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Overproduction of recombinant human bone morphogenetic protein-7 in Chinese hamster ovary cells

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ABSTRACT

Bone morphogenetic protein-7 is a multifunctional growth factor involved in various cellular processes such as osteogenesis, kidney and eye development, brown adipogenesis, and bone metastasis, and thus has been considered to have therapeutic potential for treating various diseases. In this study, we established a Chinese hamster ovary (CHO) cell line stably overexpressing recombinant human BMP-7 (rhBMP-7). Over the course of a 14-day fed-batch culture process in a 7.5-l bioreactor (5-l working volume) using chemically defined medium, the established cells could produce over 188 mg/l of rhBMP-7 protein. The rhBMP-7 was purified to homogeneity from the culture supernatant using a two-step chromatographic procedure that resulted in a recovery rate of approximately 55%, with protein purity greater than 95%. The purified rhBMP-7 was further demonstrated to be functionally active by measuring the proliferation of MC3T3-E1 cells, revealing a half-maximal effective concentration of 28.31 ng/ml.

1. Introduction

Bone morphogenetic protein-7 (BMP-7) is a 35 kDa homodimeric glycoprotein that belongs to the transforming growth factor beta (TGFβ) superfamily [1]. Originally identified by its functions in osteogenesis and chondrogenesis [2,3], BMP-7 has been shown to be involved in various cellular processes such as kidney and eye development [4], brown adipogenesis [5], and bone metastasis [6]. The recombinant human BMP-7 (rhBMP-7) produced in Chinese hamster ovary (CHO) cells was approved for revision posterolateral lumbar spine fusion and for the treatment of long bone nonunion fractures after successful clinical trials [7–10]. Recent studies using animal disease models have suggested the additional therapeutic potential of BMP-7 in various diseases including chronic kidney disease, obesity, and metastatic diseases [11,12].

The human BMP-7 gene encodes 431 amino acids including the N-terminal signal sequence (29 amino acids). The active and mature BMP-7 was shown to be initially expressed in a precursor form (pro-BMP-7) consisting of two monomeric pro-BMP-7 proteins (402 amino acids) containing the C-terminal mature BMP-7 (139 amino acids) and the N-terminal prodomain, which is subsequently cleaved from the precursor form by furin-like protease in the trans-Golgi network [13,14]. The mature BMP-7 is a glycoprotein with three potential N-glycosylation sites and has a highly folded structure, known as a cysteine knot [15], with seven cysteine residues involved in three intramolecular disulfide bonds and one intermolecular disulfide bond to form the homodimer. There have been several attempts to express rhBMP-7 in mammalian cells [13,14,16,17], but the levels of expression achieved were low and the production technologies were too premature for large-scale production. Microbial expression systems have also been used to express biologically active rhBMP-7 protein [18,19], but the expression levels reported were no better than those reported in mammalian expression systems. In the present study, a Chinese hamster ovary (CHO) cell line overexpressing biologically active rhBMP-7 was successfully developed, along with a pilot scale manufacturing process using a 7.5-l bioreactor (5-l working volume) that can be employed for large-scale production of rhBMP-7.

Abbreviations: BMP-7, bone morphogenetic protein-7; CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; EC_{50}, effective dose 50; FBS, fetal bovine serum; HRP, horseradish peroxidase; Ig, Immunoglobulin; kDa, kilodalton; MTX, methotrexate; MW, molecular weight; PCR, polymerase chain reaction; Pro-BMP-7, precursor form of BMP-7; rhBMP-7, recombinant human BMP-7; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TGFβ, transforming growth factor beta.

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2. Materials and methods

2.1. Construction of the rhBMP-7 expression plasmids

An rhBMP-7 expression plasmid was constructed by introducing a cDNA insert encoding human BMP-7 into a pMSG vector containing the human β-globin matrix attachment region, under the control of an SV promoter [20]. The cDNA encoding the protein coding region of human BMP-7 cDNA was obtained by polymerase chain reaction (PCR) with the Gene Pool™ cDNA (Liver, Human Normal cDNA) (Invitrogen) as a template using the following oligonucleotide primers: 5′-CTAGCTAGCCACCATGCACGTGCGCTCACTGCG-3′ (sense primer: NheI recognition site underlined) and 5′-CCGCTCGAGCTAGTGGCAGCCACAGGCCCG-3′ (antisense primer: XhoI recognition site underlined). The sense primer contains the Kozak sequence (GCCACC) for an efficient translation initiation [21]. The amplified DNA fragment was digested with NheI and XhoI, and then ligated to the pMSG vector, which was digested with the same restriction enzymes. The resulting expression plasmid construct, pMSG-rhBMP-7, was completely sequenced to confirm that it contained the entire human BMP-7 coding region (GenBank Accession No. M60316.1).

2.2. Development of stable rhBMP-7-expressing CHO cell lines

Dihydrofolate reductase (DHFR)-defective CHO cells (DG44) already adapted to suspension culture with chemically defined medium, EX-CELL® CD CHO (Sigma–Aldrich) supplemented with 4 mM L-glutamine (Sigma–Aldrich), were used as the host for the expression of rhBMP-7. The cells were co-transfected with 2 μg DNA that consisted of a 100:1 molar ratio of the expression plasmid, pMSG-rhBMP-7, and the selection marker plasmid, pDCH1P [22], using DOSPER (Roche Life Science), as previously described [23,24]. The transfected cells were grown for approximately two weeks in selection medium, EX-CELL® CD CHO (without hypoxanthine and thymidine) (Sigma–Aldrich) supplemented with 4 mM L-glutamine, and then the stable transfectants were maintained in the presence of Methotrexate (MTX) (Sigma–Aldrich) at a concentration increased in a stepwise fashion (10 nM → 100 nM → 1 μM). The single-cell-derived and most productive clone was isolated from the stable transfectants adapted to 1 μM MTX and grown in the same selection medium. After the cells were harvested, they were resuspended in serum-free Cell Freezing Medium (Sigma–Aldrich) and frozen in a Nalgene Cryo 1 °C Freezing Container (Thermo Fisher Scientific) for future use.

2.3. Expression, purification, and quantitation of rhBMP-7

The 14-day fed-batch cultures of rhBMP-7-expressing CHO cell lines were established in a 7.5-l Bioreactor (5-l working volume) (New Brunswick Scientific). The cultures were started with 4.5 l HyCell CHO medium (GE Healthcare Life Sciences) supplemented with 4 mM L-glutamine and 500 ml 2X stock solution of the nutrient supplement solution, EfficientFeed™ B+ AGT™ (Life Technologies). The seeding density was 5×10^5 cells/ml, and the cells were grown at 34 °C for 14 days with the addition of 18 g glucose on day 11. 7.5% sodium bicarbonate solution was added automatically to maintain the pH of the culture above 7.0. The culture supernatant was harvested and ultrafiltrated using a 0.22 μm polyethersulfone membrane (Corning). The mature rhBMP-7 was then purified from the culture supernatant using previously described procedures [25,26] with several modifications. After adding urea and ammonium sulfate to a final concentration of 4 M and 0.5 M, respectively, the culture supernatant was loaded onto a Phenyl Sepharose High Performance column (GE Healthcare Life Science) that had been pre-equilibrated with PS-equilibration buffer (0.5 M ammonium sulfate, 4 M Urea, 0.3 M sodium chloride, 20 mM HEPES, pH 7.0). The unbound proteins were removed by washing the column with PS-equilibration buffer, and the bound proteins were eluted with ethanol gradient using PS-equilibration buffer and 70% ethanol. The fractions containing mature rhBMP-7 were collected and loaded onto a Vydac 214TP C4 reverse-phase column (GraceVydac) that had been pre-equilibrated with 0.1% trifluoroacetic acid (TFA). After washing the column with 0.1% TFA, 30% acetonitrile, the proteins were eluted with an acetonitrile gradient of 30%–55% in 0.1% TFA. The fractions containing mature rhBMP-7 were collected, mixed with 1/9 volume of 10X storage solution (12% mannitol, 10% sucrose, 0.2 M glycine, and 0.5% Tween 20 (pH 4.0)), and then lyophilized. The lyophilized protein powder was stored at 4 °C and reconstituted by dissolving in distilled water. The purified mature rhBMP-7 was quantitated by ELISA using a DuoSet Kit for BMP-7 (R&D Systems) in accordance with the manufacturer's instructions. Purity was evaluated by densitometric analysis of Coomassie blue-stained gels using a Molecular Image® Gel Doc™ XR+ System (Bio-Rad).

2.4. SDS-PAGE and Western blot analysis

The protein samples were separated by 15% SDS-PAGE under reducing or non-reducing conditions, and then analyzed by Coomassie blue staining with GelCode Blue Stain Reagent (Thermo Scientific) or by Western blot using rabbit polyclonal anti-BMP-7 antibody (Thermo Scientific) and HRP-conjugated goat-rabbit IgG antibody (Thermo Scientific). Western blot detection was performed with a SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce).

2.5. De-N-glycosylation of the purified rhBMP-7

Peptide N-Glycosidase F (PNGase F) (New England Biolabs) was used to remove the N-linked glycans from the purified rhBMP-7. The de-N-glycosylation reactions were performed in accordance with the manufacturer's instruction and analyzed by 15% SDS-PAGE under reducing conditions.

2.6. N-terminal sequencing analysis of the purified rhBMP-7

N-terminal amino acid sequencing was performed by Edman degradation using a Procise 492 protein sequencer coupled to a HAISIL PTH System (Applied Biosystems) in accordance with the manufacturer's instructions. Sequences of 5 N-terminal amino acids were analyzed.

2.7. BMP-7 bioassay

The biological activity of purified rhBMP-7 was evaluated by measuring the proliferation of MC3T3-E1 cells (C57BL/6 mouse preosteoblast cells) (ATCC) according to previously described procedures [14,27] with minor modifications. MC3T3-E1 cells were grown in growth medium that is MEM Alpha Modification (with L-Glutamine, with Ribo- and Deoxyribonucleosides) (HyClone) supplemented with 10% FBS (Fetal Bovine Serum) (Sigma–Aldrich), 1 mM sodium pyruvate, and 1X Antibiotic Antimycotic Solution (Sigma–Aldrich), then harvested and washed three times with Dulbecco's Phosphate-Buffered Saline (no calcium, no magnesium) (Thermo Fisher Scientific). The washed MC3T3-E1 cells were resuspended at 1×10^4 cells/ml in growth medium, and 200 μl aliquots of the cell suspension were added to each well of 96-well microplates and incubated for 24 h at 37 °C. After removal of the medium, the microplates were loaded with the rhBMP-7 purified