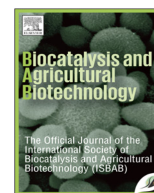
Biocatalysis and Agricultural Biotechnology 5 (2016) 199–203

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

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



Cloning and expression of human bone morphogenetic protein-2 gene in *Leishmania tarentolae*

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ARTICLE INFO

Article history:
Received 3 September 2015
Accepted 19 January 2016
Available online 21 January 2016

Keywords:
Bone morphogenetic protein 2
Morphogenesis
Growth differentiation factor 2
Osteogenesis
Spinal cord injuries
Glycosylation

ABSTRACT

Several expression hosts have been employed for the production of bone morphogenetic protein-2 (BMP-2), inducing de novo bone formation and is widely used in clinical applications. However, every expression system has its merits and demerits. For example, although *Escherichia coli* is simple and robust, but inept to make disulfide bond; hence, heterologous proteins are produced in the form of inclusion bodies, entailing in vitro refolding strategies. On the other hand yeast, which is a lower eukaryote, is able to perform glycosylation of the recombinant proteins. However, its glycosylation pattern is different from human beings. Ultimately, *Leishmania tarentolae*, a parasite protozoan of the lizard, unifying both the characteristics of prokaryotes and eukaryotes, has been introduced for the expression of glycoproteins. It is capable of making disulfide bonds, expressing correctly folded and biologically active recombinant proteins. In the current study, we successfully cloned and expressed proBMP-2 gene in *L. tarentolae*. Integration of the gene was confirmed by PCR; whereas, transcription of the coding gene was verified by back translating the target mRNA. Besides, the expression of BMP-2 was assessed by SDS-PAGE and Western blotting. A 12 kDa band was obtained, equivalent to monomeric form of BMP-2.

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1. Introduction

Bone remodeling is a natural phenomenon in which bone formation and bone resorption occur spontaneously, due to the intracellular communication of osteoblasts osteoclasts. Osteoblast cells induce the formation of bones while osteoclasts are bone resorbing cells. Bone remodeling completes in three consecutive steps. Firstly, osteoclasts digest the old bone; secondly, mononuclear cells appear on the surface of bone, and finally bone formation (Hadjidakis and Androulakis, 2006). A number of factors; such as, transforming growth factor-β (TGF-β), insulin like growth factor (IGF-1), interleukine-6 (IL-6), and bone morphogenetic proteins (BMPs) are involved in bone formation. However, only BMPs have the potentiality to induce de novo bone formation (Garimella et al., 2008). The seminal work of Urist (1965) has unveiled the notion that bone morphogenetic proteins (BMPs) are the key players in bone regeneration. Actually BMPs are multifunction cytokines, belonging to the TGF-β superfamily. Studies conducted on rodents have revealed that BMPs induced ectopic bone formation when placed under the skin of animals. It has also been demonstrated that BMPs regulate the process of embryogenesis and control the differentiation of bones, tissue, tendons, and ligaments. Due to their critical role in bone regeneration, scientists have started to study the mechanism of action of BMPs in various organs. The signaling mechanism of BMPs has been reviewed in detail (Oshin and Stewart, 2007). Some of the well-known BMPS including, BMP-2, BMP-4, BMP-6, and BMP-7 have osteoinductive properties. BMP-2 and BMP-7 have currently been approved by FDA for repairing bone fractures and spine surgery (Kamiya, 2012). Other clinical data demonstrated that BMP-2 successfully induced bone formation in osteoporotic rats (Tang et al., 2008).

Mature BMP-2 is a homodimer, each monomer contains a cysteine knot which is composed of six cysteine residues forming three intrachain disulfide bridges. The formation of hydrophobic core between monomers of BMP-2 structure makes it more stable (Scheufler et al., 1999). In the last decades, BMPs were mostly isolated and purified from bones; unfortunately, the procedure was laborious and time consuming. Repeated extraction increased the yield but reduced the biological activity of BMPs

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http://dx.doi.org/10.1016/j.bcab.2016.01.006
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both in vivo and in vitro (Hu et al., 2004). Various expression systems have been utilized for producing rhBMPs; for instance, it is possible to express rhBMP-2 in Chinese hamster ovary (CHO) cells (Israel et al., 1996, 1992, Wang et al., 1990). Attempts have also been made to express biologically active rhBMP-2 in *Escherichia coli* through in vitro refolding of inclusion bodies (Vallejo et al., 2002, Long et al., 2006). Other studies used yeast and transgenic plants for the production of heterologous BMPs in large scale. Recently *Leishmania tarentolae*, a trypanosomatid protozoan parasite of gecko has been utilized for the expression of heterologous proteins. This group of protozoa is rich in glycoproteins and its glycosylation pattern is similar to human mammals (Basile and Peticca, 2009). In current study we successfully cloned and expressed proBMP-2 gene, isolated from mammalian cell line i.e. MG-63, in *L. tarentolae.*

2. Materials and methods

2.1. Cloning BMP-2 coding cDNA

Osteosarcoma cell line, MG-63 was cultured in DMEM medium (Gibco, USA), supplemented with 10% FBS (Gibco, USA). Messenger RNA was extracted from MG-63 cell line, according to the manufacturer protocol, using GF-TR-025 RNA isolation kit (Vivantis, Germany). Concentration and purity of mRNA was determined at A260:280 and loaded on 1% agarose gel for visualization. Then 10 μg mRNA having OD $>$ 1.7 at A260/280, was reverse transcribed with 2-steps RT-PCR kit (Vivantis, Germany). From cDNA proBMP-2 encoding sequence was amplified using proBMP-2 specific forward and reverse primers, containing SalI and KpnI restriction sites (Table 1). PCR product of proBMP-2 and pTZ57R, a cloning vector both were digested with SalI and KpnI restriction enzyme (Vivantis, Germany).

2.2. Integration of proBMP-2 fragment in PTZ57R

Digests were loaded on 1% agarose gel and after electrophoresis; bands were purified using GF-1 nucleic acid recovery kit (Vivantis, Germany). Purified proBMP-2 gene fragment was then ligated in already digested pTZ57R vector, using T4 Ligase (Fermentas, Lithuania). For the confirmation of ligation, *E. coli* TOP10 strain was transformed with pTZ57R–proBMP-2 cloning vector. Transformed colonies were screened and selected on the base of blue/white colonies on LB agar plates supplemented with ampicillin and X-gal. Then a single white colony was cultured in LB broth, supplemented with ampicillin, cultured overnight and then subjected to plasmid extraction. Purified plasmid was confirmed on 1% agarose gel. Besides, ligation of proBMP-2 gene in cloning vector was also confirmed through PCR, using universal pTZ57R and proBMP-2 specific primers (Table 1).

Table 1
List of primer sequences used in this study.

Primers	Sequence (5′ to 3′)
BMP-2 forward	ATAAGCGACGCAGTTCCGGAGCTGG
BMP-2 reverse	ATAAGGTACCGCGACACCCACAACC
ptzSeq F	AGTTGGGTAACGCCAGGG
ptzSeq R	TTTCACACAGGAAACAGC
P1442	CCGACTGCAACAAGGTGTAG
A264	CATCTATAGAGAAGTACACGTAAAAG
F3001	GATCTGGTTGATTCTGCCAGTAG

2.3. Construction of expression vector

After confirmation, our next target was to subclone proBMP-2 gene in pLEXSY-hyg2 expression vector (Jena Bioscience, Germany). Both pTZ57R and pLEXSY-hyg2 expression vector were double digested with SalI and KpnI restriction enzymes (Vivantis, Germany). ProBMP-2 gene fragment and cut pLEXSY, both were purified from gel using GF-1 nucleic acid recovery kit (Vivantis, Germany). Purified proBMP-2 gene was then ligated in the multiple cloning sites of purified pLEXSY-hyg2 expression vector using T4 DNA ligase. For confirmation of proper ligation, *E. coli* XL1 strain (Stratagene), was transformed with expression vector. From transformed colonies plasmid was purified, using plasmid Mini-Prep Kit (Bioneer, Korea), and subjected to double digestion with SalI and KpnI restriction enzymes (Vivantis, Germany). Purified plasmid was further verified through PCR, using pLEXSY specific P1442 and A264 primers (Jena Bioscience, Germany), and proBMP-2 forward and reverse primers (Table 1). The gene was sequenced and checked for mutation by comparing with the reference sequence in gene bank (NC_000020.11).

2.4. Cultivation of L. tarentolae

L. tarentolae strain P10 LEXYcon2 Expression Kit (Jena Bioscience, Germany), was cultured in brain heart infusion (BHI) broth (Heimedia, India), supplemented with hemin (5 μg/ml), penicillin (50 U/ml), and streptomycin (50 μg/ml). Static cultures were cultivated in 25 ml tissue culture flasks, containing 10 ml BHI medium, and incubated at 26 °C in the dark. The medium was diluted at 1:10 into fresh medium twice a week and the number of live cells were counted on hemocytometer.

2.5. Transfection of L. tarentolae cells

The pLEXSY-proBMP2 expression vector was purified with plasmid MiniPrep Kit (Bioneer, Korea) and digested with SwaI restriction enzyme. The linearized DNA fragment and cut pLEXSY both were purified from gel using GF-1 nucleic acid recovery kit (Vivantis, Germany). For electroporation about 1×10^8 cells of *L. tarentolae,* cultured for 48 h, were centrifuged and resuspended in 1 ml pre-chilled electroporation buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 25 mM HEPES, 2 mM EDTA, and 5 mM $MgCl_2$, pH 7.6). Approximately, 5 μg of linearized DNA was used for transfecting 500 μl of *L. tarentolae* suspension in 4 mm cuvettes while in other tube cells were treated with empty self-ligated vector. Electroporation was carried out using Gene PulserXcell (Bio-Rad), by giving two pulses of 450 V and 450 μF for 3 millisecond pulse time. Electrophoresed cells were kept on ice for 10 min and then moved to BHI-hemin medium, supplemented with 100 μg/ml hygromycin. Finally, treated cells were incubated at 26 °C in the dark for one week. Transformed cells survived in the form of dense cultures on selection medium, while no growth was observed in negative control cells, transfected with empty vectors. Hygromycin resistant transformed cells were selected and used for further analysis.

2.6. Screening of transformed L. tarentolae cells

At molecular level homologous recombination was confirmed by extracting DNA from transformed *L. tarentolae* cells. Then DNA was PCR amplified with F3001 forward primer compatible with *ssu* locus of *L. tarentolae* genome, provided by LEXSYcon2 Expression Kit, (Jena Bioscience, Germany), and proBMP-2 specific reverse primers (Table 1). While for the analysis of transcription, mRNA was extracted from transformed *L. tarentolae* cells using GF-TR-025 (Vivantis, Germany). And cDNA was synthesized by using 2-steps

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