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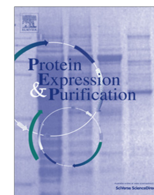

On-column refolding of bone morphogenetic protein-2 using cation exchange resin

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ABSTRACT

Refolding is often the bottle-neck step in producing recombinant proteins from inclusion bodies of *Escherichia coli*, especially for dimer proteins. The refolding process is protein specific, engaging a lot of time and cost to optimize conditions so that the thermodynamics favor protein refolding over competitive aggregation. Bone morphogenetic protein-2 (BMP-2) is a potent osteogenic agent having significant applications in bone regeneration therapy. In this study, we present a novel solid-phase refolding method for rapid and efficient refolding of recombinant BMP-2 dimer from *E. coli*. We employed a weak cation exchange resin as the adsorbing support, with decreasing gradient of denaturing agent and exposure to oxidizing conditions for adequate disulfide bond formation. Refolded BMP-2 was further purified using size exclusion chromatography and analyzed for its secondary structure and biological activity. The purified BMP-2 dimer showed dose-dependent induction of alkaline phosphatase (ALP) activity in MC3T3 pre-osteoblast cells, thus translating the success of our refolding method. This simple and rapid method can also be applied in refolding and purification of other BMP-2 like dimer proteins.

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Introduction

Recombinant DNA technology has revolutionized the development of natural and modified protein and peptide drugs resulting in growth of the biopharmaceutical industry over the last 30 years [1,2]. Knowledge and availability of different cell systems, genetic engineering of traditional hosts and advances in the techniques to eliminate process bottle necks have opened doors to efficient production at laboratory as well as industry scale [3,4].

Bone morphogenetic proteins (BMPs[1]) are an important class of therapeutic proteins involved in many biological processes of cell growth, differentiation, and regulation of cell-matrix interactions [5] thus playing an important role in morphogenesis and regeneration of tissues and organs [6]. BMPs have shown clinical significance in bone and cartilage regeneration and fracture repair therapies. US FDA has approved the use of BMP-based treatment for spinal fusions, long bone non-unions and open tibial fractures [7,8]. BMP-2 is a potent osteogenic dimer protein made of two identical beta sheet polypeptide chains linked covalently in a head-to-tail manner by a disulfide bridge. Each monomer consists of three intramolecular disulfide bonds and forms the classic cystine-knot assembly [9].

BMP-2 was first isolated and identified from demineralized bone matrix by Marshall Urist et. al. in 1970s [10]. Its extraction procedure was elaborate and laborious with poor recovery and the protein presented risks of immunological responses due to allogenic source. In late 1980s, molecular cloning was used to obtain recombinant human BMP-2 from eukaryotic expression systems like mammalian (CHO) cells [11,12]. Post-translational modifications in the eukaryotic host aids disulfide bond formation and protein folding, but has disadvantages of incomplete monomer processing, being time intensive, expensive and giving low yields with high variability [13]. Some researchers have reported BMP-2 production in transgenic plants [14] and transfected insects [15], but mentioned similar problems. Bacteria are the most popular expression hosts for the cloning of human genes. They offer many advantages such as rapid growth, high yields, low cost and ease of manipulation [3]. However, the prokaryotic system lacks inherent machinery to perform post-translational modifications and often lead to misfolding and formation of inclusion bodies (IBs). *In vitro* refolding and purification thus play a key role in restoring the native structure and biological activity of recombinant proteins extracted from IBs.

Refolding conditions can be created using renaturing aids such as 3-(1-pyridinio)-1-propanesulfonate (PPS), pyridine-3-sulfonic acid (PSA), 2-(cyclohexylamino)ethanesulfonic acid (CHES),

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[1] *Abbreviations used:* BMP, bone morphogenetic protein; ALP, alkaline phosphatase; CHO, Chinese hamster ovary; LB, Luria-Bertani medium; OD_{600}, optical density at 600 nm; IPTG, isopropyl β-D-1-thiogalactopyranoside; IB, inclusion body; PMSF, phenylmethylsulfonyl fluoride; GSH/GSSG, reduced and oxidized glutathione; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; CV, column volume; MWCO, molecular weight cut off; PVDF, polyvinylidene difluoride; TBST, tris-buffered saline with 0.1% Tween 20; CD, circular dichroism; α-MEM, minimum essential media with alpha modification; FBS, fetal bovine serum; TGF-β, transforming growth factor-β; GDF, growth differentiation factor; VEGF, vascular endothelial growth factor.

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nicotinic acid (NA) [16], oxidized-reduced glutathione [17–20], cystine-cystiene [21] , dithiothreitol [22]; along with aggregation suppressors like L-arginine [22], polyethylene glycol [23], glycerol [24]. Dilution is a simple and most commonly used refolding technique often presenting some serious limitations of long process time, multiple steps, need of large buffer volumes and vessels, and low protein loading per batch resulting in poor yields. More recently developed chromatography-based refolding approaches, such as (i) reversible adsorption onto resin followed by removal of denaturing agents, (ii) solvent-exchange by size exclusion chromatography, and (iii) refolding on column with immobilized chaperones, have demonstrated to be effective in producing high yields of refolded protein [25].

In an attempt to minimize the process time and number of steps with improvement in refolded protein quality and yield, we investigated the design and development of a solid phase refolding technique for the production of recombinant BMP-2 dimer. This study describes an on-column refolding method using weak cation exchange resin based on the principle of adsorption of unfolded BMP-2 monomer *via* weak ionic interactions. BMP-2 monomer exhibits low binding affinity towards the column resin as compared to dimer. This concept was manipulated to favor dimerization and used for efficient isolation of the refolded product. It is a novel approach highlighting the use of ion exchange chromatography for refolding of BMP-2 dimer with multiple disulfide linkages. This protocol can be readily adapted, with customized modifications, for rapid *in vitro* refolding and purification of other dimer proteins.

Materials and methods

Cloning of BMP-2 gene

The cDNA of human BMP-2 gene was purchased from Origene, MD. BMP-2 gene was amplified using forward and reverse primers by PCR. The forward primer contained Nde I restriction site and sequence of 5′–3′ CCAGGCATATGCAAGCCAAACACAAACAG. The reverse primer contained Xho I and a sequence of 3′–5′ TATCTTCTCGAGCTAGCGACACCCACAACCCTC. The amplified product was cloned into pET-41b(+) vector (Novagen, MA) and the new plasmid was named pBMP-2. The cloned BMP-2 gene was verified by DNA sequencing.

Expression of BMP-2 in Escherichia coli

The engineered pBMP-2 plasmid was transformed into BL21(DE3) *E. coli* (Novagen, MA) strain. Positive clones were obtained on LB agar plates containing 50 μg/ml kanamycin as antibiotic selector. Pre-cultures were prepared by picking a single colony and transferring it to 100 ml LB medium containing 50 μg/ml kanamycin. The pre-cultures were incubated at 37 °C overnight at 250 rpm. Volume of pre-culture equivalent to final OD_{600} of 0.1 was used to seed 1 L LB medium containing 50 μg/ml kanamycin. The cultures were grown at 37 °C, at 250 rpm agitation for 2–3 h until OD_{600} reached 0.6. Thereafter, BMP-2 expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM and further cultured for 3 h at 37 °C. Protein expression levels were monitored by SDS–Polyacrylamide gel electrophoresis (SDS–PAGE) with Coomassie blue detection.

Cell harvesting and isolation of inclusion bodies

BMP-2 overexpressed cells were collected by centrifugation at 5000 rpm for 15 min at 4 °C. Cell pellet was washed with detergent buffer (50 mM Tris–HCl pH 8.0, 500 mM NaCl, 1 mM EDTA, 0.1% Triton-X) and detergent-free buffer (without Triton-X) thrice each, to remove membrane proteins and other cellular impurities. The washed pellet was stored at −80 °C until further used. Two grams of the frozen cell pellet was resuspended in 10 ml of detergent free buffer containing lysozyme (1:100) and PMSF (1:200) on ice for 60 min to lyse the cell membrane. Further the cell suspension was subjected to 2 freeze–thaw cycles of 15 min each. The cells were disrupted by brief sonication for 15 s followed by incubation on ice for 1 min. This was repeated 4 times until the cell suspension became viscous. To aid separation of the lysed cell fractions, DNA digestion was carried out using Benzonase® (1:2000) and 2 mM $MgCl_2$ at 4 °C for 60 min. Inclusion bodies were isolated by centrifuging the lysed cell suspension at 12,000 for 30 min at 4 °C. IBs were washed using buffer containing 50 mM Tris–HCl pH 8.0, 1 M NaCl, 1 mM EDTA, 0.05% Triton X-100. IBs were solubilized for 24 h at room temperature with constant stirring in 20 ml solubilization buffer containing 50 mM Tris–HCl pH 8.0, 100 mM DTT, 1 mM EDTA with 8 M urea as denaturing agent. Insolubilized fractions were separated by centrifugation at 12,000 rpm for 30 min. The solubilized supernatant was recovered and stored in working aliquots at −80 °C.

Refolding of BMP-2 on cation exchange column

The solubilized BMP-2 was refolded on a cation exchange column by gradually removing urea in the presence of reduced (GSH) and oxidized (GSSG) glutathione. Bio-rex® 70 CM (Bio-rad, CA) resin (2 ml) was packed in disposable poly-prep column and balanced using 10× column volume (CV) of 50 mM Tris HCl pH 8.0, 50 mM NaCl, 100 mM DTT, 8 M urea. Solubilized protein was applied at concentration of 0.5 mg/ml of column resin and allowed to bind overnight at 4 °C. Unbound protein concentration was determined by colorimetric assay. The column was subjected to 10× CV refolding buffer consisting 50 mM Tris–HCl pH 8.0, 10 mM NaCl, 1 mM oxidized glutathione (GSSG), 1 mM (condition 1) or 3 mM (condition 2) reduced glutathione (GSH), 10% glycerol and urea in decreasing steps from 8 M to 4 M to 2 M. The three refolding buffers were introduced one after other at a constant flow rate of 4 ml/h. Thereafter, the refolded protein was eluted using 2× CV elution buffers with step increase in salt concentration at 0.15, 0.5, 1 and 2 M NaCl. Eluted protein fractions were concentrated using ultrafiltration tubes MWCO 5000 (Millipore Corporation, MA) and dialyzed against 100× volume of dialysis buffer (with 3 buffer changes) containing 20 mM Tris–HCl pH 8.0, 150 mM NaCl, 10% glycerol and 2 M urea to remove excess salt. Protein concentration was determined by Bio-rad Bradford assay and refolding efficiency was assessed by SDS–PAGE with silver staining.

Protein polishing using size exclusion chromatography

The dialyzed refolded BMP-2 protein pools were further polished by superose 12 HR 10/30 (GE Healthcare, PA) size exclusion column. The column was first calibrated using molecular weight protein standards (Sigma, PA). One hundred microliters of 0.185 mg/ml sample was loaded on to the column and was run using 20 mM Tris–HCl pH 8.0, 150 mM NaCl, 10% glycerol and 2 M urea at a flow rate of 0.5 ml/min. Fractions were collected and analyzed using immunoblotting. Fractions containing BMP-2 dimers were concentrated and dialyzed against 10 mM citrate buffer pH 3.6 with 10% glycerol and stored at −20 °C.

Protein concentration analysis

Protein concentration was measured by Bradford protein assay (Bio-rad, CA). Absorbance was detected at 595 nm using UV/vis

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