Weiwei Shi, Ping Qin, Yuyin Li, Aipo Diao *

School of Biotechnology, Tianjin University of Science and Technology, Key Lab of Industrial Fermentation Microbiology of the Ministry of Education, Tianjin 300457, China

ARTICLE INFO

Article history:
Received 11 March 2015
and in revised form 13 July 2015
Accepted 8 August 2015
Available online 11 August 2015

Keywords:
rhBMP7-2
Inclusion body
Protein refolding
Dialysis
Osteogenesis

ABSTRACT

Bone morphogenetic proteins (BMPs) have been applied in bone regeneration therapy due to their significant osteogenic activity, however, the complicated processing and high cost in producing recombinant BMP have limited their use in the clinic. In this study, we have developed a simple method to prepare recombinant human BMP7–BMP2 fusion protein with a flexible peptide linker (rhBMP7-2). The rhBMP7-2 protein is expressed efficiently in *Escherichia coli*, and the denatured protein purified by anion exchange chromatography then refolded by dialysis. The yield was about 6.8 mg per gram of wet cell weight. The bioactivity of re-folded rhBMP7-2 was measured by alkaline phosphatase assay and alizarin red staining using both C2C12 and MC3T3-E1 cells, and also using the rat subcutaneous ectopic bone formation model. High level osteogenic activity was found in all the assays tested demonstrating the production of corrected folded and active rhBMP7-2 protein.

1. Introduction

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor superfamily classified by characteristic features in their protein sequence. More than 20 members have so far been identified and characterized [1,2]. BMPs have roles involving in many biological processes, such as cell growth, differentiation, morphogenesis and regeneration of tissues and organs. BMP-2 is the best studied members in the BMP family, due to its prominent ability to induce bone regeneration. BMP2 was used for a variety of clinical situations, including osteointegration of metallic implants, cartilage repair and spinal fusions [3–6], and its therapeutic use was approved by the FDA in 2002 [7]. BMP7 has also been applied to the repair of injured kidney tissue due to its high activity in promoting osteoblastic differentiation [8,9]. However, the complicated processes to produce recombinant BMP2 or BMP7 proteins and the large demand for clinical treatment has resulted in high costs. Therefore, it is necessary to simplify the production process to enhance production of biological active protein.

BMPs have previously been isolated directly from bovine bones. However, because of expensive cultivation, complicated purification scheme and risks of immunological responses, the application of BMPs from bovine has been severely limited [10]. Alternatively, the active form of recombinant human BMPs have been obtained from eukaryotic hosts, such as CHO, insect cells, tobacco, however, the yields of the protein obtained are low [11–13]. The bacteria expression system is used on the whole to produce a high yield of recombinant protein with low costs, however, the biologically active BMPs generated in these systems are achieved only by extensive refolding involving several time-consuming steps and expensive reagents. Therefore, it is necessary to optimize the protein refolding conditions to boost the yield of biologically active BMPs.

Nowadays, the majority of studies have focused on expressing the recombinant BMP homodimers, while two or more BMP proteins are often co-expressed to form heterodimers in bone and other organs in vivo [14–16]. BMP2-7 heterodimers produced by co-transfection and co-expression of *BMP2* and *BMP7* cDNA in A549 cells show that the heterodimers have higher activity than their respective homodimers [17].

In this study, the recombinant BMP7–BMP2 fusion protein is linked by a flexible peptide linker (rhBMP7-2) and expressed in *Escherichia coli*. A simplified and highly efficient method for refolding rhBMP7-2 was established, and a variety of activity tests both in vivo and in vitro have validated their functional efficacy in a selection of osteogenic activity assays.

* Corresponding author at: School of Biotechnology, Tianjin University of Science and Technology, No. 29, 13th Avenue, Tianjin Economic and Technological Development Area (TEDA), Tianjin 300457, China.
E-mail address: diaoaipo@tust.edu.cn (A. Diao).

http://dx.doi.org/10.1016/j.pep.2015.08.010
1046-5928/

MSTGSKQRSQ NRSKTPKNQE ALRMANVAEN SSSDQRQACK KHELYVSFRD
LGWQDWIIAP EGYAAYYCEG ECAFPLNSYM NATNHAIVQT LVHFINPETV
PKPCCAPTQL NAISVLYFDD SSNVILKKYR NMVVRACGCH GGGGSGGGGS
GGGGSQAKHK QRKRLKSSCK RHPLYVDFSD VGWNDWIVAP PGYHAFYCHG
ECPFPLADHL NSTNHAIVQT LVNSVNSKIP KACCVPTELS AISMLYLDEN
EKVVLKNYQD MVVEGCGCR

BMP7- $(Gly_4Ser)_3$ -BMP2

Fig. 1. The amino acids sequence of recombinant human BMP7–BMP2 fusion protein with a flexible peptide linker of (Gly4Ser)3 (underlined).

2. Material and methods

2.1. Construction of the BMP7–BMP2 fusion construct

The gene splicing overlap extension PCR (the SOE PCR) were used to generate the construct. To amplify *BMP7* cDNA and *BMP2* cDNA, two pairs of PCR primers were designed. One pair was composed of a 5′-BMP7 primer with restriction site *Nde* I (5′-GGAATTC CATATG TCCA CGGGGAGCAA ACAGCG-3′), and a 3′-BMP7 primer tagged with a linker encodes $(Gly_4\ Ser)_3$ (5′-CAGGCCCGGACACCG ACGGTGCCTCCTCCTCCTAGCCCTCCTCCTCCTAGCCCTCCTCCTCCTAG C-3′). The other pair includes 5′-BMP2 primer preceded by the same linker (5′-GGAGGAGGAGGATCGGGAGGAGGAGGATCGGGAG GAGGAGGATCGCAAGCCAAACACAAACAG-3′), and 3′-BMP2 primer with restriction site *Eco*R I (5′-CTCCCAACACCCACAGCGATTCTTAA GG-3′). Firstly, One fragment consisting of *BMP7* -linker and another fragment containing linker followed by *BMP2* were produced by PCR, then PCR reaction was performed again using 5′ BMP7 primer and 3′ BMP2 primer to amplify the DNA fragment encodes the BMP7–BMP2 fusion protein linked by $(Gly_4\ Ser)_3$. The DNA fragment was cloned into expression vector pHis-NusA and named pBMP7-2.

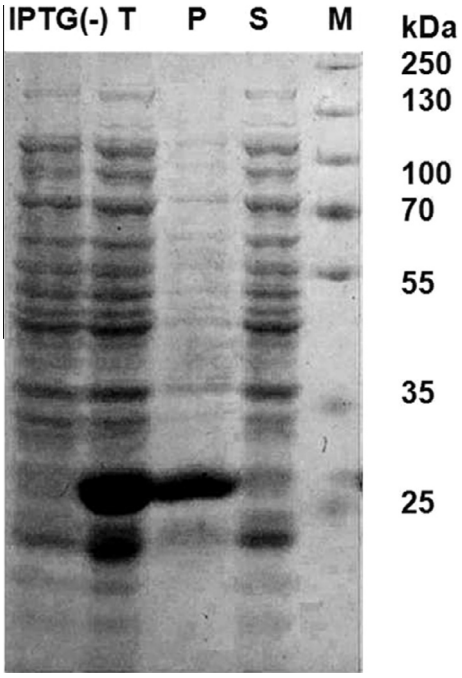

Fig. 2. Analysis of expression of the recombinant human BMP7-2 protein in *E. coli* by SDS–PAGE and Coomassie blue-staining: Lane IPTG(−), cultures before IPTG induction; Lane T, the total cell lysate; Lane P, the cell pellet after centrifugation; Lane S, the soluble cell lysate after centrifugation; Lane M, protein markers.

2.2. Expression of rhBMP7-2 in E. Coli

The pBMP7-2 plasmid was transformed into the BL21 (DE3)-RILP *E. coli* strain and cultured on LB agar plates containing 100 μg/ml ampicillin. A single colony was picked and transferred to 50 ml LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. After incubated at 37 °C overnight, 20 ml pre-cultures were used to seed to 1 l of LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. The cultures were grown at 37 °C with shaking until OD_{600} reach 0.6. Thereafter, rhBMP7-2 protein expression was induced by adding isopropyl b-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM and further cultured for 5 h at 37 °C.

2.3. Purification and refolding of rhBMP7-2

Cells were harvested by centrifugation at 5000 rpm for 15 min at 4 °C, 8 g wet weight cells were collected from 1 l of LB culture and then re-suspended in 100 ml buffer containing 100 mM Na_3PO_4 (pH 7.4), 250 mM NaCl and 10% glycerol. Lysozyme was added at a concentration of 0.1 mg/ml followed by incubation at 4 °C for 30 min and cells were lysed by sonication for 10 min (on 5 s, off 10 s), and centrifuged at 12,000 rpm for 15 min at 4 °C. Samples were analyzed by SDS–PAGE for protein expression and densitometry was carried out with Image J software.

The pellet was re-suspended in 100 ml washing buffer containing 100 mM Na_3PO_4 (pH 7.4), 250 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 4 M urea, and then incubated at 4 °C for 1.5 h. After centrifugation at 12,000 rpm for 15 min at 4 °C, the pellet was incubated in 20 ml buffer containing 20 mM Tris–HCl (pH 8.5), 10 mM DTT, and 8 M urea at 4 °C overnight and centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant which contained

Table 1
Yields and purities of rhBMP7-2 achieved at each step of purification from 1 l *E. Coli* culture.

Step	Total protein[a](mg)	rhBMP7-2 (mg)	Purity[c] (%)	Yield (%)
Cell lysate[b]	800	224	28	100
IBs wash	224	179	80	80
Anion exchange	116	110	95	49
Refolding	57	54	95	24

[a] Total protein was determined by Bradford method.
[b] Lysate from a 1 l culture yielding approximately 8 g wet cells.
[c] Purity was determined by densitometry Image J software with Image J software.