Properties of dimeric, disulfide-linked rhBMP-2 recovered from *E. coli* derived inclusion bodies by mild extraction or chaotropic solubilisation and subsequent refolding

Bastian Quaas^a, Laura Burmeister^{b,c}, Zhaopeng Li^a, Manfred Nimtz^d, Andrea Hoffmann^{b,c}, Ursula Rinas^{*a, d}

^a Leibniz University of Hannover, Technical Chemistry – Life Science, Hannover, Germany

^b Hannover Medical School, Laboratory of Biomechanics and Biomaterials, Department of Orthopedic Surgery, Hannover, Germany

^c Lower Saxony Center for Biomedical Engineering, Implant Research and Development, Hannover, Germany

^d Helmholtz Centre for Infection Research, Braunschweig, Germany

*Corresponding author:

Helmholtz Centre for Infection Research, Inhoffenstraße 7, D-38124, Braunschweig, Germany. Tel: + 49 531 6181 7014, Fax: + 49 531 6181 7099, Email:

Ursula.Rinas@helmholtz-hzi.de

ABSTRACT

Recombinant human bone morphogenetic protein-2 (rhBMP-2), a cystine-knot containing disulfide linked homodimer, was produced in form of inclusion bodies (IBs) using *E. coli* BL21 (DE3) and SHuffle T7 Express. Non-reducing SDS-PAGE analysis revealed that rhBMP-2 was present within IBs in both strains as monomer and in form of disulfide-linked dimers. Purified dimeric disulfide-linked rhBMP-2 was obtained from IBs by two different methods. The first method involved classical solubilisation using strong denaturants and subsequent refolding. The second method involved mild extraction without refolding. Both rhBMP-2 dimer variants were purified by Heparin-affinity chromatography. Mildly extracted rhBMP-2 was further purified by size-exclusion chromatography. The resulting dimeric rhBMP-2 variants were studied regarding bioactivity and folding status. In contrast to the rhBMP-2 dimer obtained by classical refolding it was shown that the disulfide-linked dimer obtained by mild extraction was not correctly folded e.g. the hydrophobic core not correctly formed. Moreover, refolded dimeric rhBMP-2 was bioactive and the mildly extracted dimeric rhBMP-2 did not show any bioactivity. Disulfide-bond analysis revealed that the intricate disulfide-bond pattern of the complex cystine-knot scaffold was not present in the IB embedded disulfide-linked rhBMP-2 dimer but was formed later during classical refolding of the reduced protein under appropriate redox conditions.

Keywords:

BRE-Luc Assay, cystine-knot, disulfide bond, Heparin affinity chromatography, mild extraction, purification, refolding, recombinant human bone morphogenetic protein-2

1. Introduction

Bone morphogenetic protein-2 (BMP-2) is a disulfide-connected, homodimeric cystine-knot protein and an important member of the transforming growth factor- β (TGF- β) family [1]. BMP-2 is well known for its ectopic bone inducing properties in mammals. These osteogenic properties are employed in many clinical applications which range from fracture healing to spinal fusions [2,3]. It is also needed for research in tissue engineering related areas (i.e. on more complex implants) as well as ingredient for cell culture media for osteogenic induction of appropriate cell types [4,5].

Each BMP-2 monomer contains six disulfide bonds forming a so-called cystine-knot [1]. The cystine-knot is an eight-membered ring structure, in which two disulfide bonds participate and through which a third disulfide bond is formed. BMP-2 as well as other members of the TGF- β family lack the common hydrophobic core of globular proteins rendering the rigid cystine-knot necessary for stabilization of the entire structure. The structure is further stabilized through dimerization which creates a hydrophobic core between the monomers. Finally, the two monomers are linked by an additional disulfide bond. BMP-2 is a very stable protein when disulfide bonds are properly formed [6,7]. However, correct folding and disulfide bond formation in BMP-2 is a slow process *in vitro* even under appropriate redox conditions [8].

BMP-2 can be extracted from bones of diverse mammals with low yields [9,10]. Moreover, recombinant production has been successfully carried out using various expression systems including mammalian cell culture (Chinese Hamster Ovary) [11], silk worms [12], tobacco plants [13] and *E. coli* [14-16]. The highest yields were achieved in *E. coli* [17]. Interestingly, *E. coli* derived rhBMP-2 induces ectopic bone formation at lower concentrations compared to cell culture derived rhBMP-2, maybe due to the lack of glycosylation [18].

Disulfide-bond containing proteins tend to form inclusion bodies (IBs) and the cytoplasm of *E. coli* is considered as reducing, thus not promoting the formation of disulfide bonds [19].

Production of recombinant human BMP-2 (rhBMP-2) in *E. coli* also leads to the formation of IBs and bioactive rhBMP-2 is usually recovered by refolding under appropriate redox conditions of solubilized IBs [14,15,17]. Many different renaturation techniques such as dialysis [20-22], dilution [14,15,17,23,24] and on column refolding [25] have been reported for the generation of bioactive rhBMP-2 from IBs. Although successful, refolding of rhBMP-2 from *E. coli* derived IBs is still a costly, time and material consuming process. Thus, a more straightforward procedure to generate bioactive rhBMP-2 using *E. coli* as expression host would be desirable.

Recently, procedures involving mild solubilisation of proteins embedded in IBs raised attention. In many cases it was shown that proteins were extracted in bioactive form from IBs by employing low concentrations of chaotropic reagents (i.e. urea) or mild detergents (i.e. N-lauroylsarcosine) [26]. If successful, mild extraction methods are advantageous compared to refolding methods as less chemicals, lower process volume and less time are required [20]. Mild extraction procedures have been also applied to the purification of different recombinant BMPs from *E. coli* derived IBs [27,28] including rhBMP-2 [29]. In all these cases, purified BMPs were claimed to be bioactive. These studies prompted us to investigate mild extraction procedures to produce rhBMP-2 from IBs of *E. coli* and to characterize the properties of refolded and mildly extracted rhBMP-2.

rhBMP-2 was produced in form of IBs using two different *E. coli* strains, *E. coli* BL21 (DE3) and *E. coli* SHuffle T7 Express. *E. coli* BL21 (DE3) is a robust common production strain [30,31] and *E. coli* SHuffle T7 Express is especially designed for the cytoplasmic production of disulfide-rich proteins [32]. Dimeric disulfide-bonded rhBMP-2 purified from both strains using either classical refolding or mild extraction was analyzed regarding folding status and biological activity.

2. Material and methods

2.1. Strains and plasmids

E. coli BL21 (DE3) and *E. coli* SHuffle T7 Express lysY (New England Biolabs, Frankfurt) were employed as expression hosts. The codon-optimized mature hBMP-2 gene was inserted into the *NdeI* und *BamHI* multiple cloning side of pET29c (Merck Millipore, Frankfurt, Germany). Transformants were obtained by heat shock and the correct insert was verified by colony PCR and sequencing using T7-primers.

2.2. Cultivation

A preculture of *E. coli* BL21/SHuffle:pET29c-hBMP-2 was inoculated in 10 ml LB medium containing 50 µg/ml kanamycin and grown overnight at 37°C and 180 rpm. The main culture was inoculated in 2 L shaking flasks containing 250 mL defined glucose-supplemented mineral salt medium (DNB, [33]) with a starting OD_{600nm} of 0.05. Cells were grown to an OD_{600nm} of 0.6-0.9 at 30°C and then induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). If not otherwise indicated, the temperature was set to 25°C after induction and growth continued for additional 18 hours prior to harvest. Cell pellets were stored at -80°C prior to further analysis or purification of rhBMP-2.

2.3. Cell fractionation for rhBMP-2 purification

The cells were resuspended 1:20 (w/v) in lysis buffer (100 mM NaCl, 1 mM EDTA, 1.13 mM MgCl₂, 6.17 mM NaH₂PO₄, 18.83 mM Na₂HPO₄ (pH 7.35) at 6°C) and disrupted using a homogenizer (M-110L Microfluidizer, Microfluidics, Newton, USA). After cell disruption the suspension was centrifuged for 1 h at 17,000 x g. The pellet fraction was stored at -80°C.

2.4. Cell fractionation for SDS-PAGE analysis

Aliquots of the *E. coli* cultures were taken at different time points of the cultivation. Cell samples were collected by centrifugation and soluble and insoluble fractions prepared according to the provided manual using BugBuster™ (Merck Millipore, Darmstadt, Germany).

2.5. Preparation and purification of refolded rhBMP-2

Refolding and purification of refolded rhBMP-2 was performed as described previously [14,17]. Briefly, 1 g of rhBMP-2 IBs was solubilized in 15 ml 6 M guanidine-HCl, 100 mM DTT, 5 mM EDTA, 100 mM Tris (pH 8.5). Refolding was carried out for 7 days by dilution of 5 ml of solubilized rhBMP-2 into 250 ml of 500 mM guanidine-HCl, 750 mM CHES, 5 mM EDTA, 2 mM reduced glutathione, 1 mM oxidized glutathione, 50 mM Tris (pH 8.5). Prior to Heparin affinity chromatography the refolding solution was mixed (1:1) with 8 M urea, 25 mM Tris (pH 8) resulting in a solution with the total volume of 500 ml and the following composition: 4 M urea, 250 mM guanidine-HCl, 375 mM CHES, 2.5 mM EDTA, 37.5 mM Tris (pH 8.25). Subsequent Heparin affinity chromatography and dialysis were carried out as described below for the purification of mildly extracted rhBMP-2.

2.6. Preparation and purification of mildly extracted rhBMP-2

rhBMP-2 was extracted from IBs (~166 mg) overnight (~16 h) in 100 ml 4 M urea, 250 mM guanidine-HCl, 375 mM CHES, 2.5 mM EDTA, 37.5 mM Tris (pH 8.25) at 10°C with continuous stirring. Please note that the composition of the extraction buffer applied for mild extraction of rhBMP-2 from *E. coli* derived IBs equals the composition of the solution in which conventionally refolded rhBMP-2 was subjected to Heparin affinity chromatography (except for the traces of the redox system). Afterwards the suspension was centrifuged for 1 h at 17,000 x g and the supernatant filtered using a 0.2 µm polyethersulfone (PES) sterile filter (Sartorius, Germany). The rhBMP-2 containing filtrate was applied to a HiPrep Heparin FF 16/10 column (GE Healthcare, USA) equilibrated with 5 column volumes (CV) equilibration buffer (4 M urea, 20 mM Tris, pH 8) using an ÄKTA Pure system (GE Healthcare, USA). Subsequently, the column was washed with 5 CV equilibration buffer. An isocratic elution

was used to separate the monomeric and dimeric rhBMP-2. The first elution step was done with a buffer containing 320 mM NaCl, 4 M urea, 20 mM Tris (pH 8) for 5 CV. The second elution step was performed using the same buffer with 420 mM NaCl. The fractions were analyzed by SDS-PAGE and those containing mainly dimeric rhBMP-2 were merged and concentrated by ultrafiltration (10 MWCO, Vivaspın 20, Sartorius, Germany) to 1 ml. The concentrated rhBMP-2 dimer solution was applied to a Hiloal 16/600 superdex 75 pg column (GE Healthcare, USA) and further purified using running buffer (4 M urea, 0.15 M NaCl, 25 mM Tris, pH 8). The elution fractions containing protein were analyzed by SDS-PAGE. The fractions containing rhBMP-2 dimer were dialyzed using a Zellutrans membrane with a cut-off of 6,000-8,000 MWCO (Carl Roth, Germany). The dialysis was performed stepwise, starting with a ~1:500 (v/v) dilution in 2 M urea, 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pH 5) for about 16 hours, followed by dialysis using the same volume against 50 mM MES (pH 5) for 24 hours. The final rhBMP-2 dimer containing dialysate was used for bioactivity measurements.

2.7. SDS-PAGE analysis

SDS-PAGE analysis using 15 % polyacrylamide gels was performed in the Mini-PROTEAN 3 Cell (Bio-Rad, USA) according to standard procedures and instructions from the manufacturer. BugBuster™ protein extraction reagent with rLysozyme and Benzonase (Merck Millipore, Darmstadt, Germany) was used to generate inclusion body fractions according to the instructions from the manufacturer. The composition of reducing sample buffer was as follows: 50 mM Tris (pH 6.8), 5 % (w/v) sodium dodecyl sulfate (SDS), 20% (v/v) glycerol, 0.1 mg/mL bromophenol blue, 2 % (v/v) β -mercaptoethanol. For preparation of non-reduced samples addition of β -mercaptoethanol was omitted. After electrophoresis, proteins were visualized by Coomassie Blue staining [34].

2.8. Protein quantification

Protein quantification was performed by densitometry analysis of Coomassie Brilliant Blue-stained gels, using the software ImageJ with bovine serum albumin (BSA) as standard.

2.9. Bioactivity measurements

BMP Responsive Element Luciferase (BRE-Luc) assay. The assay was conducted essentially as described previously [35] with the following modifications. To generate a stable reporter cell line, the mouse muscle satellite cell line C2C12 was transfected with the BRE-firefly luciferase reporter plasmid containing an inhibitor of the differentiation promotor-luciferase construct from Korchinsky *et al.* [36]. Positive cell clones were selected with 750 µg/ml G418 (Geneticin, Thermo Fisher Scientific, USA). For assaying rhBMP-2 activity, cells were seeded at a density of 35,000 cells/well in 24 well-plates using standard growth medium (DMEM F0445 (Biochrom, Germany), 10 % FBS South American Origin (Invitrogen), 4 mM glutamine (Biochrom, Germany), 1 % penicillin/streptomycin (Biochrom, Germany), 1 ml/well). After four hours, the medium was changed to serum-free growth medium without G418 (500 µl/well) and cells were allowed to incubate overnight. Subsequently, rhBMP-2 containing or control samples were added and incubation continued for 24 h. Afterwards, cells were washed with PBS and lysed with 70 µl Luciferase Cell Culture Lysis Reagent (Promega, USA). The lysate was centrifuged at 4°C for 10 min at 20,000 x g. Luciferase activity of the supernatant was measured in triplicate using 7.5 µl lysate and 25 µl Steady-Glo luciferase reagent (Promega, USA). All measurements were performed after 5 min incubation with sensitivity being set to 135 and 1 s integration time (Synergy 2, Biotek, USA).

Alkaline phosphatase activity assay using C2C12 cells. C2C12 cells were seeded at a density of 30,000 cells per well in 96 well plates using standard growth medium (DMEM F0445 (Biochrom, Germany), 10 % FBS South American Origin (Invitrogen, USA), 4 mM L-glutamine (Biochrom, Germany), 1 % penicillin/streptomycin (Biochrom, Germany), 100 µl/well). After four hours, the medium was changed to medium containing only 2 % FBS and cells were allowed to incubate overnight. Subsequently, rhBMP-2 containing or control

samples were added and incubation continued for additional 3 days with media changes every second day. Afterwards, cells were washed with 150 µl PBS and incubated with 110 µl lysis buffer (1 mM MgCl₂, 1 mM ZnCl₂, 1 % (v/v) Igepal CA-630, 0.1 M glycine, pH 9.6) for 1 h at room temperature (RT), followed by centrifugation for 5 min at 100 x g. Alkaline phosphatase activity of the supernatant was measured in triplicate using 100 µl supernatant and 100 µl p-nitrophenolphosphate solution (1 mg/ml, Sigma Aldrich, Germany). The solution was incubated for 15 min at RT and the optical density determined at 405 nm.

Alkaline phosphatase activity assay using human bone marrow-derived mesenchymal stem cells (MSCs). Human bone marrow-derived MSCs isolated by density gradient centrifugation were seeded at a density of 18,000 cells per well in 96 well plates using standard growth medium (DMEM FG0415 (Biochrom, Germany), 10 % FBS Hyclone South America (GE Healthcare, USA), 25 mM HEPES (Biochrom, Germany), 1 % penicillin/streptomycin (Biochrom, Germany), 2 ng/ml FGF-2 (Peprotech, Germany), 100 µl/well). After 4 hours, the medium was changed to test medium (DMEM FG0415 (Biochrom, Germany), 10 % FBS Hyclone South America (GE Healthcare, USA), 25 mM HEPES (Biochrom, Germany), 1 % penicillin/streptomycin (Biochrom, Germany), 10 mM β-Glycerophosphate (Fisher Scientific, Germany), 50 µg/ml Ascorbic acid (Roth, Germany), 100 µl/well) and cells were allowed to incubate overnight. Subsequently, rhBMP-2 containing or control samples were added and incubation continued for another 14 days with media changes every second day. Afterwards, the cells were washed with 150 µl PBS and incubated with 110 µl lysis buffer (1 mM MgCl₂, 1 mM ZnCl₂, 1 % (v/v) Igepal CA-630, 0.1 M glycine, pH 9.6) for 1 h at RT, followed by centrifugation for 5 min at 100 x g. Alkaline phosphatase activity of the supernatant was measured in triplicate using 100 µl supernatant and 100 µl p-nitrophenolphosphate solution (1 mg/ml, Sigma-Aldrich, Germany). The solution was incubated for 15 min at RT and optical density was determined at 405 nm.

2.10. Thermal Shift Assay

Thermal shift assays were performed to analyze temperature-induced unfolding using 22.5 μ l rhBMP-2 dimer solution (50 μ g/ml rhBMP-2 dimer in 50 mM MES buffer, pH 5) and 2.5 μ l SYPRO Orange solution (Sigma Aldrich, Germany, diluted 1:25 in MES buffer, pH 5). All samples were analyzed in triplicates in 96 well plates using a iQ5 Real time PCR (Biorad, USA) and monitoring the fluorescence using a 545-585 nm cut-off filter. The temperature was raised stepwise from 25°C to 100°C by 1°C per minute.

2.11. Disulfide-bond analysis

For analysis of disulfide bonds, protein samples were treated with iodoacetamide leading to carboxamidomethylation of free cysteines. For disulfide bond analysis in IBs, cell lysis was performed in the presence of lysis buffer additionally containing 25 mM iodoacetamide to prevent disulfide bond formation during cell disruption. The IB containing fraction (400 mg) was solubilised overnight in 50 ml 6 M guanidine HCl, 25 mM iodoacetamide and 50 mM Tris (pH 8) and subsequently extensively dialysed against 50 mM MES (pH 5). The refolded rhBMP-2 was incubated overnight in 8 M urea, 25 mM iodoacetamide and 50 mM Tris (pH 8). Afterwards, both samples were subjected to non-reducing SDS-PAGE analysis without boiling and gel pieces containing the rhBMP-2 dimer were treated with 50 μ L of 50 mM tris(2-carboxyethyl)phosphine (TCEP) for 1 h at 60°C to reduce remaining disulfide bonds. Freshly formed free cysteines were subsequently derivatised with 30 μ L of 200 mM methanethiosulfonate (MMTS) for 10 min at RT. In-gel digestion of the protein with trypsin was carried out overnight at 37°C (Promega, USA). Obtained peptides were extracted and purified with reversed-phase C18 ZipTips (Millipore, USA).

LC-MS/MS analyses of desalted peptides were performed on a Dionex UltiMate 3000 n-RSLC system connected to an Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Scientific). Peptides were loaded onto a C18 pre-column (3 μ m RP18 beads, Acclaim, 75 μ m x 20 mm), washed for 3 min at a flow rate of 6 μ L/min and separated on a C18 analytical column (3- μ m, Acclaim PepMap RSLC, 75 μ m x 50 cm, Dionex) at a flow rate of 200 nl/min

via a linear 60 min gradient from 97% buffer A (buffer A = 0.1% formic acid in water) to 25% B (buffer B = 0.1% formic acid in 80% acetonitrile), followed by a 15 min gradient from 25% buffer B to 62% buffer B. The effluent was electro-sprayed by a stainless steel emitter (Thermo Scientific). The mass spectrometer was controlled and operated in the data-dependent mode using the Xcalibur software allowing the automatic selection of 2-4 fold charged peptides and their subsequent fragmentation (top speed mode) using the ion routing multipole with nitrogen as collision gas (HCD) and orbitrap detection (resolution 15K). Every 3 seconds a MS survey scan was performed (resolution 120K). The maximum collection time for peptides was set to 200 ms. Dynamic exclusion was set to 6 sec. MS/MS raw data files were processed via the Proteome Discoverer program Version 1.4 (Thermo Scientific, Dreieich, Germany) on a Mascot server (V. 2.3.02, Matrix Science) using the NCBI "homo sapiens" database. The following search parameters were used: enzyme, trypsin; maximum missed cleavages: 1, variable modifications: MMTS (C) and CAM (C); peptide tolerance, 10 ppm; MS/MS tolerance, 0.03 Da. For relative quantification of peptides of interest, ion traces of the respective molecular ions with a mass tolerance of 10 ppm were used.

2.12. rhBMP-2 structure visualization

The three-dimensional structure of rhBMP-2 was visualized using the program Chimera [37] and structural data from the RCSB Protein Data Bank (PDB ID 3BMP).

3. Results and discussion

3.1. Presence of disulfide-linked rhBMP-2 dimers in inclusion bodies

SDS-PAGE analysis of inclusion bodies (IBs) produced in *E. coli* BL21 and SHuffle carrying the plasmid pET29c-hBMP-2 revealed the presence of monomeric rhBMP-2 at the expected molecular mass of approx. 13 kDa (Fig. 1). Interestingly, a prominent band corresponding to the molecular mass of ~26 kDa was additionally observed in IB samples prepared under non-reducing conditions. This band disappeared when IBs were analyzed under reducing conditions suggesting that the ~26 kDa band corresponds to a disulfide-linked rhBMP-2 dimer. Moreover, a positive control, refolded and purified rhBMP-2, revealed the same migration position during SDS-PAGE corresponding to a ~26 kDa protein under non-reducing conditions. The identity of the ~26 kDa band was finally verified as dimeric rhBMP-2 using LC/MS (data not shown). The relative amount of disulfide-bonded dimeric rhBMP-2 was similar in *E. coli* BL21 and in the thioredoxin reductase (*trxB*) deficient SHuffle strain (Fig. 1).

Although it is commonly accepted that disulfide bonds are usually not formed in the reducing environment of the *E. coli* cytoplasm [19], some studies do show the presence of disulfide-bonded proteins in the cytoplasm [38]. Likewise, using a mutant green fluorescent protein (GFP) with redox-sensitive cysteines it was shown that approx. half of the mutant GFP contained disulfide bonds in the cytoplasm of *E. coli* BL21 [39]. Utilization of a *trxB* deficient *E. coli* strain further increased the content of disulfide-bonded GFP. Here, production of rhBMP-2 in *E. coli* BL21 and in the *trxB* deficient SHuffle strain resulted in similar amounts of disulfide-bonded rhBMP-2. Interestingly, rhBMP-2 itself contains thioredoxin like motifs such as the -CXXXC- motif as well as a -CXC- motif (see also Fig. 7A for complete sequence of rhBMP2). Both motifs are known to be involved in disulfide isomerase activities [40-42] and thus might contribute to the formation of disulfide bonds in the cytoplasm of *E. coli*.

3.2. Purification of dimeric rhBMP-2 obtained by refolding or mild extraction using Heparin affinity chromatography

For comparative studies on the properties of dimeric disulfide-bonded rhBMP-2 obtained either by conventional refolding or by mild extraction from *E. coli* derived IBs both rhBMP-2 variants were purified under identical conditions using Heparin affinity chromatography (Fig. 2).

For the preparation of conventionally refolded rhBMP-2 IBs were solubilized under reducing conditions using high concentrations of denaturant (6 M guanidine-HCl) and rhBMP-2 subsequently refolded under appropriate redox conditions as described earlier [14,17]. After refolding and directly prior to Heparin chromatography the solution was mixed 1:1 with a sample buffer containing 8 M urea. For mild extraction of rhBMP-2 from IBs, a solubilisation buffer was used which was identical to the buffer in which refolded rhBMP-2 was applied to the Heparin column (for details of the purification scheme see also Materials and Methods and Fig. 2).

Refolded and mildly extracted rhBMP-2 were applied to the same column using exactly the same loading and elution steps (sample application, 5 column volumes (CV) washing, 5 CV first elution with 320 mM NaCl, 5 CV second elution with 420 mM NaCl). Afterwards, eluate fractions were analyzed by non-reducing SDS-PAGE (Fig. 3). The flow through fraction of refolded rhBMP-2 only contained the monomer and the flow through of the mildly extracted IBs additionally contained dimeric rhBMP-2. The first eluate fractions (320 mM NaCl) of refolded and mildly extracted rhBMP-2 both contained monomeric and dimeric rhBMP-2. However, the eluate fractions of mildly extracted rhBMP-2 contained a higher proportion of the dimer and additionally higher molecular mass contaminants presumably presenting oligomeric forms of rhBMP-2. The fractions of the second elution (420 mM NaCl) of refolded rhBMP-2 exclusively contained dimeric rhBMP-2 and those of the mildly extracted IBs additionally contained higher molecular mass contaminants.

In summary, the dimeric rhBMP-2 recovered by mild extraction exhibits lower binding affinity towards Heparin and the eluate fractions contain more higher molecular mass contaminants, presumably oligomeric forms of rhBMP-2.

3.3. Further purification of mildly extracted rhBMP-2 by size exclusion chromatography

For detailed analysis of the properties of dimeric rhBMP-2 obtained either by chaotropic solubilisation and subsequent refolding or by mild extraction from IBs protein preparations of adequate purity were required. The rhBMP-2 dimer obtained after Heparin chromatography from the refolded protein mixture was of sufficient purity and dialyzed against MES (pH 5) prior to further analysis. The mildly extracted dimeric rhBMP-2 recovered from the Heparin column needed further purification. Thus, the concentrated second eluate fraction (420 mM NaCl) containing the mildly extracted rhBMP-2 dimer was subjected to additional size exclusion chromatography. Non-reducing SDS-PAGE analysis of eluate fractions revealed successful purification of the rhBMP-2 dimer from earlier eluting higher molecular mass contaminants, presumably disulfide-bonded rhBMP-2 oligomers, and later eluting monomeric rhBMP-2 (Fig. 4). Pure rhBMP-2 dimer containing fractions were dialysed against MES (pH 5) prior to further analysis.

3.4. Bioactivity of dimeric rhBMP-2 obtained by refolding or mild extraction

The biological activity of pure dimeric rhBMP-2 in MES (pH 5) obtained either by refolding or mild extraction was analyzed using the Alkaline Phosphatase (ALP) assay. Moreover, the 100-fold more sensitive BMP Responsive Element Luciferase (BRE-Luc) assay [43] was additionally employed for activity measurements. For both assays the commonly employed murine cell line C2C12 was used (Fig. 5A and B). Both assays clearly proved that only the disulfide-bonded rhBMP-2 dimer generated by refolding revealed convincing bioactivity. The purified rhBMP-2 dimer recovered by mild extraction from IBs of *E. coli* BL21 or SHuffle did not show any biological activity. The different purified rhBMP-2 dimer preparations were additionally tested regarding their ALP inducing activities using bone marrow derived human

mesenchymal stem cells (MSCs) corroborating above results (Fig. 5C). In total, our results clearly show that refolded disulfide-bonded rhBMP-2 is biologically active but the dimeric disulfide bonded rhBMP-2 recovered by mild extraction did not exhibit biological activity. Moreover, purification of dimeric disulfide-bonded rhBMP-2 from IBs by mild extraction using arginine as proposed by Bessa *et al.* [29] also led to a biologically inactive protein (see Supplementary material for more details).

3.5. Thermal unfolding of dimeric rhBMP-2 obtained by classical refolding or mild extraction

To better understand the structural background why the mildly extracted rhBMP-2 dimer did not exhibit biological activity thermal unfolding studies were carried out using the fluorescent dye SYPRO Orange which binds to hydrophobic residues. Protein unfolding and concomitant exposure of hydrophobic amino acid residues from the core to the protein surface becomes detectable through a peak in fluorescence [44] which reflects the melting or denaturing temperature of the protein at given conditions [45]. Refolded rhBMP-2 as well as the mildly extracted variants from *E. coli* BL21 and SHuffle were exposed to increasing temperature and the SYPRO Orange fluorescence was recorded (Fig. 6). Interestingly, all rhBMP-2 preparations exhibited already at low temperature a high basic SYPRO Orange fluorescence, presumably because of the unusual high content of hydrophobic amino acids on the surface of rhBMP-2 [1]. Upon further temperature increase, the SYPRO Orange fluorescence increased sharply in the preparation of refolded rhBMP-2 with an inflection point at 59°C corresponding to the melting or denaturing temperature of rhBMP-2. However, mildly extracted rhBMP-2 did not show any apparent fluorescence peak upon further temperature increase, neither in the preparation from *E. coli* BL21 nor from SHuffle, indicating an unstructured conformation and the absence of a defined hydrophobic core in the entire temperature range investigated. These results clearly show that the lack of biological activity of mildly extracted rhBMP-2 is connected to a lack in conformational integrity.

3.6. Cystine knot analysis of dimeric disulfide-bonded rhBMP-2

All members of the TGF- β superfamily, including rhBMP-2, contain a structure-stabilizing cystine-knot in their native conformation [46,47]. Thus, the disulfide-bond pattern of dimeric disulfide-bonded rhBMP-2 within IBs was assessed by sequential derivatisation of free and bound cysteines and subsequent mass spectrometric analysis of tryptic peptides. To preserve the disulfide bond structure of rhBMP-2 within the IBs subsequent air oxidation was prevented by blocking free cysteines by iodoacetamide addition during cell lysis and IB handling. The IB fraction was then analyzed by non-reducing SDS-PAGE and the dimeric-disulfide-bonded rhBMP-2 subjected to peptide-mass fingerprinting after tryptic digestion, reduction and derivatisation of freshly reduced cysteines with methanethiosulfonate. Using this technique, it was possible to distinguish formerly free cysteines and cysteines which were originally part of a cystine bond. The former cysteines were derivatised by iodoacetamide and the latter by methanethiosulfonate leading to a difference in mass by 11 Da per cysteine residue easily detectable by mass spectrometry.

As an example, the mass analysis of the C-terminal peptide NYQDMVVEGCGCR containing the cysteines 111 and 113 is given (Fig. 7). Both cysteines are part of the cystine bonds forming the basic ring structure of the cystine-knot [1]. The data revealed that more than 95% of both cysteines were carboxamidomethylated by iodoacetamide thus representing formerly free cysteines of the disulfide-bonded, dimeric rhBMP-2 embedded within the IBs. In contrast, in refolded and bioactive rhBMP-2 the majority of these cysteines were involved in disulfide bonds. Our data clearly show that the basic ring structure of the cystine-knot is not formed within the IB contained disulfide-bonded rhBMP-2 dimer presumably the cause for the unstructured conformation and the missing bioactivity of the mildly extracted rhBMP-2 dimer. Thus, refolding under appropriate redox conditions is required to generate a bioactive rhBMP-2 dimer [48].

4. Conclusions

Production of rhBMP-2 in *E. coli* *trxB* deficient SHuffle but also in the common strain BL21 leads to the formation of cytoplasmic IBs containing monomeric as well as disulfide-bonded dimers of the growth factor. Solubilisation of rhBMP-2 IBs using strong denaturants and subsequent refolding under appropriate redox conditions results in the dimeric, correctly folded and bioactive growth factor. Recovery of the disulfide-bonded rhBMP-2 dimer by mild extraction from IBs, however, does not yield bioactive growth factor. Thermal shift assays revealed that the mildly extracted rhBMP-2 dimer does not contain the properly folded hydrophobic core found in the correctly folded and biologically active growth factor. Disulfide-bond analysis also revealed that the intricate disulfide bond pattern of the complex cystine-knot scaffold was not present in the inclusion body embedded disulfide-linked rhBMP-2 dimer but was formed later during classical refolding of the reduced protein under appropriate redox conditions. These results show that mild extraction procedures might be limited to less complex proteins.

Acknowledgements

The authors gratefully acknowledge funding through the Forschergruppe “Gradierte Implantate” FOR2180 and the Exzellenzcluster “Rebirth” EXC62, both Deutsche Forschungsgemeinschaft (DFG). In addition, the authors acknowledge Thilo Flörkemeier, Department of Orthopaedic Surgery (Annastift), for providing bone marrow for isolation of the human mesenchymal stem cells.

References

- [1] C. Scheufler, W. Sebald, M. Hülsmeier, Crystal structure of human bone morphogenetic protein-2 at 2.7 Å resolution, *J. Mol. Biol.* 287 (1999) 103-115.
- [2] W.F. McKay, S.M. Peckham, J.M. Badura, A comprehensive clinical review of recombinant human bone morphogenetic protein-2 (INFUSE Bone Graft), *Int. Orthop.* 31 (2007) 729-734.
- [3] J.M. Granjeiro, R.C. Oliveira, J.C. Bustos-Valenzuela, M.C. Sogayar, R. Taga, Bone morphogenetic proteins: from structure to clinical use, *Braz. J. Med. Biol. Res.* 38 (2005) 1463-1473.
- [4] M. Nakashima, A.H. Reddi, The application of bone morphogenetic proteins to dental tissue engineering, *Nat. Biotechnol.* 21 (2003) 1025-1032.
- [5] T. Katagiri, A. Yamaguchi, T. Ikeda, S. Yoshiki, J.M. Wozney, V. Rosen, E.A. Wang, H. Tanaka, S. Omura, T. Suda, The non-osteogenic mouse pluripotent cell line, C3H10T1/2, is induced to differentiate into osteoblastic cells by recombinant human bone morphogenetic protein-2, *Biochem. Biophys. Res. Commun.* 172 (1990) 295-299.
- [6] J.M. Wozney, V. Rosen, A.J. Celeste, L.M. Mitsock, M.J. Whitters, R.W. Kriz, R.M. Hewik, E.A. Wang, Novel regulators of bone formation: molecular clones and activities, *Science* 242 (1988) 1528-1534.
- [7] T.K. Sampath, A.H. Reddi, Dissociative extraction and reconstitution of extracellular matrix components involved in local bone differentiation, *Proc. Natl. Acad. Sci. USA* 78 (1981) 7599-7603.
- [8] L.F. Vallejo, U. Rinas, Folding and dimerization kinetics of bone morphogenetic protein-2, a member of the transforming growth factor- β family, *FEBS J.* 280 (2013) 83-92.
- [9] S.J. Sibiya, E.I. Olivier, N. Duneas, High yield isolation of BMP-2 from bone and in vivo activity of a combination of BMP-2/TGF- β 1, *J. Biomed. Mater. Res. A* 101 (2013) 641-646.
- [10] M.R. Urist, A. Lietze, H. Mizutani, K. Takagi, J.T. Triffitt, J. Amstutz, R. DeLange, J. Termine, G.A. Finerman, A bovine low molecular weight bone morphogenetic protein (BMP) fraction, *Clin. Orthop. Relat. Res.* 1982) 219-232.
- [11] D.I. Israel, J. Nove, K.M. Kerns, I.K. Moutsatsos, R.J. Kaufman, Expression and characterization of bone morphogenetic protein-2 in chinese hamster ovary cells, *Growth Factors* 7 (1992) 139-150.
- [12] N. Ishida, M. Tsujimoto, T. Kanaya, A. Shimamura, N. Tsuruoka, S. Kodama, T. Katayama, S. Oikawa, M. Matsui, T. Nakanishi, Expression and characterization of human bone morphogenetic protein-2 in silkworm larvae infected with recombinant *Bombyx mori* nuclear polyhedrosis virus, *J. Biochem.* 115 (1994) 279-285.

- [13] G. Suo, B. Chen, J. Zhang, Y. Gao, X. Wang, Z. He, J. Dai, Expression of active hBMP2 in transgenic tobacco plants, *Plant Cell. Rep.* 25 (2006) 1316-1324.
- [14] L.F. Vallejo, M. Brokelmann, S. Marten, S. Trappe, J. Cabrera-Crespo, A. Hoffmann, G. Gross, H.A. Weich, U. Rinas, Renaturation and purification of bone morphogenetic protein-2 produced as inclusion bodies in high-cell-density cultures of recombinant *Escherichia coli*, *J. Biotechnol.* 94 (2002) 185-194.
- [15] S. von Einem, E. Schwarz, R. Rudolph, A novel TWO-STEP renaturation procedure for efficient production of recombinant BMP-2, *Protein Expr. Purif.* 73 (2010) 65-69.
- [16] I.S. Kim, E.N. Lee, T.H. Cho, Y.M. Song, S.J. Hwang, J.H. Oh, E.K. Park, T.Y. Koo, Y.K. Seo, Promising efficacy of *Escherichia coli* recombinant human bone morphogenetic protein-2 in collagen sponge for ectopic and orthotopic bone formation and comparison with mammalian cell recombinant human bone morphogenetic protein-2, *Tissue Eng. Part A* 17 (2011) 337-348.
- [17] L.F. Vallejo, U. Rinas, Optimized procedure for renaturation of recombinant human bone morphogenetic protein-2 at high protein concentration, *Biotechnol. Bioeng.* 85 (2004) 601-609.
- [18] F.C. van de Watering, J.J. van den Beucken, S.P. van der Woning, A. Briest, A. Eek, H. Qureshi, L. Winnubst, O.C. Boerman, J.A. Jansen, Non-glycosylated BMP-2 can induce ectopic bone formation at lower concentrations compared to glycosylated BMP-2, *J. Control. Release* 159 (2012) 69-77.
- [19] F. Baneyx, M. Mujacic, Recombinant protein folding and misfolding in *Escherichia coli*, *Nature Biotechnol.* 22 (2004) 1399-1408.
- [20] A.S. Karyagina, I.S. Boksha, T.M. Grunina, A.V. Demidenko, M.S. Poponova, O.V. Sergienko, A.M. Lyaschuk, Z.M. Galushkina, L.A. Soboleva, E.O. Osidak, A.S. Semikhin, A.V. Gromov, V.G. Lunin, Optimization of rhBMP-2 active-form production in a heterologous expression system using microbiological and molecular genetic approaches, *Mol. Genet. Microbiol. Virol.* 31 (2016) 208-213.
- [21] Y. Zhang, Y. Ma, M. Yang, S. Min, J. Yao, L. Zhu, Expression, purification, and refolding of a recombinant human bone morphogenetic protein 2 in vitro, *Protein Expr. Purif.* 75 (2011) 155-160.
- [22] H. Zhang, J. Wu, N. Fu, J. Wang, S. Zhao, Optimized procedure for expression and renaturation of recombinant human bone morphogenetic protein-2 at high protein concentrations, *Mol. Biol. Rep.* 37 (2010) 3089-3095.
- [23] W. Guo, X. Zhu, J. Cai, L. Huang, P. Cen, Z. Xu, Refolding and two-step purification by hydrophobic interaction chromatography of recombinant human bone morphogenetic protein-2 from *Escherichia coli*, *Process Biochem.* 47 (2012) 960-967.
- [24] S. Long, L. Truong, K. Bennett, A. Phillips, F. Wong-Staal, H. Ma, Expression, purification, and renaturation of bone morphogenetic protein-2 from *Escherichia coli*, *Protein Expr. Purif.* 46 (2006) 374-378.
- [25] A.M. Rane, S. Jonnalagadda, Z. Li, On-column refolding of bone morphogenetic protein-2 using cation exchange resin, *Protein Expr. Purif.* 90 (2013) 135-140.

- [26] A. Singh, V. Upadhyay, A.K. Upadhyay, S.M. Singh, A.K. Panda, Protein recovery from inclusion bodies of *Escherichia coli* using mild solubilization process, *Microb. Cell Fact* 14 (2015) 41.
- [27] P.C. Bessa, M.T. Cerqueira, T. Rada, M.E. Gomes, N.M. Neves, A. Nobre, R.L. Reis, M. Casal, Expression, purification and osteogenic bioactivity of recombinant human BMP-4, -9, -10, -11 and -14, *Protein Expr. Purif.* 63 (2009) 89-94.
- [28] B. Klösch, W. Furst, R. Kneidinger, M. Schuller, B. Rupp, A. Banerjee, H. Redl, Expression and purification of biologically active rat bone morphogenetic protein-4 produced as inclusion bodies in recombinant *Escherichia coli*, *Biotechnol. Lett.* 27 (2005) 1559-1564.
- [29] P.C. Bessa, A.J. Pedro, B. Klösch, A. Nobre, M. van Griesven, R.L. Reis, M. Casal, Osteoinduction in human fat-derived stem cells by recombinant human bone morphogenetic protein-2 produced in *Escherichia coli*, *Biotechnol. Lett.* 30 (2008) 15-21.
- [30] Shiloach J, Rinas U: Glucose and acetate metabolism in *E. coli* - System level analysis and biotechnological applications in protein production processes; in Lee SY (ed): *Systems biology and biotechnology of Escherichia coli*. Berlin, Heidelberg, New York, Springer-Verlag, 2009 pp 377-400.
- [31] Z. Li, M. Nimtz, U. Rinas, The metabolic potential of *Escherichia coli* BL21 in defined and rich medium, *Microb. Cell Fact.* 13 (2014) 45.
- [32] J. Lobstein, C.A. Emrich, C. Jeans, M. Faulkner, P. Riggs, M. Berkmen, SHuffle, a novel *Escherichia coli* protein expression strain capable of correctly folding disulfide bonded proteins in its cytoplasm, *Microb. Cell Fact.* 11 (2012) 56.
- [33] Z. Li, W. Kessler, J. van den Heuvel, U. Rinas, Simple defined autoinduction medium for high-level recombinant protein production for T7-based *Escherichia coli* expression systems, *Appl. Microbiol. Biotechnol.* 91 (2011) 1203-1213.
- [34] G. Candiano, M. Bruschi, L. Musante, L. Santucci, G.M. Ghiggeri, B. Carnemolla, P. Orecchia, L. Zardi, P.G. Righetti, Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis, *Electrophoresis* 25 (2004) 1327-1333.
- [35] C. Lorenz, A. Hoffmann, G. Gross, H. Windhagen, P. Dellinger, K. Mohwald, W. Dempwolf, H. Menzel, Coating of titanium implant materials with thin polymeric films for binding the signaling protein BMP2, *Macromol. Biosci.* 11 (2011) 234-244.
- [36] O. Korchynskyi, P. ten Dijke, Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter, *J. Biol. Chem.* 277 (2002) 4883-4891.
- [37] E.F. Pettersen, T.D. Goddard, C.C. Huang, G.S. Couch, D.M. Greenblatt, E.C. Meng, T.E. Ferrin, UCSF Chimera - a visualization system for exploratory research and analysis, *J. Comput. Chem.* 25 (2004) 1605-1612.
- [38] B. Nilsson, C. Berman-Marks, I.D. Kuntz, S. Anderson, Secretion incompetence of bovine pancreatic trypsin inhibitor expressed in *Escherichia coli*, *J. Biol. Chem.* 266 (1991) 2970-2977.

- [39] H. Ostergaard, A. Henriksen, F.G. Hansen, J.R. Winther, Shedding light on disulfide bond formation: engineering a redox switch in green fluorescent protein, *EMBO J.* 20 (2001) 5853-5862.
- [40] Y. Ishikawa, H.P. Bächinger, An additional function of the rough endoplasmic reticulum protein complex prolyl 3-hydroxylase 1.cartilage-associated protein.cyclophilin B: the CXXXC motif reveals disulfide isomerase activity in vitro, *J. Biol. Chem.* 288 (2013) 31437-31446.
- [41] K.J. Woycechowsky, R.T. Raines, The CXC motif: a functional mimic of protein disulfide isomerase, *Biochemistry* 42 (2003) 5387-5394.
- [42] G.Z. Wang, X.Y. Dong, Y. Sun, Peptide disulfides CGC and RKCGC facilitate oxidative protein refolding, *Biochem. Eng. J.* 55 (2011) 169-175.
- [43] D. Logeart-Avramoglou, M. Bourguignon, K. Oudina, P. ten Dijke, H. Petite, An assay for the determination of biologically active bone morphogenetic proteins using cells transfected with an inhibitor of differentiation promoter-luciferase construct, *Anal. Biochem.* 349 (2006) 78-86.
- [44] U.B. Ericsson, B.M. Hallberg, G.T. Detitta, N. Dekker, P. Nordlund, Thermofluor-based high-throughput stability optimization of proteins for structural studies, *Anal. Biochem.* 357 (2006) 289-298.
- [45] S. Boivin, S. Kozak, R. Meijers, Optimization of protein purification and characterization using Thermofluor screens, *Protein Expr. Purif.* 91 (2013) 192-206.
- [46] N.W. Isaacs, Cystine knots, *Curr. Opin. Struct. Biol.* 5 (1995) 391-395.
- [47] N.Q. McDonald, W.A. Hendrickson, A structural superfamily of growth factors containing a cystine knot motif, *Cell* 73 (1993) 421-424.
- [48] J. Wendler, L.F. Vallejo, U. Rinas, U. Bilitewski, Application of an SPR-based receptor assay for the determination of biologically active recombinant bone morphogenetic protein-2, *Anal. Bioanal. Chem.* 381 (2005) 1056-1064.

Figure captions

Figure 1 Presence of disulfide-linked rhBMP-2 dimer in inclusion bodies. SDS PAGE analysis of rhBMP2 inclusion bodies (insoluble cell fraction) under non-reducing (lane 1-6, no β -mercaptoethanol) and reducing conditions (lanes 8-13, with β -mercaptoethanol). Production was carried out in *E. coli* SHuffle (lanes 1-3, 8-10) and in BL21 (lanes 4-6, 11-13) at 30°C. Lanes **1,8**: SHuffle before induction, lanes **2,9**: SHuffle 2 h post-induction, lanes **3,10**: SHuffle 4 h post-induction, lanes **4,11**: BL21 before induction, lanes **5,12**: BL21 2 h post-induction, lanes **6,13**: BL21 4 h post-induction, **7**: refolded and purified rhBMP-2 dimer.

Figure 2: Purification scheme for dimeric disulfide-bonded rhBMP-2 obtained from *E. coli* derived IBs either by classical refolding or by mild extraction. Process steps carried out under identical conditions (e.g. buffer composition, temperature) for both rhBMP-2 variants are gray-shaded.

Figure 3 Heparin-chromatography of refolded and mildly extracted rhBMP-2. Non-reducing SDS-PAGE analysis of eluate fractions of **(A)** refolded and **(B)** mildly extracted rhBMP-2. M: prestained Marker, F: flow through fraction, Elution 1: Fractions after elution with 320 mM NaCl, Elution 2: fractions after elution with 420 mM NaCl, S: refolded and purified rhBMP-2 dimer.

Figure 4 Size-exclusion-chromatography (SEC) of mildly extracted rhBMP-2. Non-reducing SDS-PAGE analysis of eluate fractions. Sa: Sample before SEC (concentrated and pre-purified by Heparin chromatography); Elution: protein containing fractions from SEC; S: purified and refolded rhBMP-2 dimer.

Figure 5 Bioactivity of refolded and mildly extracted rhBMP-2. The bioactivity of the different rhBMP-2 preparations were analyzed with C2C12 cells using the **(A)** BRE-Luc (100 ng/ml rhBMP-2) and the **(B)** ALP assay (500 ng/ml rhBMP-2). **(C)** Moreover, bioactivity was additionally tested with MCSs using the ALP assay (500 ng/ml rhBMP-2). The concentration of refolded and mildly extracted rhBMP-2 was determined as described in the Materials and Methods section and the concentration of commercial rhBMP-2 (Peprotech, *E. coli*-derived) was taken as supplied by the manufacturer. The activities are given as **(A)** relative light units (RLU) and as **(B,C)** relative absorbance at 405 nm. R: refolded rhBMP-2, C: commercial rhBMP-2, B and S: mildly extracted and purified rhBMP-2 dimer from *E. coli* BL21 and SHuffle, respectively; N: negative control (only buffer).

Figure 6 Thermal unfolding of refolded and mildly extracted rhBMP-2. Thermal shift assay of different rhBMP-2 preparations obtained either by classical refolding (■) or mild extraction from IBs of *E. coli* BL21 (●) and SHuffle (+). MES buffer pH 5 (▲) served as negative control. Fluorescence signals were normalized to the highest value measured in each rhBMP-2 sample. Shaded areas around data points indicate standard deviation of three biological replicates.

Figure 7 Cystine-knot analysis of dimeric disulfide-bonded rhBMP-2 (A) Primary structure of the rhBMP-2 monomer and (B) spatial organization of the cystine-knot. Cysteines 111 and 113 forming the ring structure of the cystine-knot are highlighted in red and green, respectively. (A) The underlined tryptic peptide was analyzed (C) by mass spectrometry. (C upper part) Corresponding peptide from dimeric disulfide-bonded rhBMP-2 embedded within IBs mainly contains di-carboxamidomethylated cysteines (Peak I: (m/z: 802.316 $[M+2H]^{2+}$ NYQDMoxVVEGC(S-cam)GC(S-cam)R). (C lower part) Corresponding peptide from refolded rhBMP-2 mainly contains di-methylthiolated cysteines (Peak IV: m/z: 791.282) NYQDMoxVVEGC(S-Me)GC(S-Me)R) but also mixed derivatives (Peaks II + III: carboxamidomethylated + methylthiolated cysteines (m/z: 796.799) NYQDMoxVVEGC(S-cam)GC(S-Me)R + NYQDMoxVVEGC(S-Me)GC(S-cam)R). Numbers next to peaks: left: area of peak relative to all peaks (%), right: retention time (min). The amino acid sequence and modifications of the respective peptides were verified by additional MS/MS spectra of all relevant ions (data not shown).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Please print Figure 7 in color