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Expression, purification, and refolding of a recombinant human bone morphogenetic protein 2 in vitro

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ABSTRACT

In this work, the recombinant human bone morphogenetic protein 2 (rhBMP-2) gene was cloned from MG-63 cells by RT-PCR, and the protein was expressed in *Escherichia coli* expression system, purified by Ni–NTA column under denaturing conditions and refolded at 4 °C by urea gradient dialysis. We found that the protein refolding yield was increased with the increase of pH value from pH 6.0 to pH 9.0. The yield was 42% and 96% at pH 7.4 and pH 9.0, respectively, while that at pH 6.0 was only 3.4%. The cell culture results showed that the rhBMP-2 refolded at pH 7.4 urea gradient dialysis had higher biological activity for MG-63 cell proliferation and differentiation than that refolded at pH 9.0 since pH 7.4 is closer to the conditions in vivo leading to the formation of dimers through the interchain disulfide bond. Moreover, the biological activity for MG-63 was promoted with the increase of rhBMP-2 concentration in the cell culture medium. This work may be important for the in vitro production and biomedical application of rhBMP-2 protein.

Introduction

Bone morphogenetic proteins (BMPs)[1] are the secreted component which belong to the transforming growth factor-β (TGF-β) superfamily and were initially isolated from the demineralized bone and bone extracts by Urist et al. [1–3]. In 1988, Wozney et al. [4] isolated the human cDNA clones corresponding to the BMPs, and expressed BMP-1, BMP-2 and BMP-3 in vitro which appeared to be independently capable of inducing the formation of cartilage in vivo. BMPs have been found to regulate cell adhesion, proliferation, differentiation, and apoptosis in a wide variety of tissues including bone [5–10]. In the regenerative medicine, BMPs are delivered to the site of the fracture, by being incorporated into the implant, which release the BMPs slowly and gradually, to allow the bone regeneration. Currently, BMP-2 has been approved by the Food and Drug Administration (FDA) for clinical applications (e.g., fracture of long bones, intervertebral disk regeneration), by delivery in a purified collagen matrix (which is implanted in the site of the fracture) [11,12]. By controlling the release from the matrix, BMP-2 could enhance the recruitment of osteogenic progenitor cells for generation of bone tissue [13], and promote the repairing of fracture [14]. In addition, BMP-2 was found to inhibit the tumor growth and induce the bone differentiation of stem cells [15].

BMPs are translated in vivo as the large preproproteins consisting of a signal peptide, prodomain, and mature domain. After removal of the signal peptide, the proproteins undergo dimerization, and then the specific proteolytic enzymes cleave the dimerized proprotein to generate the biologically active dimeric mature protein [16]. BMPs can been isolated directly from the bones, the yield however is very limited [17,18]. Also, the potential health risk associated with their isolation from allogeneic donor bone limits their clinical application [19]. BMPs are now produced using recombinant DNA technology. For example, the recombinant human BMPs (rhBMPs) were produced by BMP gene-transfected mammalian cell (CHO) cultures [16,20]. Although the eukaryotic expression system does not require the renaturation, the post-translational problem (incomplete monomer processing) and the low yield (ng/mL scale) are usual in these processes. Similar problems occurred during the production of rhBMP-2 in the virus infected insect cells [21]. The production of biologically active rhBMPs through in vitro refolding of *Escherichia coli* (*E. coli*) produced inclusion bodies has been reported [22,23]. However, the refolding procedure was complicated and the overall yield was low. The rhBMP-2 could be expressed as a soluble protein in *E. coli* [24] , however, the product was initially found in the insoluble pellet (fraction corresponding bacterial debris) and required solublization through alkaline lysis. In this work, we reported a prokaryotic expression system to produce the rhBMP-2 by isolating the

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[1] *Abbreviations used:* BMPs, bone morphogenetic proteins; TGF-β, transforming growth factor-β; FDA, food and drug administration; IPTG, isopropyl-D-thiogalactoside; FBS, fetal bovine serum; ALP, alkaline phosphatase.

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cDNA from the human osteosarcoma cell MG-63. The purpose is to develop an efficient method of purification and refolding to obtain a soluble form of rhBMP-2 with biological activity.

Materials and methods

Cloning of rhBMP-2 gene

Human cDNA encoding BMP-2 (GenBank Accession No. NM 001200) was cloned by reverse transcription-PCR (RT-PCR). Total RNA was isolated from the cultured human osteosarcoma cell MG-63 by Trizol (Invitrogen Corp., USA), and was reverse transcribed by RevertAid First Strand cDNA Synthesis Kit (Fermentas, Lithuania). The primers were designed to amplify the entire coding sequence of the full-length BMP-2 cDNA starting from the start codon, ATG, and ending with the termination codon, TAG. For the cloning purpose, the sense primer contained an *Eco*RV restriction site and had the sequence of 5′-TAGGATATCATGGTGGCCGG GACCCG-3′. The antisense primer contained a *Hin*dIII restriction site and had the sequence of 5′-TGCAAGCTTCTAGCGACACCCA-CAACCCTCC-3′. The amplified product with a size of 1.2 kbp was cloned into the pMD-18T vector (Takara Bio. Inc., Japan) and the recombinant plasmid was named pMD-18T-BMP2.

The coding region of the mature BMP-2 was amplified from the recombinant plasmid pMD-18T-BMP2 by PCR (sense primer, 5′-TAGGATATCCAAGCCAAACACAAACAG-3′; antisense primer, 5′-TGCAAGCTTCTAGCGACACCCACAACCCTCC-3′; amplicon = 363 bp). The PCR product was cloned into pMD-18T vector and digested with *Eco*RV and *Hin*dIII, which was then inserted into the corresponding site of expression vector pET30a (+) to generate pET30a-BMP2.

Expression and purification of rhBMP-2

The pET30a-BMP2 was transformed into *E. coli* strain BL21 (DE3). The transformants were grown at 37 °C in LB culture medium (25 μg/mL kanamycin) and induced with 0.08 mM isopropyl-D-thiogalactoside (IPTG) for another 6 h at 37 °C. The rhBMP-2 was expressed and the cells were harvested. Two grams of wet cells were resuspended in 40 mL of PBS (Phosphate Buffered Saline), and disrupted by sonication on ice. The cell lysate was centrifuged at 12,000 rpm for 30 min at 4 °C. Since all of the rhBMP-2 was found in the inclusion body, the insoluble fraction (i.e., inclusion body) from the lysate was resuspended in a solubilization buffer (8 M urea, 0.1 M Na_2HPO_4, 0.01 M Tris–Cl, pH 8.0) at 5 mL per gram of wet inclusion body, and centrifuged at 12,000 rpm for 30 min to pellet the cellular debris. Ni–NTA column was used to purify the rhBMP-2 from the supernatant of solubilized inclusion body. 1 mL of 50% Ni–NTA slurry was added into 4 mL supernatant, which was then mixed gently by shaking at 4 °C for 60 min. The mixture was loaded onto the Ni–NTA column and washed twice with 20 mL wash buffer (8 M urea, 0.1 M Na_2HPO_4, 0.01 M Tris–Cl, 50 mM imidazole, pH 6.3), followed by eluting with 2 mL elution buffer (8 M urea, 0.1 M Na_2HPO_4, 0.01 M Tris–Cl, 250 mM imidazole, pH 5.9). 12% SDS polyacrylamide gels were used for both non-reducing and reducing SDS–PAGE.

Refolding of denatured rhBMP-2 in vitro

The rhBMP-2 was refolded by four different methods: by conventional dilution refolding procedure; by urea gradient dialysis with pH 6.0 refolding buffer; by urea gradient dialysis with pH 7.4 refolding buffer; and by urea gradient dialysis with pH 9.0 refolding buffer. In the urea gradient dialysis, the refolding buffers included Dialysate I (6 M urea, 0.1 M NaCl, 0.02 M Tris–Cl, 5 mM EDTA, 0.05% β-mercaptoethanol), Dialysate II (4 M urea, 0.1 M NaCl, 0.02 M Tris–Cl, 5 mM EDTA, 0.05% β-mercaptoethanol) and Dialysate III (2 M urea, 0.1 M NaCl, 0.02 M Tris–Cl, 5 mM EDTA, 0.05% β-mercaptoethanol). For each step, rhBMP-2 elution (containing 32 mg protein) was dialyzed at 4 °C in the buffer (500 mL) for 6 h. After the refolding process, the samples were centrifuged at 12,000 rpm for 30 min and the supernatant was analyzed with a Coomassie Brilliant Blue-stained 12% SDS–PAGE. The protein concentration was determined by BCA Protein Assay Kit (Biocolor Ltd., UK) standardized with bovine serum albumin. The refolding yield was calculated as a percentage of the soluble target protein after refolding against the total target denatured protein before refolding.

Cell proliferation and differentiation

MTT assay

The effect of rhBMP-2 on the MG-63 cell proliferation was assessed with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, which measures the cell growth by measuring the mitochondrial function. The MG-63 cells were seeded at a density of 1×10^4 cells/well in the 96-well plates and maintained in DMEM medium containing 10% fetal bovine serum (FBS) (Gibco, USA). After 24 h of incubation, the cells were washed with PBS buffer three times and refreshed with DMEM containing 0.5% FBS with or without rhBMP-2. After 1 d, 3 d, 5 d, and 7 d of culture, the cell growth was determined by MTT assay. The absorbance was measured at 570 nm using a spectrophotometric microplate reader (Model 680, Bio-Rad, USA). For comparison, the blank (i.e., medium only) were also used for the cell culture.

Alkaline phosphatase (ALP) activity

To detect the cell differentiation induced by the rhBMP-2, the MG-63 cells were seeded at a density of 1×10^5 cells/well in the 12-well plates. After 24 h of incubation in DMEM containing 10% FBS, the cells were washed with PBS buffer and refreshed with DMEM containing 0.5% FBS with or without rhBMP-2. After 1 d, 3 d, 5 d, and 7 d of culture, the ALP activity in the cell lysates was measured based on the conversion of colorless *p*-nitrophenyl phosphate into colored *p*-nitrophenol (JianCheng Biotech., China). The color intensity was measured at 520 nm using the UV–Vis spectrophotometer (DU530, Beckman Coulter, USA). The amount of ALP was quantified by the comparison with a standard sample.

RT-PCR and ELISA analyses

After the stimulation of rhBMP-2 for 5 d, the total cellular RNA was isolated from MG-63 cells using Trizol (Invitrogen Corp., USA). The mRNA was reverse transcribed into cDNA with RevertAid First Strand cDNA Synthesis Kit (Fermentas, Lithuania). The primers were designed to amplify the entire coding sequence of full-length osteocalcin (GenBank Accession No. NM 199173). The sequence of sense primer was 5′-ATGAGAGCCCTCACACTCCTCG-3′, and the sequence of antisense primer was 5′-CTAGACCGGGCCGTAGAAGCG-3′. After the PCR process, the products were separated on 1% agarose gels containing ethidium bromide, and were photographed under UV light. Also, the cultured supernatants were collected and used to determine the amount of secreted osteocalcin by human osteocalcin/bone gla protein (OT/BGP) ELISA kit (Uscnlife Co., USA).

Results

Cloning of rhBMP-2 gene

We cloned the human BMP-2 gene from the human osteosarcoma cell MG-63 by RT-PCR and constructed the PCR product into

the pMD-18T vector. The coding region of the mature BMP-2 was amplified from the plasmid pMD-18T-BMP2, and cloned into the expression vector pET30a (+) to generate the pET30a-BMP2. The plasmid was confirmed by the double restriction enzyme digestion of pET30a-BMP2 with *Eco*RV and *Hin*dIII and PCR (Fig. 1), showing that the coding region of the mature BMP-2 gene was 345 bp as expected. The result was also confirmed by sequencing (data not shown).

Expression and purification of rhBMP-2

The expression host *E. coli* strain BL21 (DE3) underwent transformation with the plasmid pET30a-BMP2. After induction with IPTG, the rhBMP-2 was expressed at a expected band of about 20 kDa (with $[His]_6$ tag sequences) from the SDS–PAGE result (Fig. 2). Note that all of the rhBMP-2 was found in the inclusion body, and none in the soluble fraction after the cell disruption. To isolate the rhBMP-2, the bacterial cells were collected and disrupted with ultrasonication. The inclusion bodies were obtained by centrifugation and dissolved in the solubilization buffer. The cell debris was removed by centrifugation and the solubilized protein was purified by Ni–NTA column under the denaturing condition. After purification, a single band at around 20 kDa was observed (Fig. 3) from the SDS–PAGE result, agreeing well with the bands in Fig. 2. The yield of rhBMP-2 was 58 mg per liter of medium and about 4.8 mg per gram of wet cells.

In vitro refolding of rhBMP-2

To achieve the active rhBMP-2, the obtained protein after Ni–NTA purification was refolded by a conventional dilution refolding procedure. But the yield was very low, and no protein was detected by the SDS–PAGE (data not shown). So urea gradient dialysis was used. The conditions play a key role for the refolding of recombinant proteins, and lower temperature, e.g., 4 °C, is usually more effective than higher temperature [25]. Therefore, the refolding of rhBMP-2 was carried out at 4 °C at varied pH values, i.e., pH 6.0, pH 7.4, and pH 9.0. Fig. 4 showed the SDS–PAGE results of rhBMP-2 refolded at above conditions, and the refolding yields were summarized in Table 1. The data showed that the refolding yield at pH 9.0 was on the maximum level with 96%, while that at pH 7.4 and pH 6.0 was 42% and 3.4%, respectively.

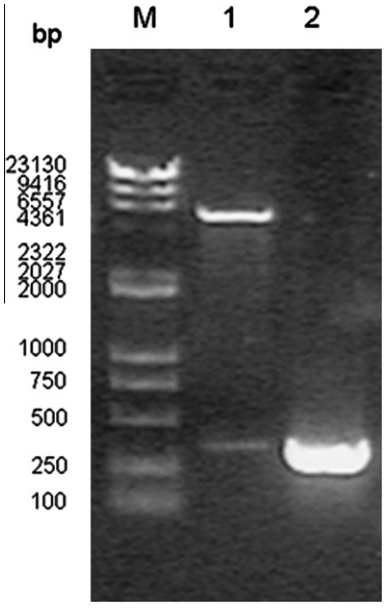

Fig. 1. Agarose gel electrophoresis analysis of BMP-2 gene. Lane M, DNA marker; Lane 1, *Eco*RV and *Hin*dIII double restriction enzyme digestion of pET30a-BMP2; Lane 2, amplified BMP-2 gene by PCR.

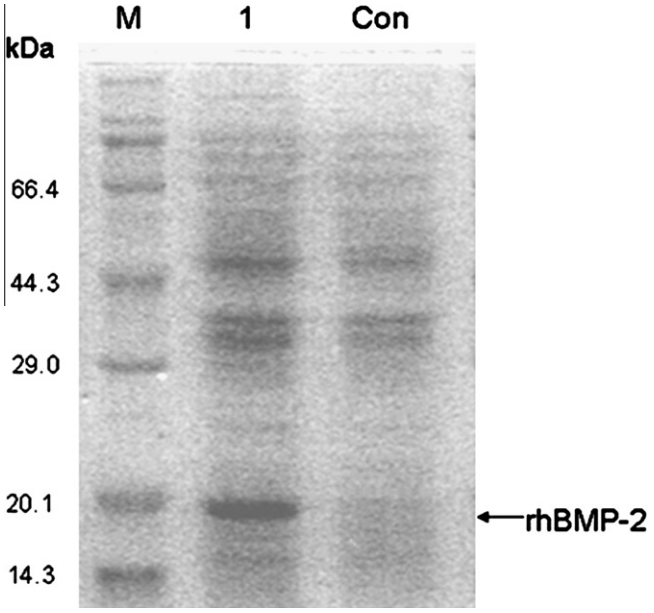

Fig. 2. SDS–PAGE analysis of the expression level of rhBMP-2 in *Escherichia coli* BL21(DE3). Lane M, Broad range protein molecular weight markers; Lane 1, induced total bacterial protein by 0.08 mM IPTG for 6 h; Lane Con, uninduced total bacterial protein.

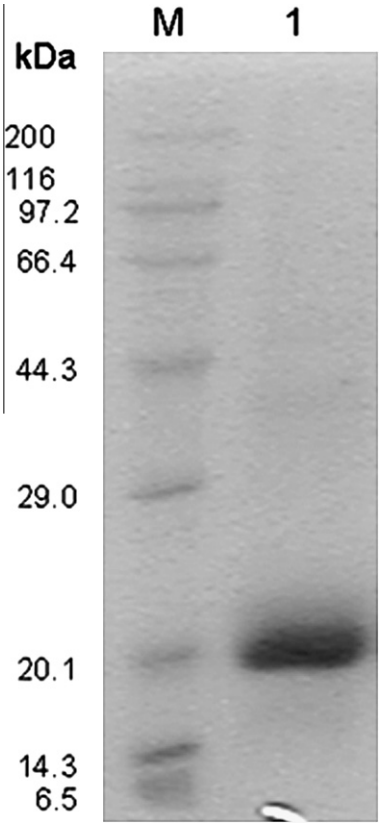

Fig. 3. SDS–PAGE analysis of purified rhBMP-2 by Ni–NTA column under denaturing conditions. Lane M, Broad range protein molecular weight markers; Lane 1, Purified rhBMP-2.

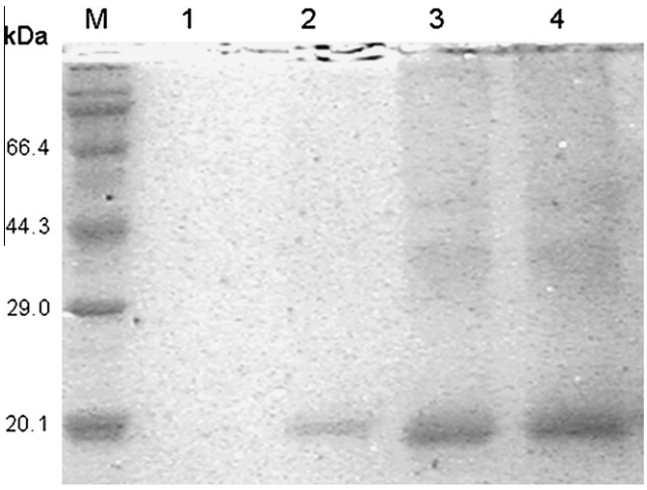

Fig. 4. SDS–PAGE analysis of rhBMP-2 refolding. Lane M, Broad range protein molecular weight markers; Lane 1, rhBMP-2 refolded at pH 6.0; Lane 2, rhBMP-2 refolded at pH 7.4; Lane 3, rhBMP-2 refolded at pH 9.0; Lane 4, denatured rhBMP-2 before refolding.

Table 1
Comparison of rhBMP-2 refolding yield by using different refolding buffers.

pH value of refolding buffer	Denatured protein (mg)	Refolded protein (mg)	Refolding yield (%)
pH 6.0	32	1.1	3.4
pH 7.4	32	13.5	42
pH 9.0	32	30.6	96

Effect of the refolded rhBMP-2 on cell proliferation

The rhBMP-2 proteins refolded at pH 9.0 and pH 7.4 as described above were used for cell culture. Fig. 5 showed the effect of refolded rhBMP-2 on the MG-63 cell proliferation by MTT assay. The MG-63 cells were cultured in 96-well plates and cultured for 1 d, 3 d, 5 d, and 7 d with different rhBMP-2 concentrations (20 μg/mL and 30 μg/mL). The control group was the cell cultured without rhBMP-2. As expected, the rhBMP-2 significantly stimulated the cell growth at 1 d of culture. With the increase of culture time, no significant difference was observed by varying the rhBMP-2 concentration and the rhBMP-2 refolded at pH 9.0 showed the lower activity for cell proliferation.

Effect of the refolded rhBMP-2 on cell differentiation

Alkaline phosphatase (ALP) activity is an important osteogenesis marker and is assumed to reflect the degree of osteogenic differentiation. Fig. 6 showed the ALP activity of MG-63 cells cultured in 12-well plates and stimulated for 1 d, 3 d, 5 d, and 7 d with different rhBMP-2 concentrations (20 μg/mL and 30 μg/mL). The rhBMP-2 refolded at pH 7.4 had a significant high activity when its concentration was 30 μg/mL.

Effect of the refolded rhBMP-2 on the osteocalcin mRNA expression and osteocalcin secretion

As a marker for the bone formation, the osteocalcin is a secreted protein in the bone, and plays an active role in the bone mineralization [26]. To determine the osteocalcin mRNA expression, the MG-63 cells were also cultured in 12-well plates and stimulated with different rhBMP-2 concentrations. The RT-PCR results were shown in Fig. 7A where the mRNA transcript of osteocalcin in the cells was stimulated by rhBMP-2, showing that the osteocalcin was amplified with the increase of rhBMP-2 concentration from 20 μg/mL to 100 μg/mL. The integrity of mRNA was confirmed by the amplification of actin mRNA. To further investigate the effect of rhBMP-2 on the secretion of osteocalcin, we measured the amount of secreted osteocalcin in the supernatants after the cells were cultured for 1 d, 3 d, 5 d and 7 d with different concentrations of rhBMP-2 refolded at pH 7.4 (Fig. 7B). The results showed that there was no significant difference among the different rhBMP-2 concentrations on the secretion of osteocalcin at 1 d of cell culture. However, due to the rhBMP-2 stimulation, the osteocalcin was significantly increased with the culture time and the rhBMP-2 concentration. The rhBMP-2 refolded at pH 7.4 could significantly promote osteocalcin mRNA expression and osteocalcin secretion.

Discussion

In this study, we cloned the human BMP-2 gene from MG-63 cells by RT-PCR and transformed it into *E. coli* strain BL21 (DE3) using pET30a (+) vector system. After induction by IPTG, the rhBMP-2 was produced, which was localized in an insoluble, inactive form in inclusion body. We tried to optimize the conditions for the prokaryotic expression system to improve the soluble expression of rhBMP-2, including the culture temperature, carbon source, IPTG concentration, and culture temperature. But we failed to

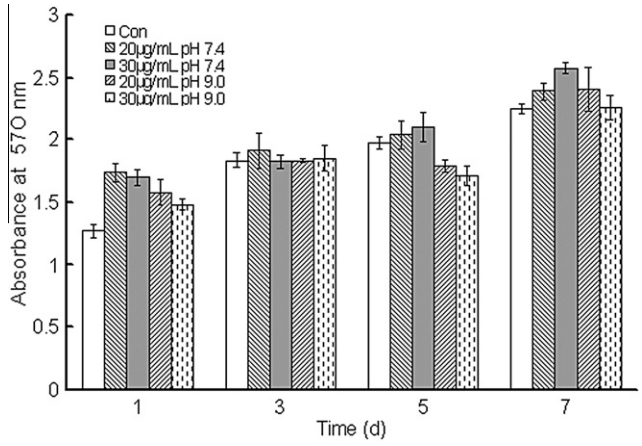

Fig. 5. MTT assay of MG-63 cultured with varied concentrations of rhBMP-2 refolded at different pH values. The control group means the cells cultured without rhBMP-2. The data reported were the mean of 9 examinations.

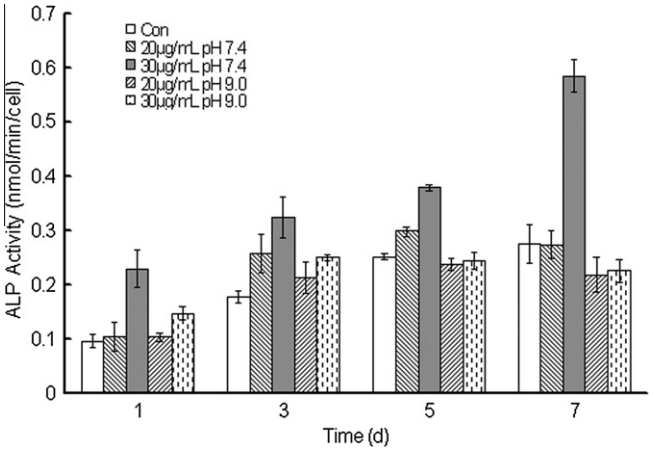

Fig. 6. ALP activity expressed by MG-63 cultured with varied concentrations of rhBMP-2 refolded at different pH values. The control group means the cells cultured without rhBMP-2. The data reported were the mean of 9 examinations.

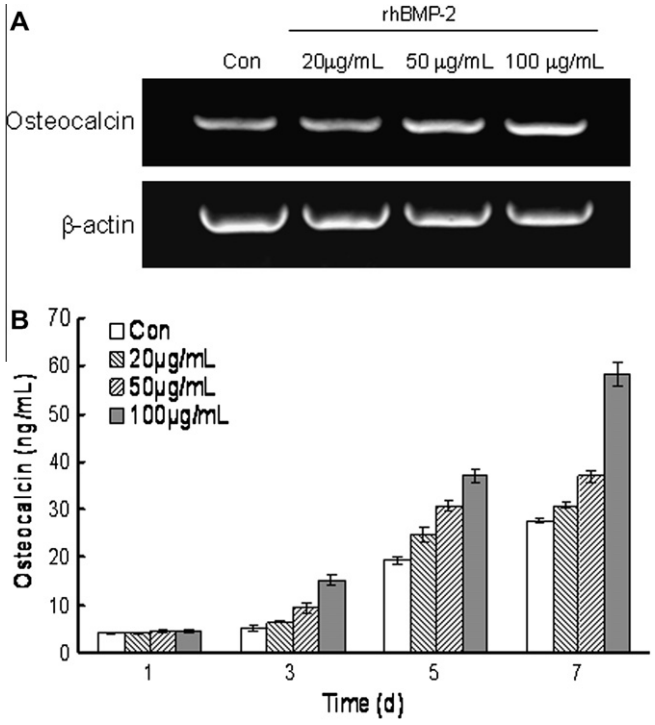

Fig. 7. (A) Agarose gel electrophoresis analysis of osteocalcin mRNA expression after MG-63 cells were cultured with varied concentrations of rhBMP-2 refolded at pH 7.4 for 5 d followed by RT-PCR using specific primers for osteocalcin and β-actin. (B) Osteocalcin production by MG-63 cultured with varied concentrations of rhBMP-2 refolded at pH 7.4. The control group means the cells cultured without rhBMP-2. The data reported were the mean of 9 examinations.

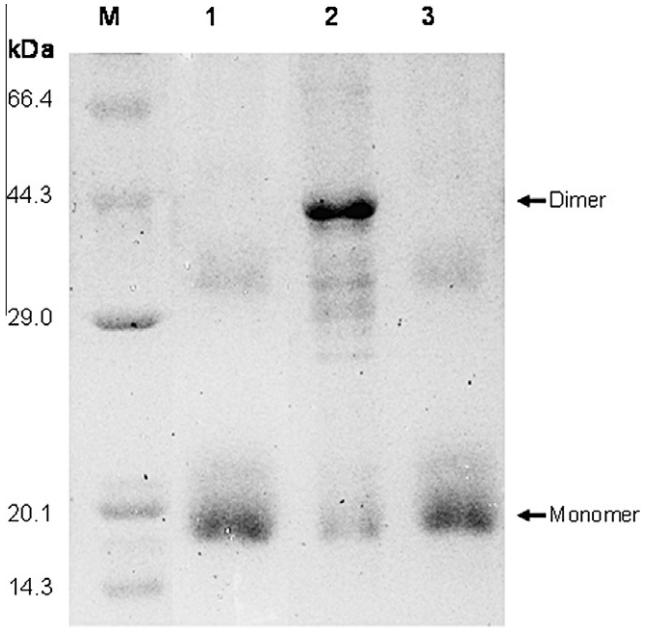

Fig. 8. Non-reducing SDS–PAGE analysis of rhBMP-2. Lane M, Broad range protein molecular weight markers; Lane 1, rhBMP-2 refolded at pH 9.0; Lane 2, rhBMP-2 refolded at pH 7.4; Lane 3, rhBMP-2 refolded at pH 9.0 followed by dialysis at pH 7.4.

obtain any soluble rhBMP-2 (data not shown). So the inclusion body was harvested and solubilized in the solubilization buffer and the denatured rhBMP-2 was purified by Ni–NTA column with high purity and high yield. In the prokaryotic expression system, refolding is critical to obtain the active rhBMP-2 by removing the denaturants. Here, we used different approaches to refold the rhBMP-2, including the conventional dilution refolding procedure, and the urea gradient dialysis. The yield of conventional dilution refolding procedure was very low, so we used the urea gradient dialysis to refold the rhBMP-2. Since the lower temperature, e.g., 4 °C, was more effective for protein refolding than the high temperature [25], we analyzed the effect of pH value of refolding buffer on the refolding yield at 4 °C. The results showed that the yield was increased with the increase of pH value from pH 6.0 to pH 9.0. The refolding yield was 42% and 96% at pH 7.4 and pH 9.0, respectively, while that at pH 6.0 was 3.4% only. This may be due to the fact that the pI value of rhBMP-2 is about 6.6, which led to the low solubility of rhBMP-2 at pH 6.0.

Since the biological activity of rhBMP-2 is the key indicator for the successful protein refolding, we compared the cell proliferation and differentiation of rhBMP-2 refolded at pH 7.4 and pH 9.0. The data showed that the rhBMP-2 refolded at pH 7.4 had higher biological activity than that refolded at pH 9.0, since pH 7.4 is closer to the conditions in vivo, and the soluble rhBMP-2 may form the dimers through the interchain disulfide bond during the refolding process, which was proved by the non-reducing SDS–PAGE results. Fig. 8 showed that the rhBMP-2 refolded at pH 7.4 had a dominant band at about 40 kDa most likely representing dimers in addition to the monomers at about 20 kDa, while the rhBMP-2 refolded at pH 9.0 had a single band at about 20 kDa. Meanwhile, the rhBMP-2 refolded at pH 9.0 followed by dialysis at pH 7.4 also showed a single band at about 20 kDa, suggesting that the rhBMP-2 dimers were primarily formed at the refolding process. The BMP-2 dimers possess a higher level of activity than monomers [22]. Also we found that the cell proliferation was promoted by increasing the rhBMP-2 concentration, and the rhBMP-2 modulated the osteocalcin mRNA expression and the osteocalcin secretion, which agreed well with the results reported by others [27,28]. However, when compared with the high activity of BMP-2 in nanogram level reported previously [22], the activity of rhBMP-2 synthesised in this work was still low. After removing the $[His]_6$ tag sequence in the next step, more detailed refolding investigation is necessary in order to synthesize the rhBMP-2 with high activity.

Acknowledgments

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