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Soluble expression and purification of synthetic human bone morphogenetic protein-2 in *Escherichia coli*

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A 345-bp gene that encodes human bone morphogenetic protein-2 (hBMP-2) has been synthesized. The codon usage of the resulting gene was modified to include those triplets that are utilized in highly expressed *Escherichia coli* genes. The hBMP-2 gene was efficiently expressed in *E. coli* as a soluble and active protein. Since the recombinant hBMP-2 was readily solublized, no further solublization steps were required throughout purification. No additional tagging residues were introduced into the synthetic hBMP-2 gene product. The developed synthetic gene is a promising approach for scaling-up the soluble expression of hBMP-2. [BMB reports 2008; 41(5): 404-407]

INTRODUCTION

Bone morphogenetic protein-2 (BMP-2) is a low molecular weight glycoprotein belonging to a group of bone matrix proteins. The osteoinductive capacity of BMP-2 has been demonstrated in preclinical models and evaluated in clinical trials (1, 2). The mature BMP-2 represents the C-terminal sequence of a long precursor protein of 396 amino acids and shows an interchain disulfide bridge connecting the two monomers to form an active dimer (3, 4).

BMPs can be isolated directly from bones; however, the yield is low (~1 μg/kg) and the purification scheme is complex (5). Recombinant BMPs have been obtained from mammalian cell cultures, but incomplete monomer processing and low yields are usual in these processes (6). Alternatively, the production of biologically active recombinant humans BMPs (rhBMPs) through in vitro refolding of *Escherichia coli* produced inclusion bodies has been reported (4, 7). However, the refolding procedure is complicated and the overall yield is low. As well, the refolding buffer contains expensive reagents. Very recently, BMP-2 was expressed as a soluble protein in *E. coli* (8). However, their product was initially found in the insoluble pellet (fraction corresponding bacterial debris) and required solublization through alkaline lysis at pH 11 in the presence of 0.7 M L-arginine and 40 mM imidazole.

Our goal was to develop an effective method to improve the high production of the soluble form of an active BMP-2 without any refolding or solublization steps. For this purpose, a 345-bp gene that encodes human BMP-2 was synthesized and expressed in *E. coli* Rogetta-gami B(DE3). Afterwards, a high yielding method for a soluble expression of hBMP-2 with a biological activity was established.

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RESULTS AND DISCUSSION

Chemical synthesis of hBMP-2

In recent years, the results of numerous animal experiments have suggested the future therapeutic benefits of BMPs in bone reconstruction (1, 2). It is hoped that the cost drops and BMP eventually becomes as affordable as other recombinant products, enabling its use in majority of indicated patient population. To obtain a large amount of highly purified intact hBMP-2, we used the following strategies. First of all, to avoid time-consuming steps mostly used in previous studies for solublization, refolding, and alkaline lysis of recombinant hBMP-2 found as insoluble proteins in a fraction corresponding to bacterial debris, we synthesized the hBMP-2 gene with a codon usage that are utilized in highly expressed *E. coli* genes. Secondly, we co-expressed thioredoxin as a separate protein using the pET32a(+) expression vector system to increase the solubility of hBMP-2. Thirdly, we tried not to introduce unnecessary tagging residues into hBMP-2 gene product. Finally, to generate the directly folded hBMP-2 in dimeric active form, the synthetic hBMP-2 gene was expressed in *E. coli* Rogetta-gami B(DE3) allowing the formation of disulfide bridges.

The synthetic hBMP-2 gene was initially constructed as three segments using plasmid pUC18 as a cloning vector. A total of 12 synthetic oligonucleotides were used to assemble the three segments. The three segments were, respectively, a 135-bp *Eco*RI/*Bst*EII fragment, a 120-bp *Bst*EII/*Sph*I fragment, and a 117-bp *Sph*I/*Hin*dIII fragment (Fig. 1). Several isolates of each

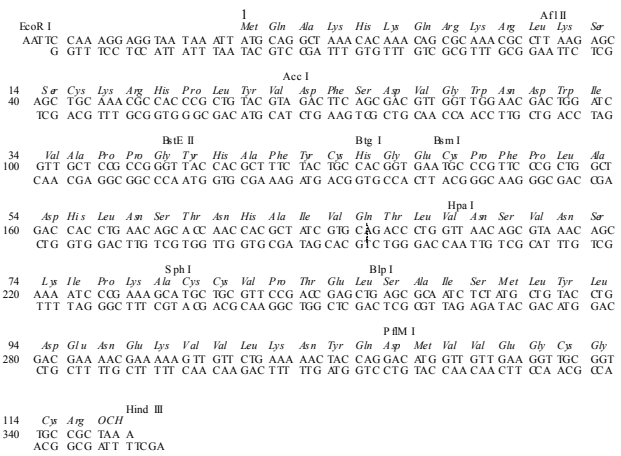

Fig. 1. DNA sequence of the synthetic hBMP-2 gene. Numbers on the left refer to amino acids (upper) and nucleotides (lower).

of the gene segments were characterized by DNA sequencing. Based on the sequencing of several isolates of each gene segment, an overall mutation frequency of 1 per 372 bp synthesized was observed and corrected to the designed sequence using a standard cassette mutagenesis procedure. The designed sequence and position of 11 restriction sites in the hBMP-2 coding region of pHBMP-2 are shown in Fig. 1.

Expression and purification of synthetic hBMP-2

The hBMP-2 gene was subcloned into pET32a(+) vector via *Eco*RI and *Hind*III sites within multi-cloning sites and transformed into Rogetta-gami B(DE3) that allows a formation of disulfide bridges. After incubation with various concentrations of IPTG at 37°C for 3 h, cell pellets were suspended in 100 mM Tris-HCl, pH 7.4 with 1 mM EDTA and lysed with a sonicator. The over production of hBMP-2 was found mainly in the supernatant as a soluble protein together with thioredoxin in two separate polypeptides. Denatured SDS/PAGE analysis (Fig. 2A) and Western blotting (Fig. 2B) of crude cell extracts showed a high level of expression of hBMP-2 at a position corresponding to a 12 kDa protein. No additional N-terminal or C-terminal tagging residues were introduced in the synthetic hBMP-2 gene product. Protein purification was performed using heparin-Sepharose and FPLC Resource-Q columns. Since the synthetic hBMP-2 was readily solublized, no detergents or no further refolding and solublization steps were required throughout the entire protein purification. The native molecular size of the purified hBMP-2 was 24 kDa as determined by FPLC gel filtration (Fig. 2C), indicating that hBMP-2 is a homodimeric protein as reported elsewhere (4).

Biological activity of synthetic hBMP-2

hBMP-2 dimer possessed biological activity as determined by the induction of alkaline phosphatase activity in C2C12 cells,

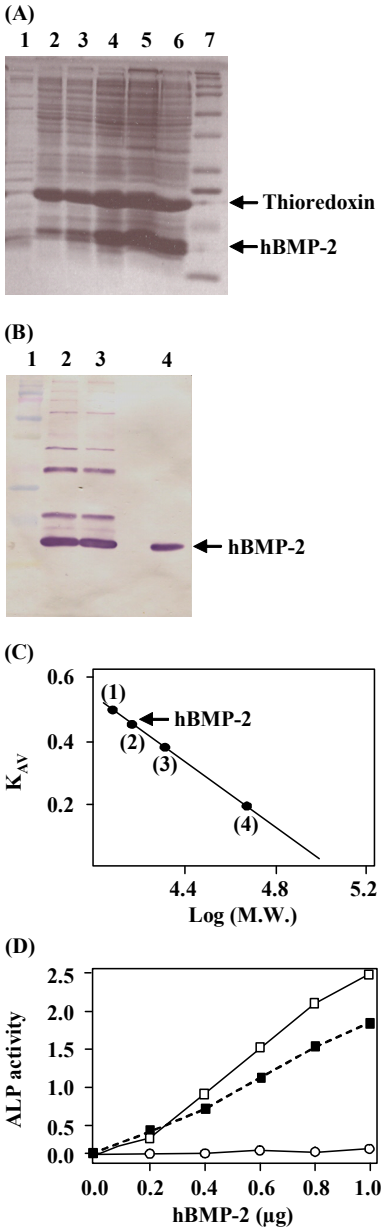

Fig. 2. Analyses of hBMP-2. (A) Expression of hBMP-2 in *E. coli* as determined by 15% SDS-PAGE. Lanes 1-6 contain crude extracts induced at respective IPTG concentrations of 0.0, 0.1, 0.2, 0.5, 1.0, and 3.0 mM. Lane 7 contains MW markers (Bio-Rad, precision plus protein standards). (B) Western blot using anti-BMP-2 antibody. Lane 1, MW markers; lane 2, crude extracts (3.0 mM IPTG); lane 3, crude extracts (0.5 mM IPTG); lane 4, purified hBMP-2. (C) Gel filtration of hBMP-2. Purified hBMP-2 was applied to FPLC Superose-12 column and eluted with 20 mM Tris-HCl, pH 8.0, 0.15 M NaCl. Calibration was performed with (1) pancreatic RNase A (13700), (2) chymotrypsinogen (25000), (3) ovalbumin (43000), and (4) bovine serum albumin (67000). Void volume was estimated with blue dextran 2000. (D) Biological activity of the purified hBMP-2. o-o, monomer, □-□, dimer, and ■-■, commercially available CHO produced hBMP-2.

whereas the hBMP-2 monomer did not show any biological activity (Fig. 2D). Construction of a synthetic gene encoding mature hBMP-2 enabled us to generate a large amount of biologically active hBMP-2 in *E. coli* without any refolding or solublization steps.

As a conclusion, we have established a new and effective method to improve the soluble expression of biologically active hBMP-2 for biochemical and clinical studies.

MATERIALS AND METHODS

Chemical synthesis and cloning of hBMP-2 gene

The design of the synthetic hBMP-2 gene was based on the amino-acid sequence of mature hBMP-2 with the following strategies. The hBMP-2 gene was assembled from three gene segments that were initially cloned into pUC18. A DNA sequence containing 11 restriction sites located approximately every 30 bp throughout the entire length of the coding region was selected from the large number of possibilities. Only those sites that are not located in pUC18 (except in the polylinker region) were included, and the gene was flanked by unique *Eco*RI and *Hind*III sites. The codon usage of the resulting hBMP-2 gene was modified to include those triplets that are utilized in highly expressed *E. coli* genes while retaining the largest possible number of unique restriction sites. A ribosome-binding site (AGGAGG) was added 10 bases upstream of the coding region to direct the initiation of translation in *E. coli*. The sequence adjacent to the ribosome binding site included an A at position -3 relative to the ATG, and the spacer region (-1 to -9) was made A + T rich to reduce potential mRNA secondary structure in the vicinity of the translation start site. Addition of a ribosome-binding site made the synthetic hBMP-2 gene portable to any of a number of commonly available plasmid vectors that carry inducible *E. coli* promoters.

Expression and purification of hBMP-2

The synthetic hBMP-2 gene was subcloned into pET32a(+) vector (Novagen, San Diego, CA) via *Eco*RI and *Hind*III restriction sites without fusion into thioredoxin and expressed in *E. coli* Rogetta-gami B(DE3) (Novagen). After incubation with isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.1-3.0 mM) at 37°C for 3 h, cell pellets were suspended in 100 mM Tris-HCl, pH 8.0 with 1 mM ethylenediaminetetraacetic acid and lysed with a ultrasonicator three times for 15 s with intervals of 1 min on ice, and supernatant and pellet fractions were collected by centrifugation at 4,000 g for 30 min at 4°C. The supernatant was applied to a heparin-Sepharose column (Amersham, Buckinghamshire, UK) equilibrated with 20 mM Tris-HCl, pH 8.0 (buffer A). The column was washed extensively with buffer A and BMP-2 was eluted with a linear gradient of NaCl. The hBMP-2 fractions were eluted at 0.5 M NaCl with a flow rate of 1 ml/min and desalted with buffer A. The hBMP-2 containing fractions were further purified by fast protein liquid chromatography (FPLC) Resource-Q column equilibrated with buffer A with a linear gradient of NaCl. Human BMP-2 was eluted at 0.3 M NaCl with a flow rate of 6 ml/min. Samples were analyzed by 15% sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (9) with mouse anti-human BMP-2 polyclonal primary antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:500 followed by goat anti-mouse alkaline phosphatase conjugated anti-KLP secondary antibody diluted 1:5,000.

Biological activity of hBMP-2

Biological activity of the purified hBMP-2 was measured by the induction of alkaline phosphatase activity in C2C12 cells at 10^5 cells/ml per well in a 24-well plate according to an established procedure (7) with a slight modification. Cells were attached overnight in Dulbecco's modified Eagle's medium with 1% fetal calf serum at 37°C with 5% CO_2 in a humidified environment. Cells were incubated for 5 days with the purified BMP-2. A commercially available CHO produced hBMP-2 (R&D Systems, Minneapolis, MN) was used as a standard.

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