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Expression and Purification of Biologically Active Human Bone Morphogenetic Protein-4 in Recombinant Chinese Hamster Ovary Cells

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Bone morphogenetic protein-4 (BMP-4) is considered to have therapeutic potential for various diseases, including cancers; however, the high expression of biologically active recombinant human BMP-4 (rhBMP-4) needed for its manufacture for therapeutic purposes has yet to be established. In the current study, we established a recombinant Chinese hamster ovary (rCHO) cell line overexpressing rhBMP-4 as well as a production process using 7.5-l bioreactor (5 L working volume). The expression of the mature rhBMP-4 was significantly enhanced by recombinant furin expression. The combination of a chemically defined medium and a nutrient supplement solution for high expression of rhBMP-4 was selected and used for bioreactor cultures. The 11-day fed-batch cultures of the established rhBMP-4-expressing rCHO cells in the 7.5-L bioreactor produced approximately 32 mg/l of rhBMP-4. The mature rhBMP-4 was purified to homogeneity from the culture supernatant using a two-step chromatographic procedure, resulting in a recovery rate of approximately 55% and a protein purity greater than 95%. The N-terminal amino acid sequences and *N*-linked glycosylation of the purified rhBMP-4 were confirmed by N-terminal sequencing and de-*N*-glycosylation analysis, respectively. The mature purified rhBMP-4 has been proved to be functionally active, with an effective dose concentration of EC_{50} of 2.93 ng/ml.

Keywords: Bone morphogenetic protein-4 (BMP-4), Chinese hamster ovary (CHO) cells, recombinant protein expression, stable cell line, bioreactor process, bioassay

Introduction

Bone morphogenetic protein-4 (BMP-4) was originally identified as one of the growth factors existing in demineralized bone extract that induced new bone formation [1, 2]. Studies with knockout and transgenic mice demonstrated that BMP-4 is involved in the development of various tissues and organs, such as mesoderm in early embryonic development, and kidney, urinary tract, and eye tissues [3, 4]. BMP-4 has also been shown to be associated with several metabolic and vascular diseases, and with cancer [5, 6]. Recent studies for targeting cancer stem cells to develop new cancer therapies have suggested a new therapeutic potential of BMP-4 in cancer treatment. The proliferations of human brain glioma cell lines have been reported to be significantly inhibited in vitro by BMP-4 [7–9]. Gene therapy using vaccinia virus expressing BMP-4 has led to excellent tumor growth inhibition and survival of mice implanted with glioblastoma cancer stem cells [10]. Similar effects of BMP-4 on colorectal cancer stem cells have also been reported [11].

The human BMP-4 is expressed as a large precursor form of 384 amino acids (pro-BMP-4) after the removal of an N-terminal signal sequence (24 amino acids) [12]. The pro-BMP-4 dimerizes in the endoplasmic reticulum, and is then cleaved to produce the active and mature BMP-4 homodimer consisting of two monomeric BMP-4 proteins (116 amino acids) by serine proteases such as furin and proprotein convertase 6 in the Golgi apparatus [12, 13].

Although increasing demands of recombinant human

BMP-4 (rhBMP-4) have been made for therapeutic purposes, efficient production procedures of biologically active BMP-4 have not yet been developed. The highest expression of rhBMP-4 using mammalian expression systems was obtained using Chinese hamster ovary (CHO) cells as the host, with a reported expression of 4–5 mg/l even after optimizing the culture conditions of recombinant CHO (rCHO) cells [14]. *Escherichia coli* expression systems have also been used to express rhBMP-4; however, these reports do not provide information about the level of expression, presumably because of the low expression [15, 16]. In a recent report, a *Pichia pastoris* expression system showed high expression of rhBMP-4 (48 mg/l); however, the purified rhBMP-4 from the culture supernatant had about 300 times lower specific activity than that of rhBMP-4 expressed and purified in a mammalian expression system [17].

The aim of this current work was to develop a production process of rhBMP-4 by establishing an rCHO cell line overexpressing biologically active rhBMP-4 and by optimization of the culture process in a 7.5-L bioreactor (5 L working volume) and through purification procedures.

Materials and Methods

Cloning of Human BMP-4 cDNA into Expression Vector

The polymerase chain reaction (PCR) was employed to obtain the protein coding region of human BMP-4 cDNA. The Gene Pool cDNA (Liver, Human Normal cDNA) (Invitrogen, USA) was used as a template for the PCR. The sense and antisense primers were 5′-CTAGCTAGCCACCATGATTCCTGGTAACCGAATG-3′ (NheI recognition site underlined) and 5′-CCGCTCGAGTCAGCGGCA CCCACATCCCTC-3′ (XhoI recognition site underlined), respectively. The sense primer was designed to contain the Kozak sequence (GCCACC) for an efficient initiation of the translation [18]. The PCR products were cloned under the mouse EF1α promoter of pMmEG(TA), which was an expression vector containing a human β-globin matrix attachment region for efficient protein expression in CHO cells [19], via the NheI and XhoI restriction sites. The resulting expression plasmid, pMmEG(TA)-rhBMP-4, was confirmed to contain the entire human BMP-4 coding region (GenBank Accession No. NM_001202) by DNA sequence analysis.

Development of rCHO Cell Lines Stably Expressing rhBMP-4

Suspension adapted CHO DG44 (*dhfr*$^-$/*dhfr*$^-$) cells, adapted to the chemically defined medium, EX-CELL CD CHO (Sigma-Aldrich, USA) supplemented with 4 mM L-glutamine (Sigma-Aldrich), were used as the host for rhBMP-4 expression. In order to evaluate the furin expression on the processing of the expressed rhBMP-4, suspension adapted CHO DG44 cells with recombinant furin expression were prepared as described previously [20] and were used as the host for rhBMP-4 expression. The two host CHO cells with and without recombinant furin expression were designated as CHO(+f) and CHO(-f), respectively. The CHO(+f) and CHO(-f) cells were co-transfected with 2 μg of DNA that consisted of a 100:1 molar ratio of the expression plasmid, pMmEG(TA)-rhBMP-4, and the selection marker plasmid, pDCH1P [21], by electroporation using the Neon transfection system (Life Technologies, USA) in accordance with the manufacturer's instructions. After transfection, the pools of transfectants were grown in the selection medium, EX-CELL CD CHO (without hypoxanthine and thymidine) (Sigma-Aldrich) supplemented with 4 mM L-glutamine. The single-cell-derived clones were isolated from pools of transfectants by limiting dilutions.

Selection of a Chemically Defined (CD) Medium and a Nutrient Supplement Solution

Four CD media and three nutrient supplement solutions that are commercially available and widely used were evaluated for the expression of rhBMP-4 by rCHO cells. The CD media used were EX-CELL CD CHO (Sigma-Aldrich), PowerCHO-2 (Lonza, USA), IS CHO-CD (Irvine Scientific, USA), and HyCell CHO (GE Healthcare Life Sciences, USA). The nutrient supplement solutions used were CHO CD Efficient Feed A, B, and C (Thermo Fisher Scientific, USA). The 50 ml batch cultures of rhBMP-4-expressing rCHO cells were formed in 125 ml Erlenmeyer flasks (Thermo Fisher Scientific) for 6 to 11 days, depending on the cell viabilities. When the cell viability reached 80%, the culture supernatant was harvested and evaluated for rhBMP-4 expression. In the case where a nutrient supplement solution was added to the CD medium, the portion of the nutrient supplement solution was 1/10 of the total culture volume. The culture temperature and the seeding density were 34°C and 5×10^5 cells/ ml, respectively.

Bioreactor Cultures and Purification of rhBMP-4

The fed-batch cultures of the rhBMP-4-expressing rCHO cell lines were established in a 7.5-L bioreactor (5 L working volume) (New Brunswick Scientific, USA) with 4.5 L HyCell CHO medium supplemented with 4 mM L-glutamine and 500 ml of CHO CD Efficient Feed C. The culture temperature and seeding density were 34°C and 5×10^5 cells/ml, respectively. The pH of the culture was maintained above 7.1 by the automatic addition of 7.5% sodium bicarbonate solution. After harvesting and ultrafiltration using a 0.22 μm polyethersulfone membrane (Corning, USA) the mature rhBMP-4 was purified from the culture supernatant using previously described procedures [22] with several modifications. The culture supernatant was loaded onto a Cellufine sulfate column (JNC Co., Japan) previously pre-equilibrated with a solution of 20 mM histidine and 0.1 M NaCl (pH 6.5). After washing the column with a solution of 20 mM histidine and 0.5 M NaCl (pH 6.5), the bound proteins were diluted with a solution of 20 mM histidine, 0.1 M NaCl, and 0.6 M arginine (pH 6.5). The fractions containing mature rhBMP-4 were collected and loaded onto a HiTrap Butyl FF column (GE Healthcare) that had been pre-equilibrated with a solution of 20 mM Tris and 0.4 M NaCl

(pH 7.8). The bound proteins were eluted with a solution of 20 mM Tris, 0.2 M NaCl, and 10% propylene glycol (pH 7.8). The purity of the purified protein was assessed by densitometric analysis of Coomassie blue-stained gels using a Molecular Image Gel Doc XR+ System (Bio-Rad, USA).

SDS-PAGE, Western Blot Analysis, and Quantitation of rhBMP-4

The culture supernatant or purified rhBMP-4 was loaded onto a 15% SDS-PAGE gel under reducing or non-reducing conditions. Upon fractionation, the proteins were analyzed using Coomassie blue staining with GelCode Blue Stain Reagent (Thermo Fisher Scientific) or western blotting. A goat polyclonal anti-BMP-4 antibody (R&D Systems, USA) and HRP-conjugated donkey-goat IgG antibody (Thermo Fisher Scientific) were used as the primary and secondary antibody, respectively. Western blot detection was performed with the ECL Prime Western Blotting Detection Reagent (GE Healthcare).

Quantitation of rhBMP-4 in culture supernatants or after purification was performed by ELISA using a DuoSet Kit for BMP-4 (R&D Systems) in accordance with the manufacturer's instructions.

N-Terminal Sequencing of Purified rhBMP-4

N-terminal amino acid sequences of purified rhBMP-4 were determined by Edman degradation using a combined system of Procise 492 protein sequencer and HAISIL PTH System (Applied Biosystems, USA) in accordance with the manufacturer's instructions. Five amino acid residues were determined.

De-*N*-Glycosylation Analysis of the Purified rhBMP-4

The *N*-linked glycans of 2 μg of purified rhBMP-4 were removed using Peptide *N*-Glycosidase F (PNGase F) (New England Biolabs, USA) in accordance with the manufacturer's instructions. The de-*N*-glycosylated protein (1 μg) was analyzed using 15% SDS-PAGE under reducing conditions.

BMP-4 Bioassay

The biological activity of purified rhBMP-4 was assessed by measuring the proliferation of MC3T3-E1 cells (C57BL/6 mouse preosteoblast cells) (ATCC CRL-2593) by using a previously described procedure [23], although we used the Premix WST-1 Cell Proliferation Assay System (Takara, Japan) in accordance with the manufacturer's instructions. The EC_{50} obtained from the purified rhBMP-4 was compared with that of the commercially available rhBMP-4 (R&D Systems).

Results and Discussion

Previous attempts to express rhBMP-4 in CHO cells showed that both unprocessed and partially processed forms of rhBMP-4 were expressed and secreted together with mature and active rhBMP-4 [12−14], suggesting that the amount of serine proteases is not sufficient for completely processing the overexpressed rhBMP-4 in CHO cells. Since only the mature rhBMP-4 form can be used for therapeutic purposes, we first evaluated the effects of furin overexpression in CHO cells on the processing of rhBMP-4. We constructed two types of rCHO cells expressing rhBMP-4 by transfecting the expression plasmid, pMmEG(TA)-rhBMP-4, into the CHO(-f) and CHO(+f) host cells (see Materials and Methods). After transfections and adaptation to the selection medium, pools of transfectants were grown in 50 ml of selection medium for 7 days at 37°C in 125 ml Erlenmyer flasks. The grown transfectants were then evaluated for processing of secreted rhBMP-4 by western blot analysis (Fig. 1). The culture supernatant obtained from the rCHO cells developed with CHO(-f) contained a small amount of unprocessed and partially processed forms of rhBMP-4 (Fig. 1, lane 1), whereas the culture supernatant obtained from the rCHO cells developed with CHO(+f) contained only the mature rhBMP-4 (Fig. 1, lane 2). The unprocessed and partially processed forms of rhBMP-4 in the supernatant obtained from the rCHO cells developed with CHO(-f) were less than 5% of all band intensities (Fig. 1, lane 1). These data

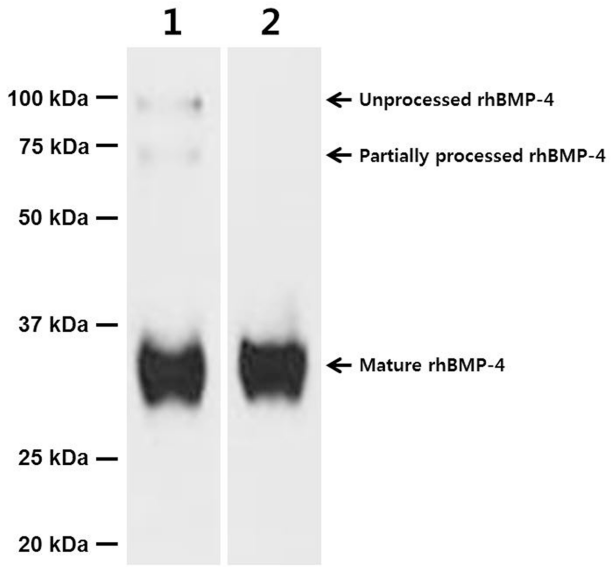

Fig. 1. Effects of recombinant furin expression on processing and expression of mature rhBMP-4.

The culture supernatants obtained from pools of transfectants were loaded onto a 15% SDS-PAGE gel under non-reducing conditions and analyzed by western blotting using goat polyclonal anti-BMP-4 antibody (see Materials and Methods): lane 1, 2 μl of culture supernatant obtained from rCHO cells developed with CHO(-f) (lane 1); lane 2, 0.4 μl of culture supernatant obtained from rCHO cells developed with CHO(+f).

are significantly different from those of a previous report, in which 35% of the total rhBMP-4 expressed and secreted in the culture medium was that of the unprocessed and processed rhBMP-4 forms [14]. Although the specific factors and conditions causing these differences are not known, the data presented in Fig. 1 suggest that the recombinant furin overexpression may not be necessary for the expression of mature rhBMP-4 for therapeutic purposes. However, the expression of rhBMP-4 by rCHO cells developed with CHO(+f) was approximately 5 times higher than that by rCHO cells developed with CHO(-f). Fig. 1 shows the differences in the loading volumes in lanes 1 and 2, suggesting that the recombinant furin expression had increased the expression of mature rhBMP-4 significantly. It is also possible that proper processing enhanced the secretion of rhBMP-4 in the culture medium. Despite that the mechanism underlying the enhanced expression of rhBMP-4 by recombinant furin expression is unknown, the rhBMP-4-expressing rCHO cells developed with CHO(+f) were chosen for use in further experiments. Therefore, the single-cell-derived rCHO cell clones with the highest expression of rhBMP-4 were isolated from the transfectant pool that was prepared by transfecting the expression plasmid, pMmEG(TA)-rhBMP-4, into CHO(+f) host cells.

In order to select a CD medium for the highest rhBMP-4 expression, we tested four CD media that are commercially available and widely used (see Materials and Methods). The optimal temperature for the rhBMP-4 expression was selected to be 34°C after comparing the expression at 32°C, 34°C, and 37°C in selection medium (data not shown). As shown in Table 1, the IS CHO-CD and HyCell media produced higher expression of rhBMP-4 than the other two CD media. These CD media were further evaluated with three nutrient supplement solutions for rhBMP-4 expression. The combination of HyCell CD and CHO CD Efficient Feed C showed the highest expression of rhBMP-4 (Table 1), and thus was chosen for use in fed-batch cultures using a 7.5-L (5 L working volume) bioreactor.

Table 1. Evaluation of commercially available chemically defined (CD) media and nutrient supplement solutions for rhBMP-4 expression.

Chemically defined medium	Nutrient supplement solution	rhBMP-4 expression (mg/l)
EX-CELL CD CHO	None	2.3
PowerCHO-2	None	12.0
IS CHO-CD	None	19.1
HyCell CHO	None	21.7
IS CHO-CD	CHO CD Efficient Feed A	9.4
	CHO CD Efficient Feed B	13.9
	CHO CD Efficient Feed C	17.0
HyCell CHO	CHO CD Efficient Feed A	18.5
	CHO CD Efficient Feed B	29.3
	CHO CD Efficient Feed C	34.0

The expression of rhBMP-4 by the selected rhBMP-4-producing rCHO cell line was assessed by establishing fed-batch cultures in a 7.5-L bioreactor (5 L working volume) (see Materials and Methods). The cell growth and expression profiles are shown in Fig. 2. The viable cell density reached the maximum level of over 0.9×10^7/ml on day 11 (Fig. 2). The rhBMP-4 level that had accumulated in the medium was the highest, although the cell viability was still over 95% on that day (Fig. 2). Therefore, the culture supernatant was harvested on day 11 for the purification of mature rhBMP-4. Three independent 11-day fed-batch cultures were performed in order to show the stabilities in rhBMP-4 expression and cell growth profile in a 7.5-L bioreactor (5 L working volume). The results are summarized in Table 2, confirming the culture process in a 7.5-L bioreactor (5 L working volume) is well established. The expression of rhBMP-4 in the 11-day fed-batch culture process was 31.8 ± 1.94 mg/l (Table 2).

Purification of the mature rhBMP-4 protein from the culture supernatant of the 11-day fed-batch culture in a 7.5-L bioreactor was performed by a two-step chromatographic purification procedure using Cellufine sulfate and HiTrap Butyl FF columns (see Materials and Methods). The purified

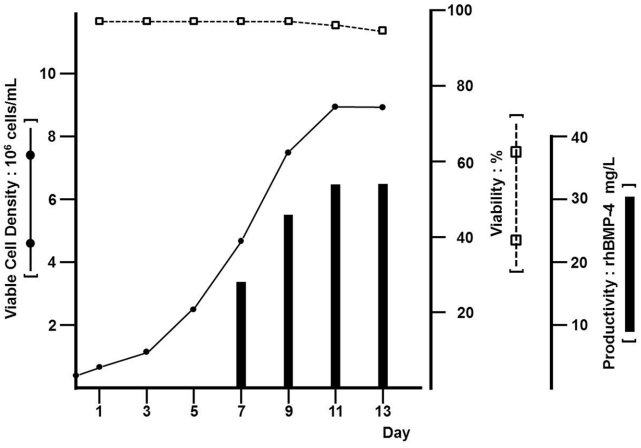

Fig. 2. Expression profile of rhBMP-4 by an rhBMP-4-expressing rCHO cell line in a 7.5-L bioreactor (5 L working volume).
The viable cell density (-●-), cell viability (-□-), and levels of accumulated rhBMP-4 in the culture medium (filled rectangle) during the fed-batch culture process are indicated.

Table 2. Producer cell performance in three 11-day fed-batch cultures in a 7.5-L bioreactor (5 L working volume).

Batch number	$q_{rhBMP-4}$ (pg/cell/day)	MVCC (10^6 cells/ml)	IVCC (10^6 cells day/ml)	Maximum rhBMP-4 concentration (μg/ml)
1[a]	0.74	9.09	43.4	32.3
2	0.71	8.65	41.8	29.7
3	0.75	9.28	44.8	33.5
Mean ± SD	0.73 ± 0.02	9.01 ± 0.32	43.3 ± 1.50	31.8 ± 1.94

The $q_{rhBMP-4}$, MVCC, and IVCC represent the specific productivity, maximum viable cell concentration, and integrated viable cell concentration, respectively.
The $q_{rhBMP-4}$ was calculated from the data obtained from day 1 to day 11.
[a]The values were obtained from the batch shown in Fig. 1.

rhBMP-4 was analyzed by SDS-PAGE, and showed several dimer bands in the 32−35 kDa range under non-reducing conditions (Fig. 3A, lane 1) and in the 17−18 kDa range under reducing conditions (Fig. 3B, lane 2). All these protein bands were positively detected with anti-BMP-4 antibody by western blotting (Fig. 3B). The protein purity of the purified rhBMP-4 was greater than 95% by densitometric analysis of the Coomassie blue-stained gels. The protein recovery rates from the Cellufine sulfate column and HiTrap Butyl FF column chromatographic steps were 76% and 74%, respectively, and thus the overall yield from the purification was about 55 %.

The mature human BMP-4 sequence has one potential *N*-linked glycosylation site. To investigate the glycosylation of the purified rhBMP-4, the purified protein was treated with PNGase F and analyzed by SDS-PAGE under reducing conditions. After PNGase F treatment, the size of the monomer protein bands in the 17−18 kDa range (Fig. 3A, lane 2) decreased to a single 13 kDa protein band (Fig. 3A, lane 3). This molecular weight (MW) matches the theoretical MW of mature BMP-4 (13.129 Da) without any posttranslational modifications when computed by the Compute pI/Mw tool [24], suggesting that the potential *N*-linked glycosylation site is glycosylated in mature rhBMP-4. The result also suggests that the various protein bands shown in both non-reducing and reducing conditions (Fig. 3A, lanes 1 and 2)

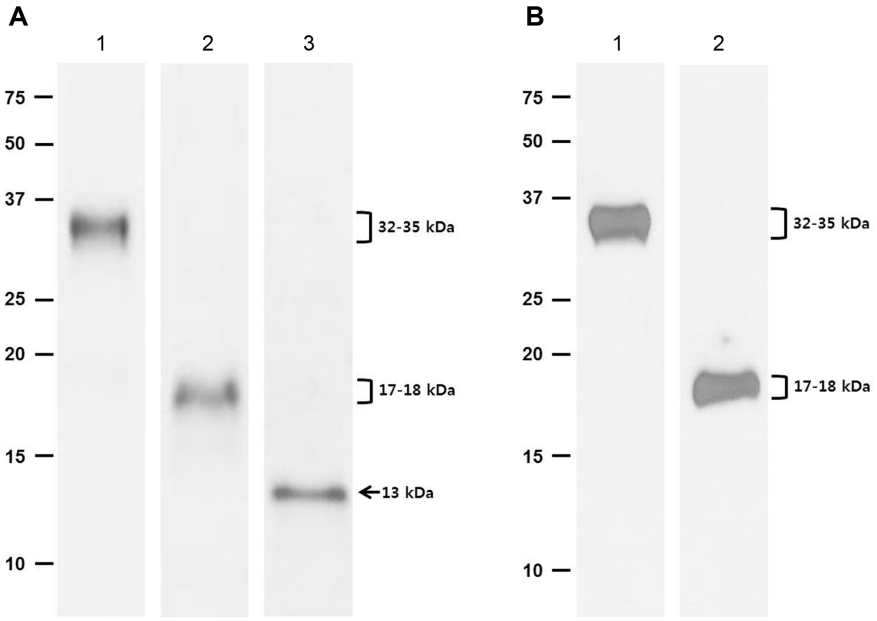

Fig. 3. SDS-PAGE and western blot analysis of the purified rhBMP-4.
(**A**) One microgram of purified rhBMP-4 was loaded onto a 15% SDS-PAGE gel under non-reducing conditions (lane 1), reducing conditions (lane 2), or reducing conditions after PNGase F treatment (lane 3), and visualized by Coomassie blue staining. (**B**) Forty nanograms of purified rhBMP-4 was loaded onto a 15% SDS-PAGE gel under non-reducing conditions (lane 1) or reducing conditions (lane 2), and analyzed by western blotting using goat polyclonal anti-BMP-4 antibody.

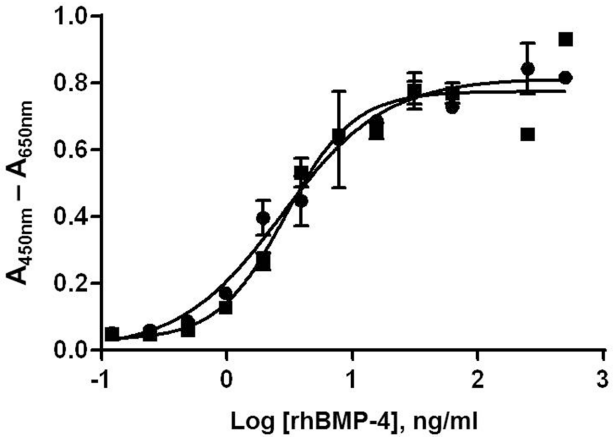

Fig. 4. Biological activity assay of the purified rhBMP-4.
The proliferation of pre-osteoblastic cells (MC3T3-E1) was assessed with the rhBMP-4 purified in this study (closed rectangles) and with rhBMP-4 purchased from R&D Systems (closed circles). The error bars indicate the standard deviation.

are due to the differential glycosylations with different carbohydrate moieties in purified rhBMP-4. The N-terminal amino acid sequences of purified rhBMP-4 were confirmed to be SPKHH by N-terminal sequencing analysis, exactly matching those of the mature human BMP-4 previously reported (GenBank Accession No. NM_001202).

A proliferation assay using the pre-osteoblastic cells (MC3T3-E1) was performed for evaluating the biological activity of the purified rhBMP-4. The proliferation activity of the rhBMP-4 purified in this study was compared with that of rhBMP-4 purchased from R&D Systems (see Materials and Methods). Both rhBMP-4 proteins activated the growth of the MC3T3-E1 cells in a dose-dependent manner (Fig. 4). The calculated ED_{50} values were 2.93 ng/ml for rhBMP-4 purified in this study and 2.76 ng/ml for the rhBMP-4 purchased from R&D Systems. Therefore, the mature rhBMP-4 obtained herein is functionally active with similar biological activity to the rhBMP-4 that is commercially available and widely used for studies related to this protein.

The recombinant protein should be produced in large scale and the manufacturing process should retain its biological activity for therapeutic purposes. Thus far, no reports have been presented that show a reasonably high expression of biologically active rhBMP-4 using mammalian expression systems as well as microbial expression systems. Only the *P. pastoris* expression system has been reported to show a high expression of rhBMP-4 [18]. However, the rhBMP-4 expressed in *P. pastoris* was reported to be not biologically active because of improper folding [18] and thus cannot be used to produce rhBMP-4 protein for therapeutic purposes. The proper folding structure of rhBMP-4 seems to be very important for its biological activity because the protein is known to have a highly folded structure, the cysteine knot [25]. Mammalian expression systems have been reported to produce the biologically active rhBMP-4; however, the highest level of expression so far reported is 4–5 mg/l [14], which is too low for the production of rhBMP-4 for therapeutic purposes. Although no reports have been presented on the importance of glycosylation in the functions of rhBMP-4, the production of mature rhBMP-4 is needed for therapeutic purposes; that is, mature rhBMP-4 is most similar to the native BMP-4 in the human body. We report the first demonstration of the production of large quantities of functionally active rhBMP-4 by CHO cells. Furthermore, no raw materials of animal origin were used during the development, from the establishment of the rhBMP-4-expressing rCHO cell line to the purification process. Therefore, the manufacturing process developed herein is applicable to establish the industrial production of rhBMP-4 for medical uses, although the culture and purification process described require successful scale up and further refinement, respectively.

Acknowledgments

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