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Recombinant Human Bone Morphogenetic Protein-2: Optimization of Overproduction, Solubilization, Renaturation and Its Characterization

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Abstract: A codon-optimized synthetic gene encoding recombinant human Bone Morphogenetic Protein 2 (rhBMP-2) fused to thioredoxin-6x-histidine tag at its amino terminus was previously constructed and the recombinant product as a monomer expressed in *Escherichia coli* BL21(DE3) was confirmed by nano-LC mass spectrometry (LC-MS/MS2) analysis. In this study, we optimized the conditions for overproduction, solubilization of Inclusion Bodies (IB) and dimerization of rhBMP-2 monomer. Overproduction was optimized at various isopropyl- β -D-thiogalactopyranoside concentrations and incubation temperatures. Different kinds of buffer at pH 8.0 to 9.0 were applied for optimization of solubilization and dimerization. Enterokinase cleavage, sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western Blot analyses were applied in protein characterization. The activity of rhBMP-2 dimer was determined by production of alkaline phosphatase on C2C12 cells. Under the optimal condition found in this study, 11.5 g cell wet weight or 0.6 g rhBMP-2 monomer per L culture was produced. The soluble monomer of 64 mg was resulted from 200 mg of IB using buffer containing 4 M urea, 0.1 M NaCl, 0.02 M tris-HCl, pH 8.0, 5 mM EDTA and 5 mM dithiothreitol. The dimer to monomer ratio of 4:5 was resulted from dimerization using buffer containing 4 M urea, 100 mM tris-HCl, 5 mM EDTA. The rhBMP-2 cleaved by enterokinase gave correct protein fragments, was recognized by monoclonal antibody against rhBMP-2 and the protein was proved to be biologically active. In conclusion, this study we provide evidence that after optimized solubilization and renaturation steps, rhBMP-2 fusion protein expressed from synthetic gene was biologically active.

Key words: Bone-morphogenetic protein-2, fusion protein, solubilization, dimerization

INTRODUCTION

Recombinant human Bone morphogenetic protein 2 (rhBMP-2) and animal or human bone-derived BMP-2 can induce differentiation of osteoprogenitor cells into mature osteoblasts both *in vitro* and *in vivo* (Wozney, 1992; Um *et al.*, 2006; Ishikawa *et al.*, 2007; Bessa *et al.*, 2008). Due to its osteoinductive capability, rhBMP-2 has been used to treat bone and periodontal defects (King *et al.*, 1997; Kirker-Head, 2005; Schmoekel *et al.*, 2005), regenerates dentin (Iohara *et al.*, 2004) and suppresses cancer cells (Arnold *et al.*, 1999; Kawamura *et al.*, 2000). One limitation of using rhBMP-2 for therapeutic purposes is relatively expensive for its production. However, efforts for overproduction of rhBMP-2 have been reported in prokaryotic expression system to obtain pure and biologically active protein. The yield of active rhBMP-2 was varied depending on a number of variables such as

vector and host expression systems, production conditions, protein isolation methods, refolding, dimerization and purification steps (Vallejo *et al.*, 2002; Vallejo and Rinas, 2004; Long *et al.*, 2006; Zhang *et al.*, 2010, 2011; Von Einem *et al.*, 2010). The condition of production for the cultivation of recombinant protein in *Escherichia coli* is significantly influenced by several parameters such as carbon and nitrogen sources and their concentrations, temperature, pH and oxygen consumptions. Carbon source is obtained usually from glucose or glycerol, while peptone or yeast extract is used as nitrogen source (Schmidt, 2004; Graumann and Premstaller, 2006; Tripathi *et al.*, 2009). Other components are also added to increase the production of recombinant proteins such as NaCl (Bhandari and Gowrishankar, 1997) or isopropyl- β -D-thiogalactopyranoside (IPTG) (Roy *et al.*, 2007; Guillen *et al.*, 2007) as inducers depending on the induction systems (Tripathi *et al.*, 2009).

In *E. coli* system, human proteins with disulfide bond are commonly produced as insoluble proteins in denatured or inactive state and forms IB (Schmidt, 2004; Graumann and Premstaller, 2006). The high recovery of soluble monomer from IB can be achieved using buffer at alkaline pH containing chaotropic agents such as urea or guanidine-HCl. Other agents are also added to increase the efficiency of IB solubilization such as detergents (sodium dodecyl sulfate or SDS, Triton X-100) and reducing agents (dithiothreitol or DTT, β -mercaptoethanol) (Patra *et al.*, 2000; Singh and Panda, 2005; Freydel *et al.*, 2007). Some previous studies have developed buffers for rhBMP-2 IB solubilization (Vallejo *et al.*, 2002; Vallejo and Rinas, 2004; Long *et al.*, 2006; Zhang *et al.*, 2010; Von Einem *et al.*, 2010; Zhang *et al.*, 2011). The yield of solubilization was not explicitly reported (Long *et al.*, 2006; Zhang *et al.*, 2010; Zhang *et al.*, 2011) except in one study (Von Einem *et al.*, 2010) but the purity of more than 60% of the solubilized rhBMP-2 was mostly achieved (Long *et al.*, 2006; Zhang *et al.*, 2010, 2011).

The rhBMP-2 in denaturing condition is in monomeric form, while native BMP-2 is active as homodimer (Scheufler *et al.*, 1999). All published studies reported that refolding and dimerization or in brief renaturation is a prerequisite step to obtain bioactive BMP-2 (Vallejo *et al.*, 2002; Vallejo and Rinas, 2004; Long *et al.*, 2006; Zhang *et al.*, 2010; Von Einem *et al.*, 2010; Zhang *et al.*, 2011). Naturally, mature BMP-2 consists of 114 amino acids of monomeric BMP-2 and has seven cysteine residues. Six cysteines are responsible for folding of the monomeric molecule and the seventh cysteine forms a disulfide bridge that links the two monomers. The covalently linked dimer is critical for BMP-2 activity (Scheufler *et al.*, 1999; Hillger *et al.*, 2005). Previous studies demonstrated that renaturation yields of rhBMP-2 were varied and mainly influenced by different technique, the amount of protein, buffer compositions and pH of the renaturation process (Vallejo *et al.*, 2002; Vallejo and Rinas, 2004; Long *et al.*, 2006; Zhang *et al.*, 2010, 2011; Von Einem *et al.*, 2010). Then, alkaline pH was the best condition for BMP-2 renaturation (Vallejo and Rinas, 2004; Zhang *et al.*, 2011). One simplest technique used for small scale renaturation process is dilution method, i.e., unfolded protein is diluted directly or indirectly (in a dialysis membrane) in renaturation buffer (Singh and Panda, 2005; Jungbauer and Kaar, 2007).

Most of studies used complementary DNA (cDNA) for expressing rhBMP-2 in *E. coli* (Vallejo *et al.*, 2002; Vallejo and Rinas, 2004; Long *et al.*, 2006; Zhang *et al.*, 2010, 2011; Von Einem *et al.*, 2010). In our previous study, a codon-optimized Open Reading Frame

(ORF) encoding human BMP-2 was constructed and the gene product was overproduced in *E. coli* BL21(DE3), purified, partially characterized and confirmed to be rhBMP-2 by nano-LC mass spectrometry (LC-MS/MS2) analysis. The rhBMP-2 was overproduced as a mature monomeric BMP-2 at high level as a 30.6 kDa fusion protein with thioredoxin-6x-histidine (rhBMP-2-6His-trx). Optimization of rhBMP-2 overproduction of our construct in *E. coli* BL21(DE3) and optimization of solubilization and renaturation have not been previously done. Optimizations are required to be done prior to high scale production and as mentioned above it crucially needed to gain high yield of active rhBMP-2. Therefore, the aim of present study was to optimize the overproduction of our rhBMP-2 fusion protein in *E. coli* BL21(DE3), the solubilization of rhBMP-2 from IB, the dimerization of soluble monomeric form of rhBMP-2, to further characterize rhBMP-2 dimer using protein approach and to determine its biological activity using C2C12 cell line.

MATERIALS AND METHODS

Host strain and plasmids: A previously constructed recombinant pET32b plasmid carrying codon-optimized ORF of gene encoding human BMP-2 (pET32b-hBMP-2) maintained in *E. coli* BL21(DE3) was used in this work and was designated as *E. coli* (pET32b-hBMP2).

Optimization of recombinant *E. coli* growth and expression of hBMP-2: The growth of *E. coli* (pET32b-hBMP2) was studied in 20 mL scale using three liquid media i.e., Luria Bertani (LB), Terrific Broth (TB) and Super Optimal Broth (SOB) containing 100 $\mu\text{g mL}^{-1}$ ampicillin. The composition of each medium was made according to Sambrook and Russell (2001). The volumetric ratio between medium to flask was 1:5. A single colony from agar plate was inoculated to liquid medium and incubated for 18 h at 150 rpm 37°C. After overnight incubation, 3% of inoculum was transferred to fresh medium. To determine optimal incubation temperature, incubation was done in two temperatures, 28°C (room temperature) and 37°C. The expression of the rhBMP-2 ORF was induced by the addition of IPTG in three concentrations (0, 0.5 and 1 mM) to the culture at an Optical Density (OD) 600 nm of 0.6-0.7. After four hours of induction, cells were harvested by centrifugation at 4°C and 11000 g for 10 min. Cell pellets were stored at -20°C until used.

Cell pellet was added to 900 μL of protein loading buffer and 100 μL of 1 mM DTT (Sambrook and Russell, 2001). Resuspended cell pellet was heated in a water bath at 75°C for 10 min, cooled at room temperature and centrifuged at 11000 xg for 2 min. Ten microliters of

supernatant was analyzed by 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were stained with Coomassie Brilliant Blue. Bacterial growth or cell biomass was determined based on Cell pellet Wet Weight (cww).

Isolation and solubilization of inclusion bodies: To isolate IB, 406 mg of wet cells pellet was resuspended in 5 mL of lysis buffer containing 20 mM tris-HCl (pH 8.0), 500 mM NaCl and 0.1% Triton X-100 (Long *et al.*, 2006). Then, 1 mM of phenylmethanesulfonyl fluoride was added to the resuspension and the mixture was sonicated on ice using Ultrasonic homogenizer (Virsonic 300) at output power of 15% for 30 sec and repeated for 10 times with interval of 30 sec between sonication. Centrifugation was done to separate pellet and supernatant. Pellet or IB was stored at -20°C for further analysis or solubilized directly. Three solubilization buffers, i.e., buffer 1 (4 M urea, 0.1 M NaCl, 0.02 M tris-HCl, pH 8.0, 5 mM EDTA, 5 mM DTT) modified from (Long *et al.*, 2006), buffer 2 (6 M urea, 150 mM NaCl, 0.02 M tris-HCl, pH 7.5, 5 mM DTT) (Long *et al.*, 2006), or buffer 3 (6 M guanidine HCl, 0.1 M tris-HCl, pH 8.5, 1 mM EDTA, 0.1 M DTT (Von Einem *et al.*, 2010) was each used to determine the optimal recovery of soluble rhBMP-2. Two hundred milligrams of IB was dissolved in each solubilization buffer, stirred at 8°C for three hours, centrifuged at 9500 g, 4°C for 20 min. The soluble fraction was subjected to protein analysis.

Renaturation of rhBMP-2 fusion protein: Refolding of monomeric rhBMP-2 and its dimerization were done in the same renaturation buffer according to dialysis method (Bollag *et al.*, 1996). We used dissolved IB instead of affinity purified rhBMP-2 as a source for renaturation steps. Soluble monomer rhBMP-2 (12.93 mg) was dialyzed against renaturation buffer at 8°C and stirred for 72 h. The buffer contained 4 M urea, 100 mM tris-HCl, 5 mM EDTA and was adjusted to three different pH i.e., 8.0, 8.5 and 9.0. The ratio between protein solution and buffer was 1: 10 (v/v). The buffer was changed every 24 h or at least three times during renaturation.

Enterokinase cleavage of rhBMP-2 fusion protein: Our rhBMP-2 fusion protein contains enterokinase cleavage site that is located at its amino terminus between thioredoxin-6x-histidine tag and rhBMP-2. In order to confirm that the fusion protein could be cleaved by enterokinase to give a 12 kDa rhBMP-2, 1 U of enterokinase (EKMax, Invitrogen) was used in the reaction. Affinity purified rhBMP-2 for enterokinase cleavage was obtained from our previous study. The

cleavage steps were done as described in manufacturer protocol (Invitrogen).

Immunodetection of rhBMP-2 fusion protein: After SDS-PAGE separation, proteins were analyzed by Western blot to confirm the immunoreactivity of rhBMP-2 in monomeric, dimeric or enterokinase-cleaved forms with a monoclonal antibody against hBMP-2. Monoclonal anti BMP-2 (Sigma) was diluted 1: 2000 in phosphate buffer saline pH 7.4 containing 0.1% Tween 20 (PBS-T). Western blot was performed as described previously (Bollag *et al.*, 1996; Sambrook and Russell, 2001).

Activity Assay for rhBMP-2 fusion protein: Our preparation containing rhBMP-2 was tested for its biological activity using C2C12 cells (ATCC No. CRL-1772) according to previous method with minor modification (Katagiri *et al.*, 1994; Vallejo *et al.*, 2002). C2C12 cells were plated in 24-well plates at a density of 1×10^4 cells cm^{-2} and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Chemical Co.) supplied with 10% fetal bovine serum (Invitrogen), 5 mg mL^{-1} penicillin-streptomycin and 50 $\mu\text{g mL}^{-1}$ gentamicin. The medium was changed every 2-3 days. After reaching confluency (observed as day 0), the medium was changed by fresh DMEM (1 mL) containing rhBMP-2 at several amounts (125, 250, 375 and 500 ng) or without rhBMP-2. After 6 days of incubation, the cultured cell was fixed with 4% of formaldehyde for 10 min, washed with PBS and stained for Alkaline Phosphatase (ALP) expression using Fast red TR/mL naphthol AS-MX (Sigma Chemical Co.). For quantitative analysis, the presence of ALP activity on C2C12 myoblast cell was determined using spectrophotometer (Beckman DU-7000) at λ 405 nm. C2C12 cell lysate per well was added 10 mM p-nitrophenyl phosphate for 30 min and the reaction was stopped by the addition of 50 μL of 2 M NaOH. The yellow color produced at the end of reaction was measured as ALP activity (Katagiri *et al.*, 1994; Vallejo *et al.*, 2002). ALP of calf intestine (Roche Diagnostics GmBH, Germany) was used as a standard.

Protein and statistical analysis: The protein total concentration was determined by Bradford method using spectrophotometer (Beckman DU 7000) at λ 595 nm according to Bollag *et al.* (1996). Proteins were characterized using 15% SDS-PAGE, generally under reducing condition except for rhBMP-2 dimer under non-reducing condition (in the absence of reducing agent such as DTT). The amount of rhBMP-2 in gel was measured based on the intensity of 30.6 kDa protein band using ImageJ software Version 1.43 (Gallagher, 2010).

Bovine Serum Albumin (BSA) (Sigma) was used as a protein standard and the amount of rhBMP-2 was calibrated to linear regression of the amount of BSA.

All data from optimization experiments were obtained from three independent measurements, performed as mean and standard deviation and analyzed using Minitab16 software (license No. 16.1.1.1).

RESULTS

The growth of recombinant *E. coli* BL21(DE3): Studying bacterial growth key parameters in liquid culture is one strategy to improve the volumetric productivity of recombinant protein that can be calculated from cell biomass produced (Beshay *et al.*, 2003; Romano *et al.*, 2009; Tripathi *et al.*, 2009). The growths of *E. coli* (pET32b-hBMP-2) in LB, TB and SOB complex media and in the absence of IPTG are presented in Fig. 1a. The growth of recombinant *E. coli* was not significantly influenced by growth media and induction temperatures

($p < 0.05$). The result showed that the biomass production from bacterial growth in three media and two induction temperatures (28 and 37°C) without IPTG induction were similar. The yields were 9.93 g cww L⁻¹ culture, 9.90 g cww L⁻¹, 9.17 g cww L⁻¹ culture in SOB, TB and LB medium, respectively (Fig. 1a).

Since IPTG was used for induction of rhBMP-2 production, the growth of recombinant *E. coli* was also investigated in its presence. IPTG significantly influenced the growth of recombinant *E. coli* (pET32b-hBMP-2) ($p < 0.05$). The growth of *E. coli* (pET32b-hBMP-2) was best obtained in the presence of 0.5 mM IPTG, while the growth was inhibited by the addition of 1 mM IPTG (Fig. 1a). The highest biomass production after 4 h in the presence of 0.5 mM IPTG was 11.57 g L⁻¹ culture in SOB medium at temperature induction of 37°C. Therefore, the production of biomass obtained in the presence of IPTG increased almost 1.2 times than that in the absence of IPTG (9.93 g L⁻¹ medium) in SOB medium at temperature induction of 37°C (Fig. 1a).

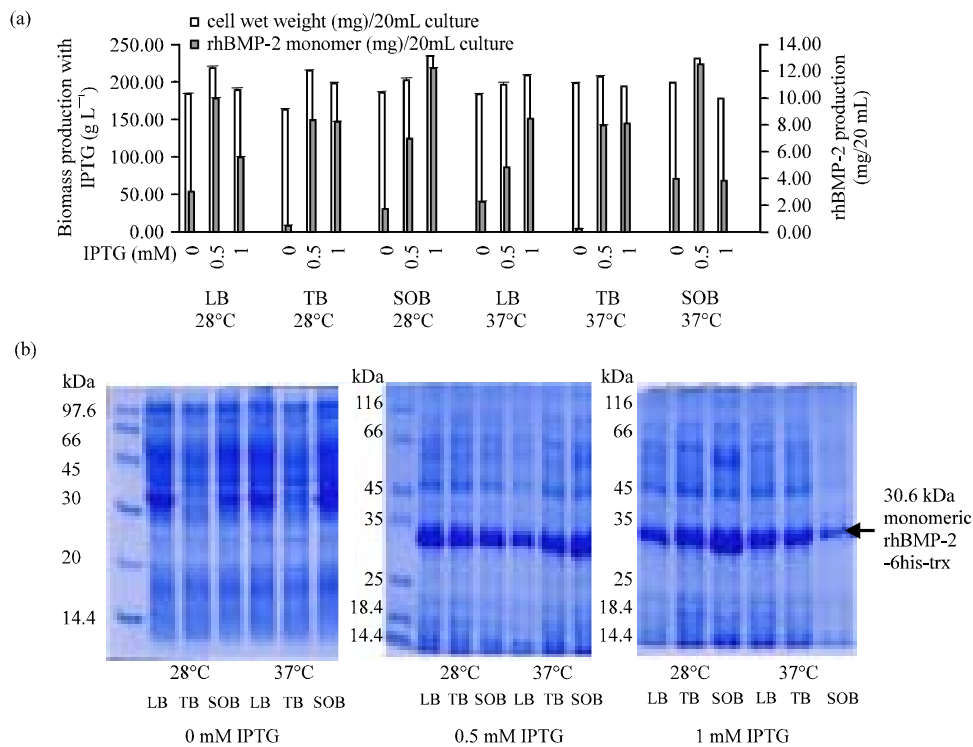


Fig. 1: The growth of *E. coli* BL21(DE3) harboring pET32b-hBMP-2 and production of its recombinant protein in 3 media, 2 incubation temperatures and with or without IPTG induction, (a) The growth was measured as amount of wet cell biomass, while recombinant protein production as rhBMP-2-6His-trx expressed and (b) Analysis of hBMP-2 ORF expression. Whole cells lysates were obtained after 4 hours IPTG induction. Total proteins were separated by 15% SDS-PAGE and stained by Coomassie Brilliant Blue. Luria Bertani (LB), Terrific Broth (TB) and Super Optimal Broth (SOB) liquid medium

Expression of hBMP-2: As mentioned in the introduction that there was varied yields in the production of rhBMP-2 in *E. coli* host. The yield can be controlled by several parameters such as medium, temperature, or inducer. The study of recombinant protein expression at small scale is a prerequisite to understand expression control parameters or to measure protein recovery at final step of production (Schmidt, 2004; Graumann and Premstaller, 2006; Tripathi *et al.*, 2009). Therefore, in this study, we also reported the expression of hBMP-2 in *E. coli* (pET32b-hBMP-2) in the optimized condition aforementioned.

Like the biomass productivity, the amount of monomeric rhBMP-2 fusion protein expressed in our recombinant *E. coli* in the absence of IPTG was also not significantly affected by growth media and induction temperatures (Fig. 1). The rhBMP-2 yield in the absence of IPTG reached 0.2 g L⁻¹ medium. On the other side, IPTG significantly induced expression of hBMP-2 on *E. coli* (pET32b-hBMP-2). Compared to 1 mM IPTG, the induction of 0.5 mM IPTG significantly increased the rhBMP-2 production in our recombinant *E. coli* (p<0.05). The highest yield of hBMP-2 expression in the presence of 0.5 mM IPTG was 0.62 g L⁻¹ in SOB medium at induction temperature of 37°C (Fig. 1).

Solubility of rhBMP-2 from IB in three kinds of buffer composition:

Like most of other studies (Vallejo *et al.*, 2002; Long *et al.*, 2006; Zhang *et al.*, 2010, 2011; Von Einem *et al.*, 2010), majority of our rhBMP-2 was also present in IB. To achieve high recovery of bioactive protein and an effective way in the production of recombinant proteins expressed in IB form, solubilization is one of crucial steps (Patra *et al.*, 2000; Singh and Panda, 2005; Freydel *et al.*, 2007). Here, we studied the solubilization of IB of rhBMP-2 using three kinds of buffer and one centrifugation strategy to gain high recovery of soluble monomer rhBMP-2 fusion protein. Buffer 1 was the best buffer, while buffers 2 and 3 have similar effect on IB solubilization (p<0.05). Based on our preliminary result, the yield of soluble rhBMP-2 fusion protein from IB was 0.19 g L⁻¹ culture. Using buffer 1, soluble monomer was obtained 0.4 g per Liter of culture or 0.3 g g⁻¹ IB (Table 1, Fig. 2). The optimization of solubilization buffer increased only two times of yield of soluble rhBMP fusion protein.

Renaturation of monomeric rhBMP-2 at various pH:

Zhang *et al.* (2011) reported that *in vitro* renaturation of rhBMP-2 using refolding buffers of pH 6.0, pH 7.4 and pH 9.0 gave the highest yield when using buffer of pH 9.0. On the other side, refolded BMP-2 at pH 7.4 had the

highest activity than those at pH 6.0 and pH 9.0. Here, we studied the activity of our refolded rhBMP-2 in the range of pH 7.4 and 9.0, i.e. in three renaturation conditions of pH (pH 8.0, pH 8.5 and pH 9.0) for comparing our yield to previous study (Zhang *et al.*, 2011). As reported earlier, initially we used affinity purified rhBMP-2 protein for renaturation but the yield of dimer was low. Furthermore, it required several times to concentrate the protein, hence it was time consuming and led to protein degradation (data not shown). Meanwhile solubilization result revealed that the homogeneity of rhBMP-2-6His-Trx was above 80% (Fig. 2b). Therefore, we used soluble monomer rhBMP-2 as protein source of renaturation.

The result showed that the pH of renaturation buffer significantly affect the yield of renaturation (p<0.05). The renaturation buffer at pH 8.5 was the best condition to dimerize monomer rhBMP-2. The amount of dimer after renaturation in buffer pH 8.5 was 3.5 fold higher (0.2 g L⁻¹) than that of before renaturation. However, renaturation still resulted in a mixture of dimer and monomer (Fig. 3). The ratio of dimer to monomer was 5:4 (w/w). The yield of renaturation in buffer pH 8.0 and pH 9.0 was similar 0.1 g L⁻¹ and ratio dimer to monomer was 4:5 (w/w).

Enterokinase cleavage and immunodetection of rhBMP-2 fusion proteins:

Since our rhBMP-2 protein is a fusion protein, in attempt to obtain rhBMP-2 without any amino terminal tag, we performed enterokinase cleavage. The enterokinase cleavage of monomer rhBMP-2-6His-trx fusion protein (30.6 kDa) resulted in 2 protein bands in an SDS-PAGE analysis with a predicted molecular weight of 12 kDa (mature BMP-2) and 18.6 kDa (thioredoxin and his-tag) (Fig. 4). This result demonstrated that enterokinase can be used to obtain rhBMP-2 without any tag in the future.

Monoclonal mouse anti BMP-2 (1: 2000) recognized monomeric rhBMP-2-6His-trx, its dimer (61.2 kDa) and the native monomer form (12 kDa) (Fig. 4b). The molecular weight of 61.2 kDa was detected not only in renaturation result (Fig. 4b, lane 3) but also in dissolved IB (Fig. 4b, lane 1) and protein bound to Ni-TED affinity column (Fig. 3b, lane 2). *E. coli* BL21 (DE3) harboring pET32b(+)-rhBMP-2 with IPTG or without IPTG induction expressed immunoreactive proteins and *E. coli* BL21 (DE3) did not (Fig. 4b, lane 4-6). The expressed recombinant protein was formed as monomer in whole cell lysate (Fig. 4b, lane 4, 5). As previously described, two bands of protein predicted to native monomer and thioredoxin resulted from enterokinase cleavage were confirmed by this analysis (Fig. 4a, lane 7). It was proved that one of two bands, the 12 kDa in size was monomer native BMP-2 (Fig. 4b, lane 7). This confirmed undoubtedly that the expressed protein was indeed rhBMP-2.

Table 1: Yield comparison of expression, solubilization and renaturation between our preliminary study and the present study

	Preliminary study*		Present study			
	No IPTG 37°C	0.5 mM IPTG 37°C	No IPTG 28°C	No IPTG 37°C	0.5 mM IPTG 37°C	1 mM IPTG 28°C
Expression	19.90%	60.24%	0.2 g L ⁻¹	0.2 g L ⁻¹	0.62 g L ⁻¹	0.61 g L ⁻¹
Yield of IB		1.39 g L ⁻¹				
Soluble rhBMP-2 from IB		188.4 mg L ⁻¹			63.99 mg/200 mg IB 0.4 g L ⁻¹ or 0.13 g g ⁻¹ IB (buffer 1) 41.32 mg/200 mg IB (buffer 2) 44.73 mg/200 mg IB (buffer 3)	
Yield of dimer		7.63 mg L ⁻¹			0.1 g L ⁻¹ , pH 8.0 0.2 g L ⁻¹ , pH 8.5 0.1 g L ⁻¹ , pH 9.0	
Dimer to monomer gram ratio		1:5.5 (15.38%) 1:5 (16.67%)			4:5, pH 8.0 5:4, pH 8.5 4:5, pH 9.0	

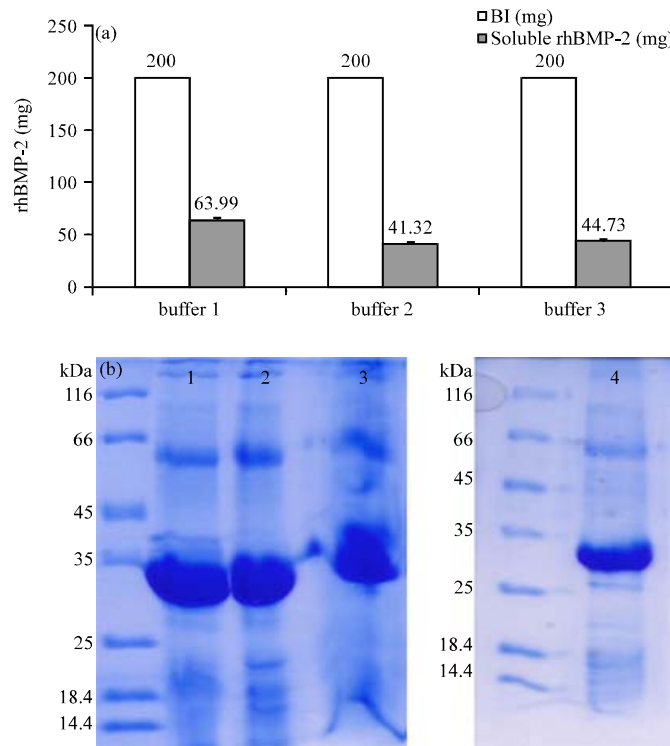


Fig. 2: Optimization of rhBMP-2 IB solubilization using 3 kinds of buffer, (a) The weight of IB before solubilization and soluble rhBMP-2 fusion protein obtained (mean±SD) after solubilization and (b) Solubilization result at reduced condition separated by 15% SDS-PAGE. Lane 1, 2, 3: Soluble rhBMP-2 in buffer 1, 2 and 3, respectively; lane 4: Soluble rhBMP-2 in buffer 3 followed by dialysis in buffer containing 4 M urea, 0.02 M tris/HCL (pH 8.0) and 5 mM EDTA for 30 min

The bioactivity of rhBMP-2-6His-trx on C2C12 myoblast cells: BMP2 converts non-osteogenic cells, such as C2C12 (myoblastic) and C3H10T1/2 (fibroblastic) cells to osteogenic cells (Katagiri *et al.*, 1994). One characteristics of the conversion of myoblast cells to osteogenic cells or

osteogenesis is the increase of alkaline phosphatase (ALP) activity. In previous study, (Zhang *et al.*, 2011) revealed that refolded rhBMP-2 obtained by using buffer at pH 9.0 had a lower activity on proliferation and differentiation of MG-63 cells than that using buffer

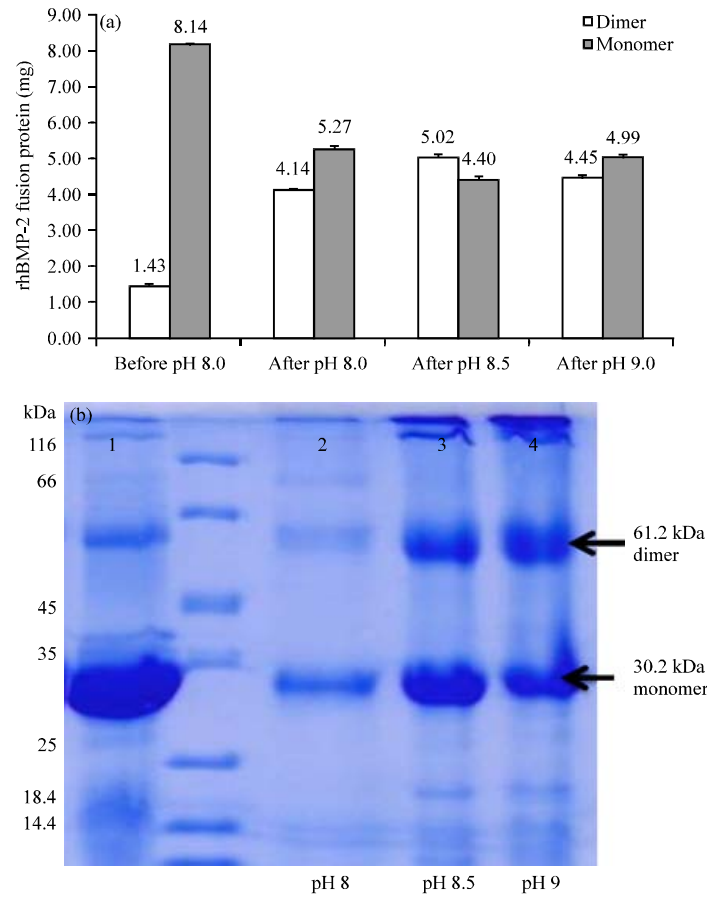


Fig. 3: Renaturation of monomeric rhBMP-2 fusion protein in three pH alkaline, (a) The ratio of dimer to monomer before and after renaturation in buffer solution and (b) Dimeric and monomeric rhBMP-2 protein before and after renaturation at 15 % SDS-PAGE. Lane 1: Soluble rhBMP-2 fusion protein (from IB solubilization) before renaturation at reduced condition, Lane 2: Protein marker, Lane 3, 4: Dimeric and monomeric rhBMP-2 (non-reduced condition) after renaturation in buffer pH 8, 8.5 and 9

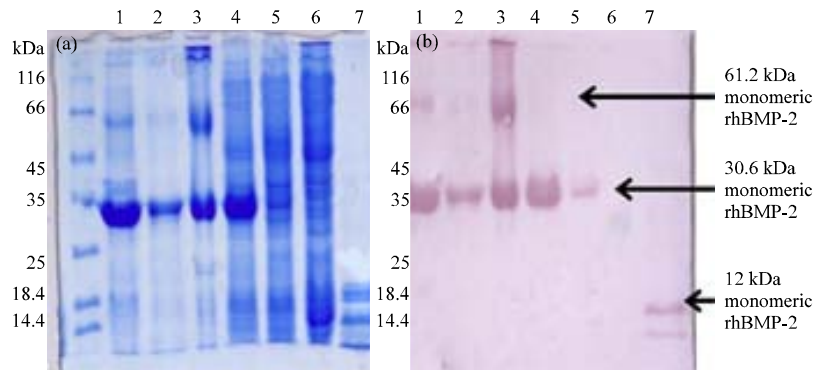


Fig. 4: Characterization of rhBMP-2 fusion protein, (a) Coomassie brilliant blue stained of proteins in a 15% SDS-PAGE analysis and (b) Western blot result using mouse monoclonal antibody against hBMP-2. Lane 1: Dissolved IB, Lane 2: Purified rhBMP-2 using Ni-TED affinity column after elution by 50 mM NaH_2PO_4 , 300 mM NaCl, 250 mM imidazole, pH 8.0, Lane 3: Dimerization result, Lane 4: Whole cell lysate after 0.5 mM IPTG induction, Lane 5: Whole cell lysate without IPTG induction; Lane 6: Whole cell lysate of total protein of *E. coli* BL21(DE3), Lane 7: enterokinase cleavage of dissolved IB rhBMP2

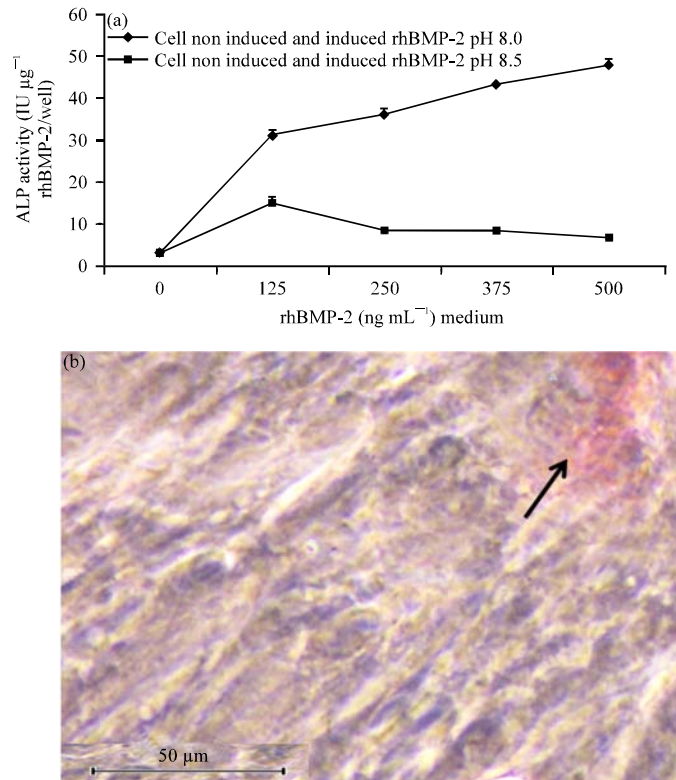


Fig. 5: The activity of dimeric rhBMP-2 fusion protein mixed with monomeric form on C2C2 myoblast cells after 6 days of incubation, (a) Alkaline phosphatase on untreated (0 ng rhBMP-2/mL) and rhBMP-2 treated cells and (b) Alkaline phosphatase (red color was pointed by black arrow) on 500 ng treated cells after Fast Red TR/Naphthol AS-MX staining

pH 7.4. Thus, in this study, we assayed the activity of rhBMP-2 in buffer at pH 8.0 and 8.5 on C2C12 myoblast cells. We used a mixture of monomeric and dimeric rhBMP-6His-Trx to determine the activity. The activity of our preparation containing dimeric form of rhBMP-2 fusion protein at pH 8.0 or pH 8.5 was measure as the activity of ALP per microgram protein (rhBMP-2 added) per well (Fig. 5). The result showed that C2C12 cells incubated for 6 days in medium containing certain amounts of dimeric rhBMP-2 at pH 8.0 or pH 8.5 buffer significantly expressed ALP ($p < 0.05$). The increase of ALP production on C2C12 myoblast cell showed as dose-dependent response (Fig. 5a). The activity of ALP in C2C12 myoblast cell treated by rhBMP-2 in buffer of pH 8.0 was higher than in buffer of pH 8.5. Untreated C2C12 myoblast cells (no rhBMP-2 added) produced ALP at low level around 3 IU per µg protein per well. The ALP production was induced slightly by 125 ng of dimer rhBMP-2 (4:5 ratio dimer to monomer) per mL medium. It increased significantly by more than 375 ng of dimer rhBMP-2 (4:5 ratio dimer to monomer) per mL medium. The

confirmation of the presence of ALP in C2C12 cells after staining by fast red TR was showed in Fig. 5b.

DISCUSSION

Our clone can grow best in all type of medium (LB, TB and SOB) in the absence of IPTG. It can be explained that those media contain rich of nitrogen as an important growth component i.e., triptone and yeast extract. Even, in the non-recombinant fungi strain (*Aspergillus heteromorphus* MTCC 9262), nitrogen sources added in the growth medium increased 3-fold of the production of pectin methyl esterase (Mandhania *et al.*, 2010). LB, TB and SOB media were medium that have been used frequently in producing recombinant protein (Vallejo *et al.*, 2002; Beshay *et al.*, 2003; Vallejo and Rinas, 2004; Long *et al.*, 2006; Zhang *et al.*, 2010, 2011; Romano *et al.*, 2009; Von Einem *et al.*, 2010). In contrast to our study, the use of TB medium was reported better than SOB or LB medium in producing the highest cell biomass of recombinant

human D-amino acid oxidase (hDAAO) (Romano *et al.*, 2009). Then, in production of recombinant β -glucanase, the highest cell dry weight was also obtained when the growth medium containing yeast extract as primary nitrogen source. Beside nitrogen, yeast extract also contains a significant amount of vitamins, growth factors and minerals and these might be responsible for the higher biomass productivity (Beshay *et al.*, 2003). IPTG at concentration of 0.5 mM increased only 1.2 times in comparison to the biomass production obtained in the absence of IPTG. However, the addition of 0.5 mM IPTG to the growth of *E. coli* (pET32b-hBMP-2) was the optimal amount of inducer, can be applicable in scale up production (growth medium more than 20 mL) and reproducible.

Similar to biomass productivity, the production of rhBMP-2 fusion protein of *E. coli* (pET32b-hBMP-2) without IPTG addition was not affected by type of medium and temperature. The optimization of productivity of rhBMP-2 fusion protein in the absence of IPTG was quite success (0.2 g per L culture). Compared to another study, the yield of rhBMP-2 without IPTG was 28.258 mg of rhBMP-2/L culture using temperature shift induction (30 to 42°C for 4 h) (Zhang *et al.*, 2010). Either growth of recombinant *E. coli* or its recombinant protein expression tend to be increased after 0.5 mM IPTG induction. Due to the addition of 1 mM IPTG was quite costly and residual IPTG could be toxic when producing human therapeutic protein (Schmidt, 2004), the use of 0.5 mM IPTG was advantage for obtaining the best yield of rhBMP-2 (0.62 g L⁻¹). Interestingly, the use of IPTG concentration lower than our study as inducer has been reported. The addition of 0.08 mM IPTG was adequate to express rhBMP-2 for six hours in LB medium at 37°C (58 mg rhBMP-2/L medium or 4.8 mg g⁻¹ wet cells) (Zhang *et al.*, 2011).

Buffer 1 (4 M urea, 0.1 M NaCl, 0.02 M Tris/HCl, pH 8.0, 5 mM EDTA, 5 mM DTT) was the best buffer to solubilize IB of rhBMP-2, it may be due to protein stability. Recombinant BMP-2 was found more stable in buffer containing urea than guanidine-HCl (like in buffer 3) (Vallejo *et al.*, 2002). The cost of using 4 M urea was also relatively lower than using 6 M urea (buffer 2) and easier further preparation of active rhBMP-2. Following IB solubilization, several studies did the dialysis process to remove reducing agent (Vallejo *et al.*, 2002; Zhang *et al.*, 2011). Since 4 M urea was used either in our solubilization or renaturation step, the soluble rhBMP-2 for source of renaturation did not need dialysis process to remove reducing agent (DTT) and this would shorten the time for preparation. DTT may be removed while the protein was diluted in first round of renaturation buffer. Unlike our

study, other experiments were success to use 8 M urea in solubilizing rhBMP-2 IB (Zhang *et al.*, 2010, 2011). In current study, similar yield of rhBMP-2 from IB solubilization was also achieved using buffer 3 containing 6 M guanidine-HCl in the presence of reducing and chelating agent. The high yield may be associated with the presence of synergy effect of guanidine HCl as denaturant, the highest pH buffer (pH 8.5) and the highest reducing agent (0.1 M DTT) to maintain cysteine in reduced form. Our rhBMP-2 renaturation result strengthened the previous study that alkaline pH of buffer was at narrow range of pH 7.4 - pH 9. Vallejo and Rinas (2004) reported that the disulfide bond was not arranged in buffer pH lower than pH 7.0. Furthermore at above pH 9, BMP-2 may be are unstable and leads to inhibition of renaturation. Our renaturation in buffer pH 8.0 yielded 0.1 g active dimer rhBMP-2 per L culture. However, this result was considerably low compared to other studies which was higher in terms of the quantity and purity of their yields. Ours was in form of protein fusion and was still in a mixture of dimeric and monomeric forms and the yield was only 3-fold of amount of dimer before renaturation. Another study reported that after separating dimer from monomer using HiTrap Heparin HP affinity column, the yield of active BMP-2 dimer was 29.4 mg g⁻¹ cell wet weight (Long *et al.*, 2006). Then, Zhang *et al.* (2011) published that the percentage of refolding yield was 42% using buffer renaturation at pH 7.4. Inevitably, the yield of in vitro renaturation of rhBMP-2 fusion protein was different with other studies, it might be due to the differences of nature of BMP-2 protein, renaturation strategy, initial soluble monomer protein concentration, composition and pH of renaturation buffer, temperature and the time of renaturation.

The bioactivity assay showed that dimer rhBMP-2 fusion protein in buffer pH 8.0 was more active than in buffer pH 8.5. While, another study found that rhBMP-2 in buffer pH 7.4 was more active than in buffer pH 9 (Zhang *et al.*, 2011). It can be concluded that alkaline pH of renaturation to gain an active rhBMP-2 may be in range of pH 7-8. Jatta *et al.* (2009) also reported those phenomena in non-recombinant protein (lipase produced by *Candida albicans*). The enzyme was most active in buffer pH 7, while its activity was reduced either under or above pH 7 of buffer. Similar pattern of ALP activity on C2C12 cell found in this study was also reported in another study. It was increased significantly after addition of more than 300 ng mL⁻¹ of dimeric rhBMP-2. It means that 300 ng mL⁻¹ of BMP-2 was capable to inhibit myoblast cells (C2C12) differentiation and induce osteoblastic phenotype (Long *et al.*, 2006). However,

regarding to pharmaceutical requirement and the success of enterokinase cleavage of our recombinant protein, it will be better if in future study the native form of rhBMP-2 (12 kDa) resulted from enterokinase cleavage is carried out as source of renaturation step.

Finally, The use of medium containing high nitrogen is appropriate to growth *E. coli* BL21 (DE3) harboring pET32b(+)-rhBMP-2 in achieving high volumetric and productivity of active rhBMP fusion protein. IB solubilization and renaturation of its soluble rhBMP-2 fusion protein using simple strategy was also successful to obtain biologically active rhBMP-2 fusion protein that it has similar effect on inducing C2C12 cell to produce ALP compared to rhBMP-2 expressed from cDNA.

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