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A novel *TWO-STEP* renaturation procedure for efficient production of recombinant BMP-2

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ABSTRACT

Bone morphogenetic proteins (BMPs) stimulate bone formation and thus constitute important protein therapeutics. Here, a novel procedure is presented which allows fast and efficient production of biologically active BMP-2 via a *TWO-STEP* procedure: the conditions are designed such that the first step favors formation of monomeric species with the correct intramolecular disulfide bridges, the conditions of the second folding reaction stimulate the formation of the intermolecular disulfide bridge. The short processing times and increased yields compared to previously published procedures allow low-cost production of this important protein drug.

Introduction

The protein family of bone morphogenetic proteins (BMPs)[1] is a subgroup belonging to the transforming growth factor (TGF)-β superfamily of multifunctional cytokines. To date over 20 BMPs have been identified and characterized. BMPs are known to induce the differentiation pathway of pluripotent mesenchymal cell lines, such as C3H10T1/2 or C2C12, into osteoblastic or chondrocytic lineage [1,2]. Besides their roles in bone formation, several central biological functions have been attributed to BMPs. Depending on the tissue in which they are expressed, BMPs regulate organ development and pattern formation in primordial germ cells [3]. BMP-2 is among the best studied members in the BMP family. Its ability to induce de novo bone formation [4–7] renders BMP-2 a therapeutic protein. Application in human has been approved by the FDA in 2002. BMP-2 is commercially available under the product name InductOs®. Due to its bone inducing activity, BMP-2 is used as an alternative to bone autografting during the healing of critical fractures, for spinal fusions or the treatment of bone defects [8]. Thus, there is a large demand for biological active BMP-2.

Native BMP-2 is a homodimeric protein. Each monomer possesses a characteristic disulfide pattern, the cystine knot [9], consisting of two disulfide bridges that connect the polypeptide backbone to a ring through which a third disulfide bridge threads. This arrangement stabilizes the monomer which lacks a buried hydrophobic core that is common for most globular proteins [9]. The two monomers are arranged in a head-to-tail manner. The interface between the two monomers is stabilized by hydrophobic interactions and an intermolecular disulfide bond.

Several expression and production systems have been published for recombinant human BMP-2. It is for example possible to obtain constitutively active BMP-2 from mammalian cell cultures [10,11]. However, expensive cultivation and poor yields render this procedure cost-intensive, especially at the industrial scale. In another experimental approach, transgenic tobacco plants were constructed for eukaryotic expression system for BMP-2 [12]. However, also with this system extended growth periods and expression times, besides the requirement of a four-step purification protocol, limit its transfer to the industrial production.

Production in prokaryotic hosts is usually the method of choice when post-translational modifications – as in case of BMP-2 – do not matter. The advantages of the prokaryotic system are (i) high yield, (ii) low costs and (iii) high bio-safety. Since natively folded BMP-2 requires the existence of the correct disulfide connectivities and because disulfide bond formation does usually not occur in the reducing intracellular milieu, BMP-2 accumulates in form of inclusion bodies (IBs) in bacterial hosts. Active BMP-2 can be obtained by in vitro renaturation of solubilized IB protein. Optimization of protein refolding is crucial for industrial applications because the most efficient – low cost – strategy determines market competitiveness.

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[1] *Abbreviations used:* AP, alkaline phosphatase; BCA, bicinchononic acid; BMP, bone morphogenetic protein; FBS, fetal bovine serum; GSH/GSSG, reduced and oxidized form of glutathione; IBs, inclusion bodies; MWCO, molecular weight cut-off; PBS, phosphate buffered saline; RT, room temperature; vol., volumes.

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Here, we present a novel protocol for the oxidative folding of BMP-2 from IB protein. The protocol involves a *TWO-STEP* refolding process of which the first reaction facilitates the formation of the intramolecular cystine knot and the second reaction promotes dimer formation involving the formation of the intermolecular disulfide bridge. The protocol described below allows BMP-2 production with improved yields and reduced processing times.

Materials and methods

Recombinant expression of BMP-2 in Escherichia coli cells

Host for recombinant expression of the cDNA for BMP-2 was the *Escherichia coli* strain BL21 (DE3). Recombinant DNA for BMP-2 was inserted into pET11a (Novagen). The expression strain also possessed an additional plasmid, pUBS520, with the gene *dnaY*. The gene encodes a tRNA that recognizes the codons AGG/AGA for arginine. These codons are rare in *E. coli,* but abundant in the cDNA for BMP-2. Thus, the presence of this plasmid allowed to minimize codon usage problems [13]. Recombinant protein production was performed by fermentation as published before [14]. The fermentation medium consisted of: 54 g/l yeast extract (OHLY KAV; DHW, Cologne, Germany), 0.54 g/l NH_4Cl, and 12 g/l glycerol. The pH was adjusted to 7.0–7.4 with K_2HPO_4. Addition of 2.8 mM $MgSO_4$ and 0.01% thiamine occurred by sterile filters. Ampicillin and kanamycin were present at final concentrations of 100 and 50 μg/ml, respectively. Feeding was started at $OD_{600} \sim 20$. Gene expression was induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at $OD_{600} \sim 60$. Three hours after induction, cells were harvested by centrifugation and stored at −80 °C.

Inclusion body preparation and solubilization

Inclusion body (IB) isolation and solubilization was performed as described previously [14,15]. In a typical experiment, 20–30 g cell wet weight was homogenized in 100–150 ml 0.1 M Tris/HCl, pH 7, 1 mM EDTA at 4 °C. Lysozyme was added at a concentration of 1.5 mg per gram biomass followed by an incubation at 4 °C for 30 min. Cell disruption was carried out by high pressure homogenization. In order to digest DNA, $MgCl_2$ was added to a final concentration of 3 mM and Benzonase® (MERCK KGaA, Darmstadt, Germany) to a final concentration of 80 U/ml. After incubation for 30 min at room temperature 0.5 vol. of 60 mM EDTA, 6% (v/v) Triton X-100, 1.5 M NaCl, pH 7 was added to the suspension followed by another incubation for 30 min at 4 °C. Inclusion bodies were spun down by centrifugation at 31,000*g* for 10 min at 4 °C. The pellet was resuspended in 8 ml 0.1 M Tris/HCl, pH 7, 20 mM EDTA per gram cell wet weight. This centrifugation step was repeated at least 4 times. Finally, the mass of the IB pellet was determined. For solubilization and reduction, per 100 mg IB pellet, 10 ml 6 M GdmCl, 0.1 M Tris/HCl, pH 8.5, 1 mM EDTA, 100 mM DTT was added. After 2 h of incubation at RT, the pH was lowered by dropwise addition of HCl (25% w/v) to 3–4 to inhibit disulfide bond formation. Residual insoluble material was removed by centrifugation (10 min, 10,000*g*, 4 °C). DTT was completely removed by dialysis against 10- to 20-fold vol. of 5 M GdmCl, pH 3–4 at 4 °C. The dialysis step was repeated at least 4 times. Protein concentration was crudely determined by the absorption at 260 and 280 nm according to the equation:

$$c(\mathrm{mg/ml}) = 1.552 \times A_{280} - 0.757 \times A_{260}$$

Typically, the protein concentration was 20–30 mg/ml at this step of the preparation. The subsequent renaturation is detailed in the Results and discussion section.

Concentration of protein solutions by cross-flow filtration

To lower the volume of the renaturation batch, the protein solution was concentrated 5-fold using a VivaFlow 200 flipflow filtration device, MWCO 10,000 Da (Sigma–Aldrich, Munich, Germany).

Purification of native species after renaturation

After refolding, the protein solution was dialysed against 6 M urea, 100 mM Tris/HCl, 5 mM EDTA, pH 6.0. Although 6 M urea leads to denaturation of BMP-2, its presence during heparin affinity chromatography was found to be beneficial for a good separation of the dimeric from the monomeric species [14]. Since disulfide bridges remain intact under these slightly acidic conditions, the dimeric species quickly refolds upon transfer to acetate buffer. Dimeric BMP-2 eluted at a NaCl concentration of 0.7 M. Homogenous fractions were pooled, dialysed against 20 mM NH_4-acetate, pH 4.8 and lyophilized. Restoration of the freeze-dried BMP-2 was done with 50 mM Na-acetate, pH 4.8 to protein concentrations of 0.5 mg/ml.

Test for biological activity of BMP-2

Biological activity of BMP-2 was analyzed by alkaline phosphatase (AP) induction in C2C12 cells as described previously by us and others [16,17]. C2C12 cells (DSMZ, Braunschweig, Germany) were maintained in RPMI 1640 medium (PAA Laboratories GmbH, Cölbe, Germany) supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. To allow differentiation, which is accompanied by induction of AP, the serum concentration was reduced to 2%.

2×10^3 cells per well were seeded into 96-multiwell plates. After cells had been allowed to attach over night, complete medium (10% FBS) was replaced by 200 μl differentiation medium (2% FBS) supplemented with BMP-2. After 4 days, the medium was removed, cells were washed with PBS and lysed in 100 μl lysis buffer (100 mM glycine/Na^+, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 9.6, 1% Nonidet P-40) by gentle shaking at RT for 2–3 h. One hundred and eighty microliters substrate solution (9 mM *p*-nitrophenyl-phosphate in lysis buffer) was added to 20 μl of the lysis solution. The reaction mixture was incubated at 37 °C. Changes in absorption at 405 nm were followed over 30 min using an ELISA-plate reader and corrected against the total protein concentration determined by BCA-assay kit (Thermo Fisher Scientific, Bonn, Germany).

Results and discussion

TWO-STEP renaturation protocol allows production of BMP-2 with good yields

BMP-2 production from prokaryotic hosts requires refolding from IB protein because disulfide bonds do not form in the reducing cytosol. A previous protocol published by our lab involved renaturation times of up to 14 days [14]. These long incubation times are extremely cost-intensive and therefore have to be avoided in industrial processes. We speculated it was not the formation of the cystine knot, but rather the formation of the intermolecular disulfide bond when two BMP-2 monomers assemble to the dimeric species, that would constitute the rate limiting step and cause the long renaturation times (Fig. 1B). Principally, dimerization or intermolecular disulfide bond formation can be stimulated by high protein concentrations. However, high protein concentrations during renaturation often lead to protein aggregation because aggregation is usually a higher order process. The rationale of the protocol presented here was to establish refolding conditions in a way that in a first reaction the formation of the intramolecular cystine knot of BMP-2 should be favored, and in a