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Effects of Chemical Additives in Refolding Buffer on Recombinant Human BMP-2 Dimerization and the Bioactivity on SaOS-2 Osteoblasts

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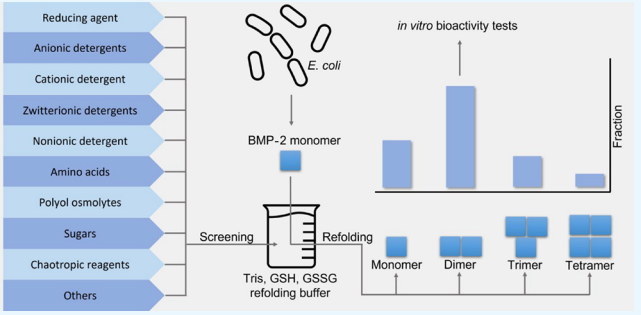
ABSTRACT: Bone morphogenetic protein-2 (BMP-2) is a promising osteogenic agent in tissue engineering. BMP-2 is usually expressed in *Escherichia coli* owing to the high yield and low cost, but the protein is expressed as inclusion bodies. Thus, the bottleneck for BMP-2 production in *E. coli* is the refolding process. Here, we explored the effects of the refolding buffer composition on BMP-2 refolding. The BMP-2 inclusion body was solubilized in urea and subjected to refolding by the dilution method. Various additives were investigated to improve the BMP-2 refolding yield. Nonreducing SDS-PAGE showed that BMP-2 dimers, the presumably biologically active form, were detected at approximately 25 kDa. The highest yield of the BMP-2 dimers was observed in the refolding buffer that contained ionic detergents (sarkosyl and cetylpyridinium chloride) followed by zwitterionic and nonionic detergents (NDSB-195, NP-40, and Tween 80). In addition, sugars (glucose, sorbitol, and sucrose) in combination with anionic detergents (sodium dodecyl sulfate and sarkosyl) reduced BMP-2 oligomers and increased the BMP-2 dimer yield. Subsequently, the refolded BMP-2s were tested for their bioactivity using the alkaline phosphatase assay in osteogenic cells (SaOS-2), as well as the luciferase reporter assay and the calcium assays. The refolded BMP-2 showed the activities in the calcium deposition assay and the luciferase reporter assay but not in the alkaline phosphatase activity assay or the intracellular calcium assay even though the dimers were clearly detected. Therefore, the detection of the disulfide-linked dimeric BMP-2 in nonreducing SDS-PAGE is an inadequate proxy for the bioactivity of BMP-2.



INTRODUCTION

Bone morphogenetic protein-2 (BMP-2) is a multifunctional secreted growth factor, which belongs to the transforming growth factor beta (TGF-β) superfamily. To date, more than 20 BMPs have been identified and characterized in humans.[1,2] BMP-2 is widely used in dental treatment, articular cartilage damage therapy, and bone fracture healing.[3,4] Mature BMP-2 consists of 114 amino acids. The N-terminus shows a potent heparin-binding property while the C-terminus is important for dimerization.[5] BMP-2 functions as a homodimeric protein, which contains seven cysteine residues. The six residues form intramolecular disulfide bonds (Cys34/Cys111, Cys47/Cys113, and Cys14/Cys79) into a cystine knot, and the remaining residue forms an intermolecular disulfide bond (Cys78).[5] The complexity of the disulfide bonds makes in vitro production of the functional protein difficult.

Due to the current and forthcoming demand of BMP-2, several recombinant systems have been used to generate biologically active BMP-2 instead of purification from the demineralized bone matrix (DBM), which is a time-consuming process with very low yield and high cost.[6] The mammalian cell is one of the expression systems that has generally been used to produce recombinant human BMPs (rhBMPs) because this system has the characteristics most similar to the endogenous BMP synthesis in human cells. The mammalian cells usually used for rhBMP production are Chinese hamster ovary cells (CHO), human embryonic kidney cells (HEK293T), and African green monkey kidney fibroblast-like cells (COS-7).[7−9] The advantages of the eukaryotic expression systems are native protein folding and post-translational modifications (PTMs), which result in the bioactive form of the protein. However, the yield of the recombinant protein in the mammalian expression systems is very low, resulting in a high cost for production.[10] Another system, which has widely been used to produce recombinant protein, is the *Escherichia coli* (*E. coli*) system. This system typically ensures very high yields with low cost for large-scale production.[11] However, the proteins synthesized in *E. coli*

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may not fold as correctly as they do in the mammalian cells and do not undergo PTMs such as glycosylation, phosphorylation, acetylation, and proteolytic cleavage. Therefore, the bacteria-derived recombinant proteins might be inactive.[10,12]

To take advantage of the high yield, rhBMP-2 is sometimes produced in the *E. coli* expression system despite no correct folding and PTMs.[13,14] However, the BMP-2 produced in *E. coli* is insoluble or aggregated into inclusion bodies (IBs). Refolding of the insoluble BMP-2 into the active form is often detected by disulfide-linked dimerization, which is indicative of its bioactivity.[15−17] Renaturation of the aggregated protein into an active form is a crucial step for the industrial-scale production. To overcome this obstacle, different chemicals and methods have been used to solubilize and renature the IB such as dialysis, dilution, chemical additives, and microfluidic chips.[18,19] Various chemical additives have been added in the expression medium or in the refolding buffers to increase the solubility of the expressed proteins.[20] Typical additives used for protein refolding are denaturants, protein aggregation inhibitors, and protein stabilizers.[18] The aggregated proteins are usually dissolved in a high concentration of chaotropic agents, such as urea and guanidine chloride (GuHCl), because a low concentration of the denaturants can stabilize the protein structure.[21,22] In addition, several compounds are classified as protein aggregation inhibitors such as arginine, cyclodextrins, and *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES).[23−25] Moreover, various reagents can stabilize the protein structure, including glycerol, nondetergent sulfobetaine 195 (NDSB-195), polyethylene glycol (PEG), and sugars (sucrose and trehalose).[26−30]

Due to the complicated and time-consuming refolding process, several studies sought to identify novel refolding methods to improve the BMP-2 production. For instance, Long et al. developed a simple method using FoldIt refolding buffers to refold BMP-2.[17] Rane et al. employed a solid-phase method for BMP-2 refolding on a weak cation exchange resin.[15] Moreover, Vallejo and Rinas studied folding kinetics and renaturation conditions of BMP-2 by optimizing various factors such as pH, temperature, redox conditions, and protein concentrations.[25,31] Due to the promising clinical use of BMP-2, this study aimed to screen and optimize BMP-2 refolding buffer compositions for a large-scale production and examine the refolding efficiency by the measurement of the disulfide-linked oligomeric states using nonreducing SDS-PAGE and analyzing the bioactivity of BMP-2. The chemical additives and the refolding condition studied in our work may be applicable to the refolding of other proteins to maximize the dimer or oligomer yield.

MATERIALS AND METHODS

Plasmid Construction and Protein Expression. Full-length BMP-2 was amplified by PCR using forward primers 5′-ATACCATGGCCAAACACAAACAGCGGAAACGCC-3′ and reverse primers 5′-CGCGGATCCTCATTAGCGACACCCACAACCCTCCACAACC-3′ and cloned into pET28a using the NcoI and BamHI restriction sites. The transformants were grown and selected on Luria Bertani (LB) agar containing 50 μg/mL kanamycin. The nucleotide sequence of BMP-2 in the resulting recombinant plasmids was verified by DNA sequencing. The expression plasmid was then transformed into *E. coli* Tuner (DE3) competent cells for the recombinant protein expression. The transformed bacteria were inoculated into 100 mL of LB medium containing 50 μg/mL kanamycin and grown overnight at 37 °C. The overnight culture (15 mL) was inoculated into 500 mL of LB medium containing the same antibiotics and incubated at 37 °C with shaking at 250 RPM until the OD600 reached 0.4−0.6. Subsequently, the expression of the recombinant proteins was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the final concentration of 0.5 mM, and the bacteria were further grown at 37 °C for 6 h. The bacterial cultures were harvested by centrifugation at 6000 × *g* at 4 °C for 10 min and washed with 0.9% NaCl. The cell pellets were stored at −80 °C before protein extraction.

Inclusion Body Isolation and Purification. The cell pellets were resuspended with 100 mM Tris-HCl, pH 8.0, 10 mM ethylenediamine tetraacetic acid (EDTA) containing 0.1 mg/mL lysozyme and incubated at 4 °C for 30 min. Thereafter, phenylmethylsulfonyl fluoride (PMSF) was added to 1 mM and sonicated for three cycles of 10 s (30% amplitude). The cell pellets were centrifuged at 16,000 × *g* at 4 °C for 15 min. The supernatant was discarded and 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% triton X-100 was added and sonicated. The cell pellets were then collected by centrifugation at 16,000 × *g* at 4 °C for 15 min, and the supernatant was discarded. Thereafter, 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1 M NaCl was added and sonicated. The IB was collected by centrifugation at 14,000 × *g* at 4 °C for 10 min, and the supernatant was discarded. The IB was then solubilized in 20 mM Tris-HCl, pH 8.5, 6 M urea, 10 mM dithiothreitol (DTT) and clarified by centrifugation at 40,000 × *g* at 4 °C for 30 min. To further purify the BMP-2, the protein was loaded onto a HiPrep SP FF column (GE Healthcare; Uppsala, Sweden) pre-equilibrated with the binding buffer (20 mM Tris, pH 7.0 and 6 M urea). The column was washed with the binding buffer containing 100 mM NaCl, and the bound protein was eluted with the binding buffer containing 500 mM NaCl. The purified BMP-2 IB was stored at −20 °C until protein refolding.

Screening of BMP-2 Refolding in Various Single Additives and Combination of Two Additives. BMP-2 was refolded in various additives as shown in Table 1 using a dilution method. Briefly, BMP-2 at a final concentration of 0.1 mg/mL was refolded in 1.5 mL microcentrifuge tubes with various additives in 100 mM Tris, pH 8.5, 2 mM reduced glutathione (GSH), and 4 mM oxidized glutathione (GSSG) at 4 °C for 20 h. In addition, an anionic detergent such as 0.05% sodium dodecyl sulfate (SDS) or 0.2% sodium *N*-lauroylsarcosinate (sarkosyl) was added into the refolding buffers to study the effect of the combination of two additives. The refolded proteins were analyzed using nonreducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with silver staining. The relative amounts of different oligomeric states of BMP-2 were quantified as band intensities using ImageJ software (NIH, USA).

Alkaline Phosphatase (ALP) Activity Assay. The alkaline phosphatase (ALP) activity assay, an early osteogenic marker, was performed on the osteosarcoma cells (SaOS-2). Briefly, the SaOS-2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco; Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin−streptomycin (HyClone Laboratories Inc., Logan, UT) and maintained in a humidified incubator at 37 °C with 5% CO_2. The SaOS-2 cells were seeded at a density of 0.8×10^3 cells/cm^2 in 24-well plates and treated either with 10 μL of 0.1 mg/mL refolded BMP-2 crude proteins in DMEM containing 2% FBS (a final concentration of 2 μg/mL crude proteins) or a 100 ng/mL commercially available rhBMP-2 (355-BM-010/CF; R&D

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Systems, Inc., Minneapolis, MN), which was purified from CHO cells, as a positive control. The media and the crude proteins were replaced every 2 days, and the cells were cultured for a 7 day time course. After removing the culture medium, the cells were washed with PBS and lysed with SDS lysis buffer (150 mM NaCl, 15 mM sodium citrate, and 0.02% SDS) for 30 min. Then 10 μL of the cell lysate was incubated with 100 μL of the *p*-nitrophenyl phosphate liquid substrate (Sigma-Aldrich; St. Louis, MO) at 37 °C for 15 min. The reaction was stopped by adding 0.02 N NaOH, and the enzyme activity was measured by the absorbance at 405 nm (BioTek Synergy H1, Agilent; Santa Clara, CA). The protein content was determined using the Bradford protein assay (Bio-Rad, Hercules, CA).

Large-Scale BMP-2 Refolding and Biological Activity Test of Crude Proteins. The BMP-2 IB (1 mg) was refolded in 1 mL of RF1 refolding buffer (Table 2). The refolding protein was incubated at 4 °C for 20 h and separated by nonreducing SDS-PAGE with silver staining. BMP-2 was then dialyzed in 10 mM acetic acid, and the protein concentration was measured by absorbance at 280 nm before biological activity tests. The ALP activity assay was performed using 100 ng/mL of the refolded BMP-2 crude protein (RF1) for 7 days as in the aforementioned method. A concentration of 100 ng/mL commercial BMP-2 (355-BM-010/CF; R&D Systems, Inc.) was used as a positive control.

Protein Purification. Purification of the refolded BMP-2 dimers was performed by HiTrap Heparin HP column chromatography (GE Healthcare). Briefly, 4 mg of the BMP-2 IB was refolded and dialyzed in 20 mM Tris-HCl, pH 8.0 and 4 M urea. The dialyzed BMP-2 was applied into a HiTrap Heparin HP column. The column was washed with the dialysis buffer, and the monomeric and dimeric forms of BMP-2 were eluted with a linear gradient of the dialysis buffer containing 0−1 M NaCl. The purified BMP-2 was dialyzed in 10 mM acetic acid before biological activity testing.

Luciferase Activity Assay. To determine whether BMP-2 could induce luciferase expression from a reporter plasmid, human embryonic kidney cells (HEK293T) were cultured in DMEM (Gibco) supplemented with 10% FBS with no antibiotics at a density of 5×10^4 cells/cm^2 in a 24-well plate. The cells were cotransfected with pGL3 BRE Luciferase, which was kindly provided by Martine Roussel and Peter ten Dijke (Addgene plasmid # 45126),[32] and pIS1, which was a gift from David Bartel (Addgene plasmid # 12179), was used as the internal control. The transfection agent was FuGENE Transfection Reagent (Promega; Madison, WI) in a serum-free medium according to the manufacturer's protocol. Thereafter, the cells were cultured in 0.1% FBS containing 100 ng/mL purified BMP-2 for 3 h. The cell extracts were then prepared for the luciferase activity assay. The cells were washed with PBS and lysed with 150 μL of luciferase lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, and 1% NP-40) at room temperature for 15 min. The luciferase activity was analyzed using the Dual-Glo Luciferase Assay System (Promega). Briefly, 75 μL of the cell lysate was mixed with 75 μL of Dual-Glo Luciferase Assay Reagent and incubated at room temperature for 10 min. The firefly luminescence was measured using a microplate reader (BioTek Synergy H1). Seventy-five microliters of Dual-Glo Stop & Glo Reagent was then added into the same plate and incubated at room temperature for 10 min before quantifying the Renilla luminescence.

Alizarin Red Assay. To evaluate extracellular calcium deposition, the SaOS-2 cells were cultured in osteogenic DMEM containing 10 mM β-glycerophosphate, 100 nM dexamethasone, 50 μM L-ascorbic acid, 10% FBS, and 1%

Table 1. List of Refolding Additives Used in This Work[a]

reagents	concentrations
Reducing Agents	
tris(2-carboxyethy)phosphine hydrochloride (TCEP)	1 mM, 5 mM, 10 mM
Anionic Detergents	
sodium dodecyl sulfate (SDS)	0.05%, 0.1%, 0.2%
sodium *N*-lauroylsarcosinate (sarkosyl) sodium deoxycholate	0.2%, 0.5%, 1% 0.1%, 0.5%, 1%
Cationic Detergents	
cetylpyridinium chloride (CPC)	0.01%, 0.05%, 1%
Zwitterionic Detergents	
3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)	10 mM, 20 mM, 30 mM
betaine	1 mM, 5 mM, 10 mM
nondetergent sulfobetaine 201 (NDSB-201)	0.1 M, 0.5 M, 0.75 M
nondetergent sulfobetaine 211 (NDSB-211)	1 mM, 5 mM, 10 mM
nondetergent sulfobetaine 221 (NDSB-221)	1 mM, 5 mM, 10 mM
nondetergent sulfobetaine 195 (NDSB-195)	1 mM, 5 mM, 10 mM
nondetergent sulfobetaine 256 (NDSB-256)	1 mM, 5 mM, 10 mM
Nonionic Detergents	
nonidet P-40 (NP-40)	0.1%, 0.5%, 1%
Tween 20	0.01%, 0.05%, 0.1%
Tween 80	0.01%, 0.05%, 0.1%
Triton X-100	0.1%, 0.5%, 1%
octylglucoside (OGP)	0.1%, 0.5%, 1%
Amino Acids	
glycine	0.25 M, 0.5 M, 0.75 M
L-arginine	0.5 M, 0.75 M, 1 M
Polyol Osmolytes	
glycerol	0.1%, 0.3%, 0.5%
polyethylene glycol 1500 (PEG1500)	0.1%, 0.3%, 0.5%
polyethylene glycol 2000 (PEG2000)	0.1%, 0.3%, 0.5%
polyethylene glycol 3350 (PEG3350)	0.1%, 0.3%, 0.5%
polyethylene glycol 4000 (PEG4000)	0.1%, 0.3%, 0.5%
polyethylene glycol 6000 (PEG6000)	0.1%, 0.3%, 0.5%
polyethylene glycol 8000 (PEG8000)	0.1%, 0.3%, 0.5%
polyethylene glycol 10,000 (PEG10000)	0.1%, 0.3%, 0.5%
Sugars	
sucrose	0.25 M, 0.5 M, 0.75 M
glucose	0.25 M, 0.5 M, 0.75 M
sorbitol	0.2 M, 0.5 M, 0.75 M
trehalose	0.2 M, 0.5 M, 0.75 M
Chaotropic Reagents	
urea	0.5 M, 1 M, 2 M
guanidine hydrochloride (GuHCl)	0.1 M, 0.5 M, 1 M
Other Additives	
N-cyclohexyl-2-aminoethanesulfonic acid (CHES)	0.5 M, 0.75 M, 1 M
ethylenediamine tetraacetic acid (EDTA)	1 mM, 5 mM, 10 mM
sodium citrate	0.2 M, 0.3 M, 0.5 M
sodium alginate	0.1%, 0.5%, 1%
α-cyclodextrin	1 mM, 5 mM, 10 mM
β-cyclodextrin	1 mM, 5 mM, 10 mM
γ-cyclodextrin	1 mM, 5 mM, 10 mM
calcium chloride ($CaCl_2$)	2 mM, 5 mM, 10 mM
magnesium chloride ($MgCl_2$)	2 mM, 5 mM, 10 mM
sodium chloride (NaCl)	0.2 M, 0.5 M, 0.5 M

[a]All additives were in 100 mM Tris, pH 8.5, 2 mM GSH, and 4 mM GSSG.

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Table 2. List of Refolding Buffers Used for the Bioactivity Tests

refolding buffer	refolding buffer composition
RF1	100 mM Tris, pH 8.5, 2 mM GSH, 4 mM GSSG, and 0.2% sarkosyl
RF2	100 mM Tris, pH 8.5, 2 mM GSH, 4 mM GSSG, 0.05% SDS, and 0.5% glycerol

penicillin−streptomycin. The cells were seeded at a density of 2.5×10^3 cells/cm^2 in 24-well plates and treated with 100 ng/mL purified BMP-2 in osteogenic DMEM containing 10% FBS. The media containing the purified protein were replaced every 2 days, and the cells were cultured in total for 15 days. After removing the culture medium, the cells were washed with PBS and fixed with 4% formaldehyde at room temperature for 15 min. The cells were then washed with PBS for three times and incubated with 40 mM Alizarin Red at room temperature for 30 min. The cells were washed with deionized water for five times. Quantification of mineralization was performed as reported by Gregory et al.[33] Briefly, the cells were incubated with 200 μL of 10% acetic acid at room temperature for 30 min and transferred into a 1.5 mL microcentrifuge tube and heated at 85 °C for 10 min. Thereafter, the cells were incubated on ice for 5 min and centrifuged at 20,000 × *g* for 15 min. The supernatants were transferred and mixed with 10% ammonium hydroxide (NH_4OH) to neutralize the acid, and the absorbance at 405 nm was measured using a microplate reader (BioTek Synergy H1).

Intracellular Calcium Assay. Late osteogenic markers were determined by measurement of intracellular calcium.[34] Briefly, the SaOS-2 cells were cultured in osteogenic DMEM as described earlier. The media and the purified protein were replaced every 2 days for 15 days. After removing the culture medium, the cells were washed with PBS and lysed with SDS lysis buffer at room temperature for 30 min. The cells were then mixed with an equal volume of 1 M hydrochloric acid (HCl) and incubated at 4 °C overnight. Thereafter, 10 μL of the samples were mixed with 1 mL of 0.88 M ethanolamine buffer, pH 11, and 100 μL of the *O*-cresolphthalein complex substrate (OCPC). The solution absorbance was measured at 570 nm.

Statistical Analysis. All quantitative data are presented as mean ± standard deviation (SD). Comparisons between two groups of samples were performed using unpaired Student's *t*-test, whereas multiple comparisons of more than two groups of samples were performed using one-way analysis of variance (ANOVA) with Tukey's post hoc test using SPSS version 16.0 (SPSS; Chicago, IL). *p*-values less than 0.05 were considered statistically significant.

RESULTS

Expression of rhBMP-2 in *E. coli*. rhBMP-2 was produced in *E. coli* Tuner (DE3) as IBs. The aggregated protein was dissolved with 6 M urea and purified using an SP FF cation exchange chromatography column to remove contaminating proteins before refolding. rhBMP-2 was eluted (fraction E1) as monomers with a molecular weight of 12 kDa (Figure 1). The BMP-2 IB obtained from 1 L of *E. coli* culture was approximately 30 mg. The IB was refolded in RF2 and purified using a heparin column. The yield of the purified BMP-2 was approximately 8.3 mg, which included dimers and the other mers. The advantage of the BMP-2 expression in the *E. coli* system is the high yield of the protein monomers compared to the mammalian expression (Table 3).

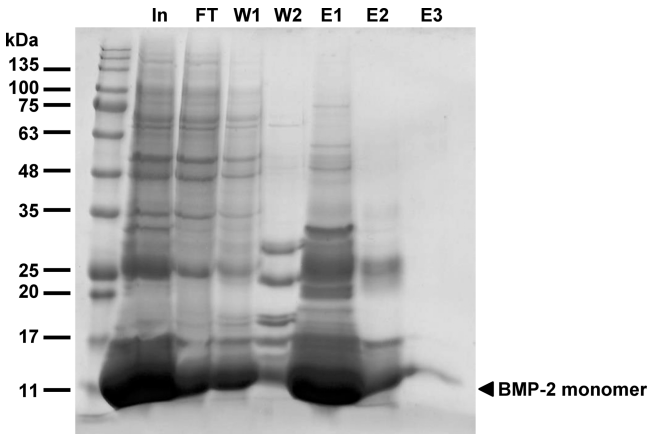

Figure 1. Expression of rhBMP-2 and IB purification. rhBMP-2 was produced in *E. coli* as IBs and purified using an SP FF column. All fractions were separated in nonreducing SDS-PAGE and stained with Coomassie brilliant blue R. Lane In = input fraction of the IB from the urea extract; FT = flowthrough; W1 = wash fraction with the binding buffer (20 mM Tris, pH 7.0, and 6 M urea); W2 = wash fraction with the binding buffer containing 100 mM NaCl; E1, E2 = elute fraction containing the binding buffer with 500 mM NaCl; and E3 = elute fraction with the binding buffer.

Table 3. Comparison of the Purified BMP-2 Yields from the Different Expression Systems

cell type	purified BMP-2 yield	reference
E. coli	8.3 mg (1 L of *E. coli*)	our result
E. coli	29.4 mg/g cell wet weight	Long et al.[17]
Chinese hamster ovary cells (CHO)	20−30 ng/mL	Israel et al.[35]

Effect of Various Single Additives on BMP-2 Refolding. To investigate the effect of the refolding buffer components on BMP-2 refolding, 43 additives were screened by adding each of them into the refolding buffer containing 100 mM Tris, pH 8.5, 2 mM GSH, and 4 mM GSSG. The results showed that 20 out of 43 additives could produce dimeric BMP-2 including SDS, EDTA, arginine, GuHCl, PEG3350, sodium citrate, α-, β-, γ-cyclodextrin, CHES, sorbitol, glycine, sucrose, glucose, Tween 80, NP-40, sodium deoxycholate, CPC, sarkosyl, and NDSB-195 (Figure 2A,B). Among the 20 additives that could produce the dimeric BMP-2, 0.2% sarkosyl showed the highest yield of the dimers (73.88%) followed by 0.1% CPC (67.89%) (Figure 2A, xii). In contrast, sugars and oligosaccharides showed low yields of the dimers. Five millimolar α-cyclodextrin resulted in the lowest yield (24.98%) (Figure 1A, vii). In addition, some additives such as CHES and sodium deoxycholate showed an equal yield of the monomers and the dimers (Figure 2A, viii, xi). Moreover, trimers, and tetramers were mostly observed when sugars such as sorbitol, sucrose, or glucose were used (Figure 2A, viii, ix).

Effect of the Combination of an Additive with 0.05% SDS on BMP-2 Refolding. For the evaluation of the effect of the refolding buffer containing 0.05% SDS, BMP-2 was refolded in a refolding buffer containing a combination of an additive and 0.05% SDS in 100 mM Tris, pH 8.5, 2 mM GSH, and 4 mM GSSG. The results revealed that 0.05% SDS with 10 mM NDSB-256 showed the highest yield of the BMP-2 dimers (56.75%)

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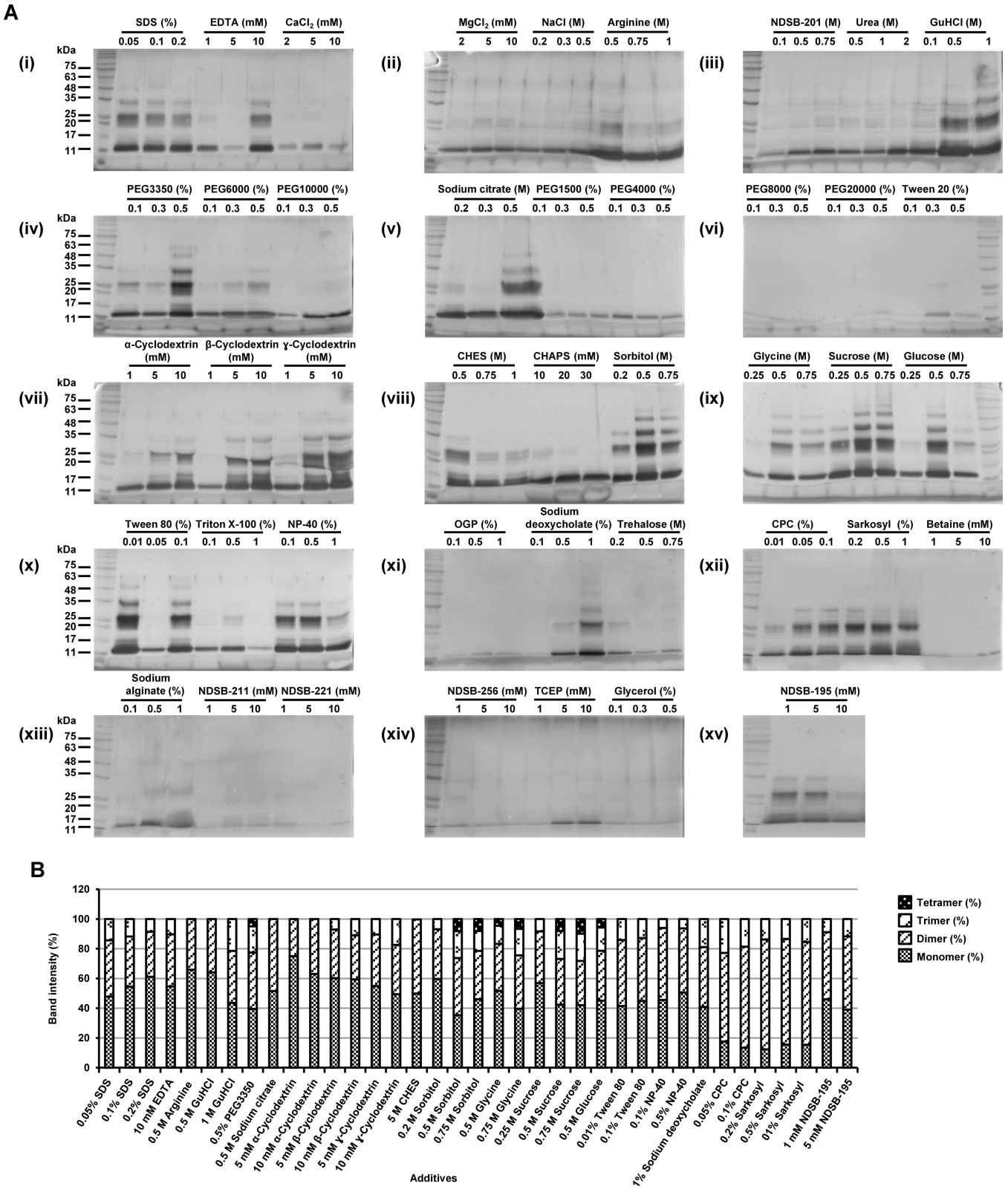

Figure 2. Effect of single additives on BMP-2 refolding. (A) BMP-2 (0.1 mg) was refolded in 1 mL of the refolding buffer containing100 mM Tris, pH 8.5, 2 mM GSH, and 4 mM GSSG with various single additives at different concentrations and separated by nonreducing SDS-PAGE with silver staining. (B) Band intensity analysis of BMP-2 from different refolding conditions using the ImageJ program.

(Figure 3A,B). Addition of an anionic detergent such as SDS leads to the BMP-2 dimer formation greater than NDSB-256 alone. The combination of 0.05% SDS with 0.5% glycerol or 0.2 M trehalose also showed high yields of the dimers, 56.75 and 55.97%, respectively (Figure 3A, ii). The equal yields of the monomers and the dimers were mostly found in the combination of 0.05% SDS with sugars such as glucose, sorbitol, and glucose (Figure 3A, iii). The lowest yield of the dimers was

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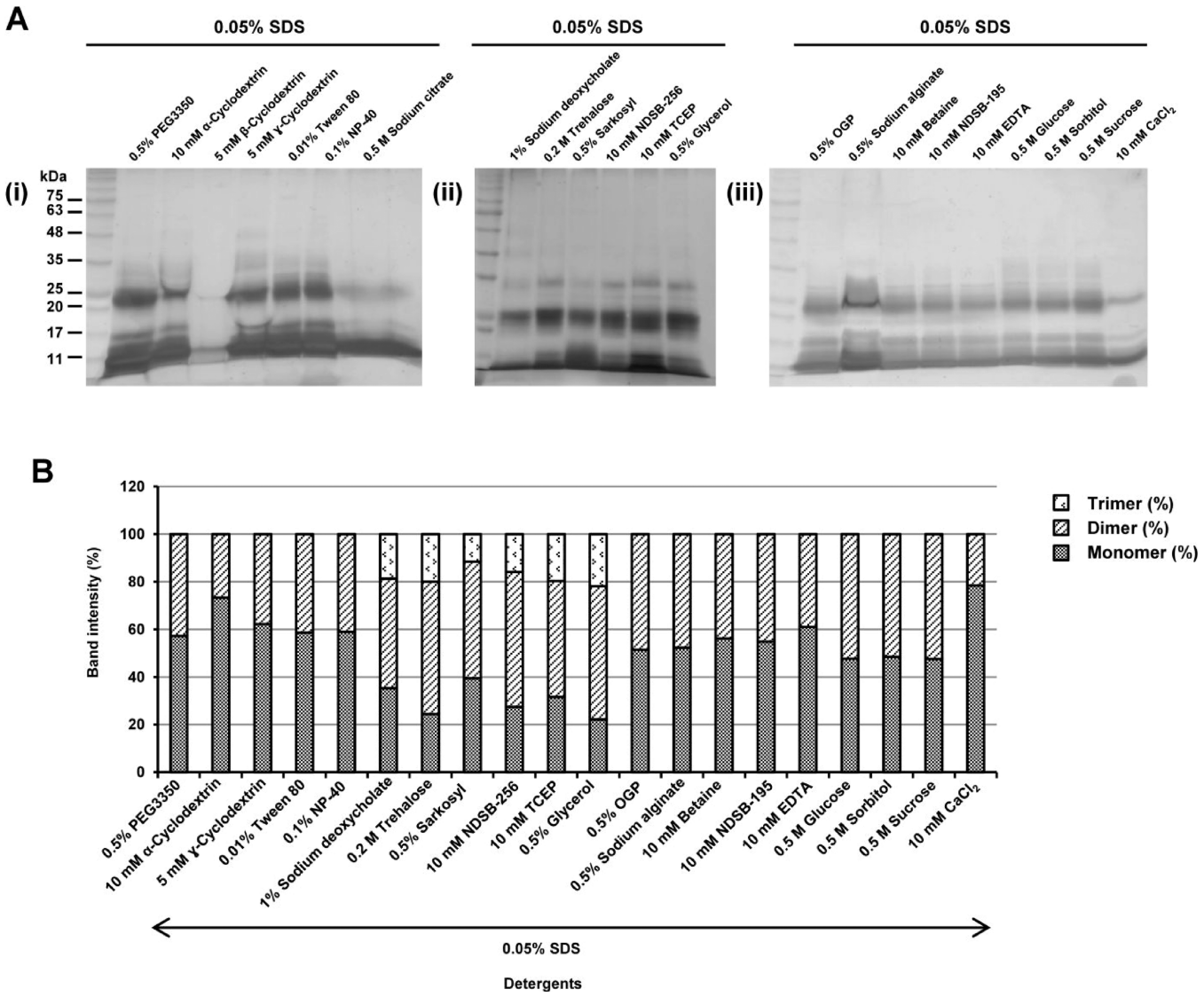

Figure 3. Effects of the combination of an additive with 0.05% SDS on BMP-2 refolding. (A) BMP-2 (0.1 mg) was refolded in combinations of an additive and 0.05% SDS in 1 mL of the refolding buffer containing 100 mM Tris, pH 8.5, 2 mM GSH, and 4 mM GSSG and separated by nonreducing SDS-PAGE with silver staining. (B) Band intensity analysis of BMP-2 from the different combinations using the ImageJ program.

observed in the combination of 0.05% SDS with 10 mM $CaCl_2$ (21.60%) (Figure 3A, iii). The BMP-2 dimer was not found in the combination of 0.05% SDS with 5 mM β-cyclodextrin or 0.5 M sodium citrate (Figure 3A, i).

Effect of the Combination of an Additive with 0.2% Sarkosyl on BMP-2 Refolding. We further explored the combination of an additive with 0.2% sarkosyl. BMP-2 refolding was carried out by dilution in a refolding buffer containing the combination of an additive with 0.2% sarkosyl in 100 mM Tris, pH 8.5, 2 mM GSH, and 4 mM GSSG. The highest yield of the dimers was found in the combination of 0.2% sarkosyl with 0.5 M glucose (59.91%) (Figure 4, i). High yields were also found in the combination of 0.2% sarkosyl with 0.5 M sorbitol or 0.5% PEG3350, 58.60% and 57.29, respectively (Figure 4, i). In fact, the combination of 0.2% sarkosyl with polyol osmolytes such as PEG3350 and PEG8000 increased the BMP-2 dimers (Figure 4, i and iii). The lowest yield was observed in the combination of 0.2% sarkosyl with 0.5 M NaCl (28.34%) (Figure 4, iii). The BMP-2 dimer was not detected in the combination of 0.2% sarkosyl with EDTA, arginine, GuHCl, CHES, or sodium citrate (Figure 4, i).

Effect of BMP-2 Crude Proteins on Alkaline Phosphatase Activity. The ALP activity was used as an early osteogenic marker. The SaOS-2 cells were treated for 7 days with various refolded BMP-2 crude proteins. The results showed that the ALP activity was not significantly different compared to the negative control (Figure 5). However, the BMP-2 crude proteins refolded in the buffers containing 0.05% SDS and 0.01% Tween 80, and α-, β-, or γ-cyclodextrin tended to have relatively high ALP activity compared to the other additives. We also found that some crude proteins from certain additives such as 0.1% CPC, 0.05% SDS and 1% sodium deoxycholate, 0.2% sarkosyl, and 0.5% PEG3350 induced cytotoxicity and cell death.

In addition, we refolded BMP-2 on a larger scale in RF1 (Table 2) that contained 0.2% sarkosyl as a single additive because this buffer showed the greatest dimer yield (Figure 2B). We then studied its bioactivity compared to the controls using the ALP assay. The result showed that the BMP-2 refolded in RF1 induced the ALP activity indifferently from the negative

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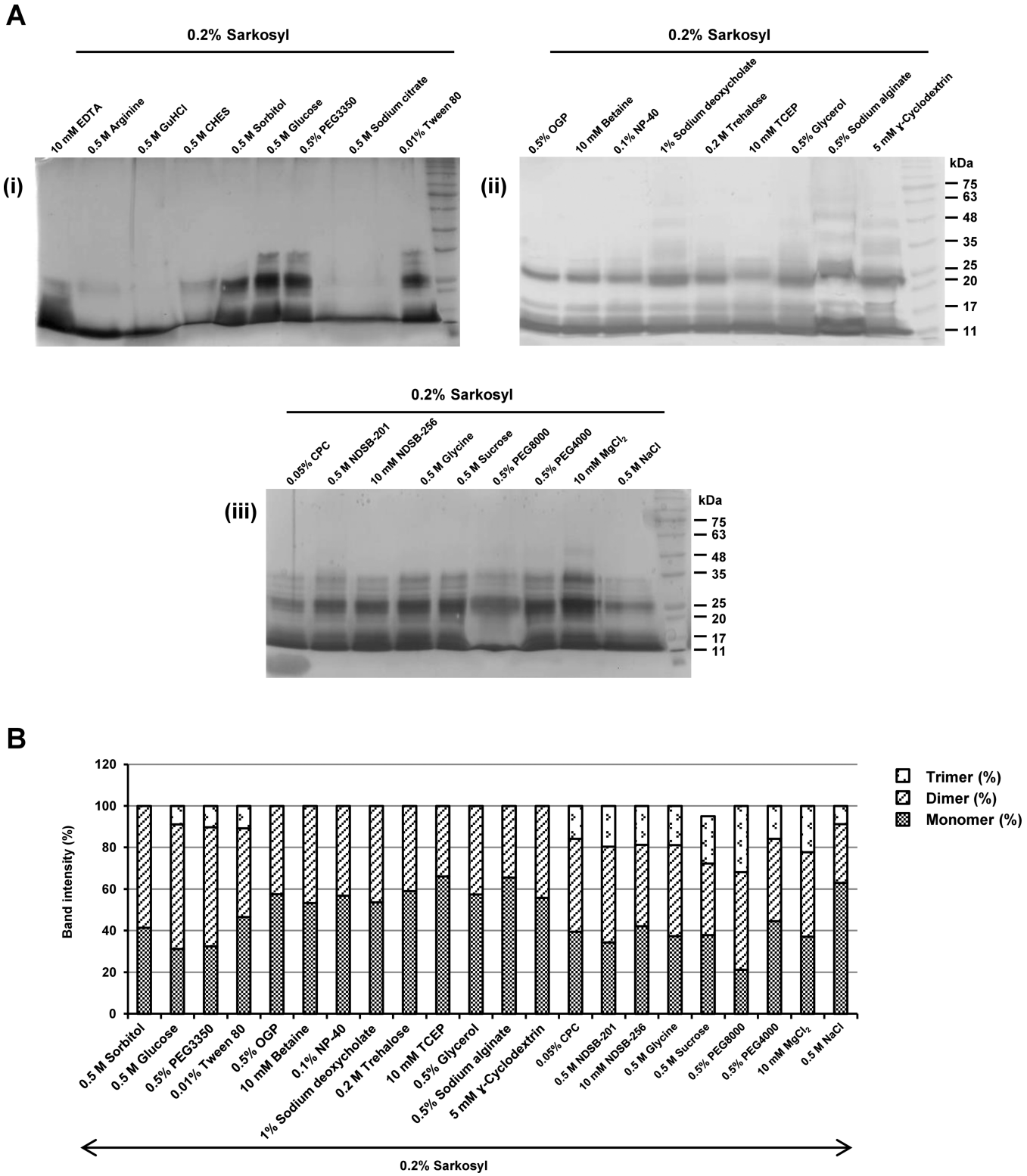

Figure 4. Effects of the combination of an additive with 0.2% sarkosyl on BMP-2 refolding. (A) BMP-2 (0.1 mg) was refolded in combinations of an additive and 0.2% sarkosyl in 1 mL of the refolding buffer containing 100 mM Tris, pH 8.5, 2 mM GSH, and 4 mM GSSG and separated by nonreducing SDS-PAGE with silver staining. (B) Band intensity analysis of BMP-2 from the different combinations using the ImageJ program.

control although the BMP-2 dimers could clearly be observed (Figure 6A,B).

Bioactivity of the Purified BMP-2 Proteins. To evaluate the bioactivity of the purified BMP-2, the protein was refolded in the selected refolding buffer containing 0.05% SDS and 0.5% glycerol (RF2; Table 2) and then purified before testing (Figure S1). The purified BMP-2 that had been refolded in RF2 was analyzed using SDS-PAGE as shown in Figure 7A. The result showed that the purification using a heparin column (henceforth designated HP-) failed to separate the dimers from the monomers (Figure 7A, lane 3). Four bioactivity tests were conducted to examine the BMP-2 activity. These include the

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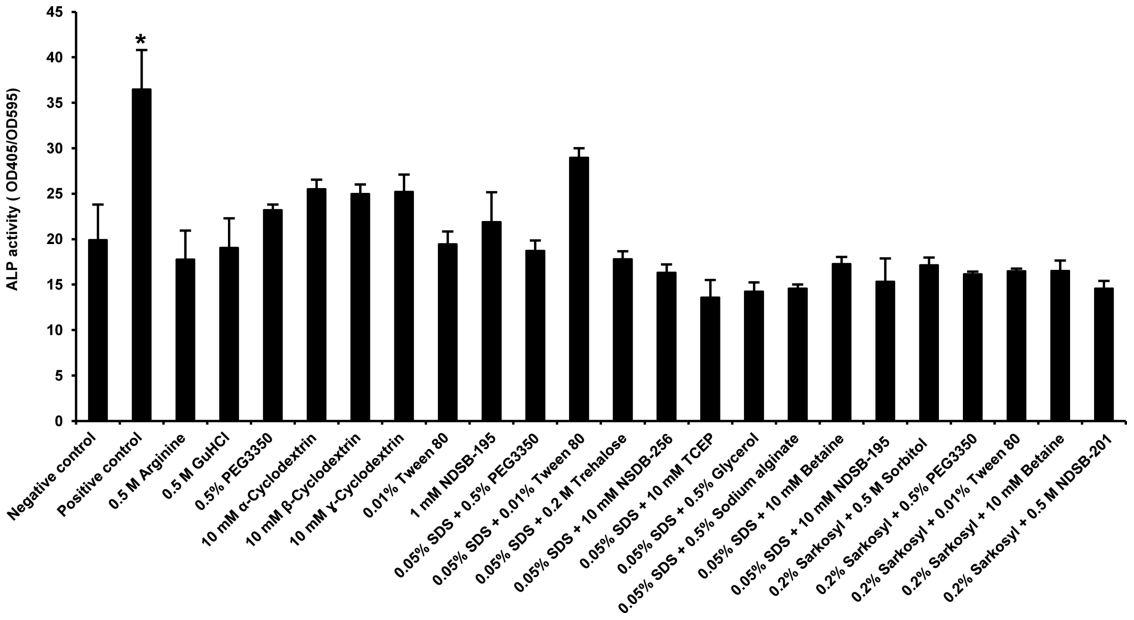

Figure 5. Effect of BMP-2 crude proteins on the ALP activity. The crude proteins (10 μL) from the different additives were added onto the SaOS-2 culture to a final concentration of 2 μg/mL, and the ALP activity was determined. The positive control was 100 ng/mL commercial rhBMP-2. The data were reported as mean ± SD (n = 3). *p < 0.05 versus the negative control.

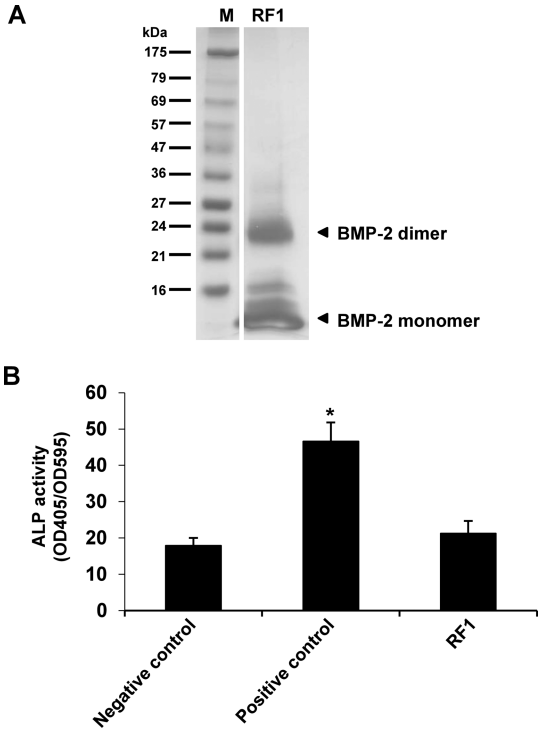

Figure 6. Large-scale BMP-2 refolding and the bioactivity of the crude protein. (A) BMP-2 (1 mg) was refolded in 1 mL of the refolding buffer containing 0.2% sarkosyl, 100 mM Tris, pH 8.5, 2 mM GSH, and 4 mM GSSG (RF1). The refolded BMP-2 was analyzed by nonreducing 12% SDS-PAGE with silver staining. (B) ALP activity of SaOS-2 cells treated with 100 ng/mL BMP-2 crude protein or 100 ng/mL commercial rhBMP-2 (positive control) for 7 days. The data were reported as mean ± SD (n = 3). *p < 0.001 versus the negative control.

luciferase reporter assay, ALP assay, Alizarin Red assay, and intracellular calcium assay. The luciferase reporter assay showed that the positive control and HP-RF2 significantly increased the luciferase activity compared to the negative control (Figure 7B). The Alizarin Red assay, which was performed to determine extracellular calcium deposition, showed that the positive control and HP-RF2 significantly increased the extracellular calcium deposition (Figure 7C). However, the ALP assay and intracellular calcium assay showed that the proteins refolded in RF2, either crude or purified, exhibited no significant difference in the bioactivity compared to the negative control (Figure 7D,E).

DISCUSSION

Expression of BMP-2 in *E. coli* mostly generates IBs that need to be purified and refolded into disulfide-linked dimers. Formation of the dimers are often indicative of its bioactivity.[16,17] Thus, the refolding is a critical step to obtain the fully functional recombinant protein. The in vitro protein refolding from IBs is affected by many factors such as protein concentrations, solubilizing methods, refolding buffer composition, pH, refolding temperature, redox conditions, and the ionic strength.[17,25,31] The dilution method is one of the refolding methods used for recovery of bioactive protein form IBs and mostly uses additives for correcting unfolded and/or misfolded protein into the correctly folded protein. In this study, we investigated various additives for BMP-2 refolding using single additives and the combinations of an additive with an anionic detergent, including SDS and sarkosyl. BMP-2 was expressed in *E. coli* as aggregated protein and dissolved in a high urea concentration, which denatured the native proteins. Purification of BMP-2 with an SP FF column revealed that soluble BMP-2 was mostly found as an inactive monomeric form (Figure 1). BMP-2 was then refolded in a minimal buffer containing only Tris buffer and glutathione (GSH/GSSG) for a redox environment. The dimer formation was screened in various additives such as reducing agents, ionic detergents, zwitterionic detergents, nonionic detergents, amino acids, polyol osmolytes, sugars, chaotropic reagents, etc. as shown in Table 1.

Among 178 refolding buffers, only 79 refolding buffers could clearly generate the BMP-2 dimers. The high yields of the BMP-

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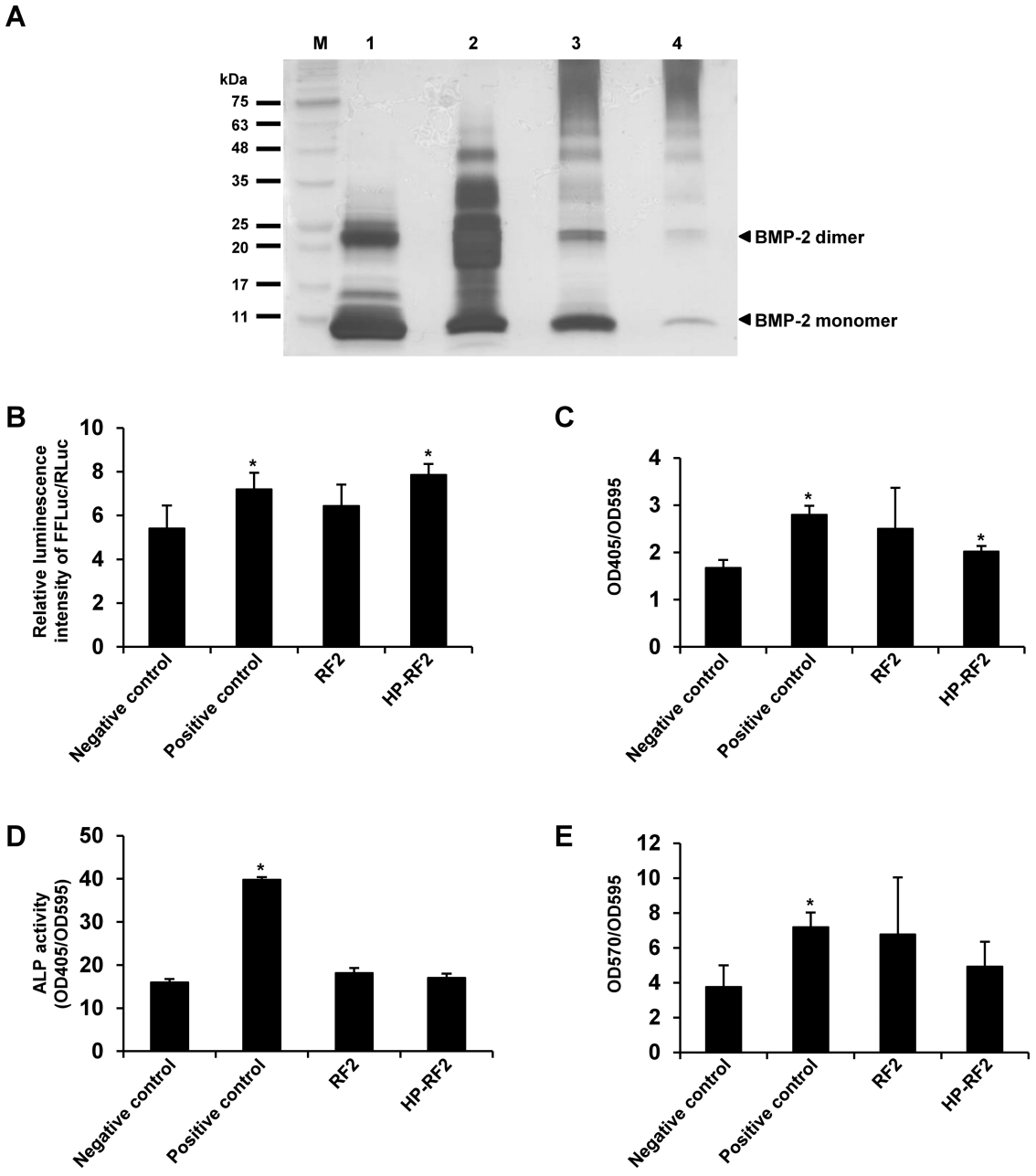

Figure 7. Purified BMP-2 and its bioactivities. (A) BMP-2 (4 mg) was refolded and purified before analyzing with nonreducing 12% SDS-PAGE with silver staining. Lane 1: the BMP-2 refolded in 0.05% SDS and 0.5% glycerol (RF2). Lane 2: the BMP-2 refolded in 0.05% SDS and 0.5% glycerol and dialyzed in 10 mM acetic acid. Lane 3: the refolded BMP-2 purified with a heparin column (HP-RF2). Lane 4: the purified BMP-2 dialyzed in 10 mM acetic acid. (B–E) Bioactivity assays when the SaOS-2 cells were treated with 100 ng/mL crude (RF2), or purified BMP-2 (HP-RF2), or 100 ng/mL commercial BMP2 as a positive control, and the untreated cells were the negative control. Each bar represents mean ± SD of the data obtained from three independent experiments. *$p < 0.05$ versus negative control. (B) Luciferase assay. (C) Extracellular calcium assay. (D) ALP activity assay. (E) Intracellular calcium assay.

2 dimers were observed under various refolding conditions, which were affected by types and concentrations of the refolding additives (Figures 234). The highest yield of the dimers was observed in the refolding buffers that contained an ionic detergent such as sarkosyl or CPC (Figure 2A, xii). Gabrielczyk et al. refolded the fructosyltransferase enzyme and found renaturation of the unfolded protein depending on the ionic strength and the cosolvent charge density.[36] Mizukami et al. reported that increasing the ionic strength could accelerate refolding and slow down unfolding of SAMP1 proteins.[37] These results suggest that the high refolding yield might involve the ionic strength and the ion charge density of the proteins and the refolding additives. Conversely, refolding buffers containing α-, β-, or γ-cyclodextrin attenuated the BMP-2 dimer formation and led to trimer formation (Figure 2A, vii). Cyclodextrins are cyclic oligosaccharides with hydrophobic cavities that could interact with unfolded proteins. Cyclodextrins have been used for protein refolding because they could inhibit protein aggregation during the refolding process.[24,38]

In addition, the refolding buffers that contained sugars (sorbitol, sucrose, and glucose) could induce BMP-2 oligomer formation (Figure 2A, viii, ix), and the addition of 0.5% SDS and

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0.02% sarkosyl increased the dimer fraction and reduced the oligomer fractions (trimers and tetramers) (Figures 3 and 4). The addition of 0.02% sarkosyl into the refolding buffers containing polyol osmolytes such as PEG3350 or PEG8000 could increase the percentage of the BMP-2 dimers. Sugars and polyol osmolytes are known to enhance the protein stability.[26,39,40] Previous literature studies have reported that glycerol, sorbitol, and trehalose are the efficient cosolvents for protein refolding because they reduce protein aggregation.[40,41] Similar results were also observed in our study where 0.5% SDS was added into the refolding buffer containing glycerol, trehalose, glucose, sorbitol, or sucrose (Figure 3A,B). SDS has generally been considered as a strong denaturing surfactant. However, a report suggested that the presence of SDS in the refolding buffers could improve protein refolding due to the reduction of electrostatic repulsion.[42] The experiments by Jafari et al. indicated that the SDS molecules could stabilize the protein native state at low temperature.[43] In contrast to SDS, sarkosyl is a mild detergent used for protein solubilization, and it does not have the denaturing effect. Sarkosyl has been reported to improve the yield of bioactive proteins from IBs.[44]

The bioactivity test was conducted using crude BMP-2 which had been refolded under various conditions. We did not find any significant difference of the ALP activity induced by the crude proteins (Figure 5). Noteworthy, the crude proteins might contain monomeric, dimeric, and oligomeric forms of BMP-2. We also noticed that the ALP activity tended to increase in the refolding buffers containing 0.05% SDS, 0.01% Tween 80, and α-, β-, or γ-cyclodextrin. All of these results showed that the ALP activity was not promoted even though the BMP-2 dimers were present. The positive control, however, significantly induced the ALP activity. We hypothesized that the cysteine knot might not properly form even though the protein dimerized. This implied that correct folding of the BMP-2 dimers may be more important than the dimer yield.

To confirm that the lack of the ALP activity was not due to unequal amounts of the dimeric BMP-2, we performed the ALP activity assay, the luciferase reporter assay, and the calcium assays using the BMP-2 purified from a heparin column at the same concentration as the positive control. Similar to the crude proteins, the purified BMP-2 did not exhibit biological activities in the ALP or intracellular calcium assay (Figure 7D,E). Although the heparin column did not enrich the dimers, there clearly was still a significant amount of dimers. This observation suggested that the dimeric BMP-2 that we obtained was not identical to the biologically active dimeric BMP-2 that could be enriched by a heparin column.[17] We treated the cells in our bioassays with 100 ng/mL of either purified (HP-RF2) or commercial BMP-2 (positive control; made from the CHO cell line). Growth factors can typically work at low concentrations; thus, we expected our BMP-2 to show its activity in the ALP assays at the concentration used. However, the refolded BMP-2 did not show an activity as high as the positive control. This could be because our purified protein contained a smaller fraction of the biologically active BMP-2 dimers than the positive control. Our refolded BMP-2 dimers might be a mix of correctly and incorrectly folded BMP-2. Moreover, our purified protein did show the activity in the luciferase assay (Figure 7B) and the extracellular calcium assay (Figure 7C). This further supports our hypothesis that our protein contains both active and inactive forms. Therefore, a better separation technique will be required to separate the active dimers from the inactive form although the dimeric fraction can be enhanced by our refolding conditions. It is also worth noting that nonreducing SDS-PAGE is a rapid technique to detect BMP-2 dimers, but it may not be suitable for distinguishing the different folding states of BMP-2 because it comprises many cysteines that could incorrectly form disulfide-linked dimers.

In conclusion, we have screened single and combinations of additives for BMP-2 refolding with the aim to identify novel conditions that improve the BMP-2 dimer yield. The refolding buffers that contained ionic detergents could increase the yield of BMP-2 dimers. Moreover, using ionic detergents such as sarkosyl or SDS in combination with other additives seemed to reduce the oligomeric fractions. We exhaustively screened common additives used for protein refolding. However, we examined only one additive at a time during the initial screening. Therefore, a combination of multiple additives could be attempted. Still, as demonstrated in our work, SDS-PAGE is a suboptimal assay to screen for the biologically active BMP-2. Thus, a higher throughput bioactivity assay for functional BMP-2 should be employed. For example, refolded BMP-2 solution could be added to BRITER cells.[45] Functional BMP-2 should result in detectable luminescence signals. Our results highlighted that the BMP-2 dimers, either crude or purified, should not be assumed functional. Hence, the nonreducing SDS-PAGE is an inadequate technique to judge the functionality of the disulfide-linked BMP-2 dimers.

CONCLUSIONS

We screened each of 178 chemical additives added into a minimal refolding buffer and found that only 49 additives could give the BMP-2 dimers detected in nonreducing SDS-PAGE. Sarkosyl and CPC gave the highest dimer yield, whereas sugars, oligosaccharides, and cyclodextrins resulted in low yields. Combinations of sarkosyl or SDS with other additives could reduce the trimers and tetramers, but the combinations must be selected well because some combinations such as sarkosyl and EDTA or SDS and β-cyclodextrin did not give dimers.

Our refolded BMP-2 showed the activities in the calcium deposition assay and the luciferase reporter assay but not in the alkaline phosphatase activity assay or the intracellular calcium assay. These results suggest that the BMP-2 dimers detected in SDS-PAGE should not be assumed functional, and the nonreducing SDS-PAGE is an inadequate technique to judge the functionality of the disulfide-linked BMP-2 dimers.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c05802.

Percentage of BMP-2 oligomers in various refolding buffers (Table S1) and purification of BMP2 on HiTrap Heparin HP (Figure S1) (PDF)

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Notes

The authors declare no competing financial interest.

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