Supplementary material

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

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Mild extraction of rhBMP-2 from IBs using arginine

The method based on mild extraction of rhBMP-2 from *E. coli* derived IBs using arginine [1] was also employed and the obtained rhBMP-2 tested for bioactivity.

Materials and methods

Briefly, rhBMP-2 was extracted from ~166 mg IBs using 100 ml 0.7 M L-arginine, pH 11 over-night at room temperature and continuous stirring. The suspension containing solubilized rhBMP-2 was centrifuged at 4.000 x g for 30 min. The supernatant was taken and the pH adjusted to pH 8.5 with 0.1 M HCl resulting in a final concentration of ~0.3 M L-arginine. This solution was filtered using a 0.22 µm PES filter and loaded onto a HiPrep Heparin 5 ml column (GE Healthcare, USA) equilibrated with 5 column volumes (CV) of 0.35 M L-arginine, 30 mM Tris, pH 8.5. Afterwards the column was washed with 5 CV equilibration buffer. Elution was carried out with 0.5 M L-arginine, 0.7 M NaCl, 30 mM Tris, pH 8.5. In contrast to the protocol by Bessa *et al.* [1] the buffer was not changed to PBS during washing and elution as rhBMP-2 precipitates in PBS (at pH 6 and above) as described by El Bialy *et al.* [2] and also according to our experience. The elution fractions containing rhBMP-2 were merged and dialyzed against 2 M urea, 50 mM MES, pH 5 overnight. Following, a dialysis against 50 mM MES, pH 5 was performed for 24 h. Analytical methods including SDS-PAGE and biological activity analysis are given in the Materials and methods section of the main manuscript.

Results and discussion

SDS-PAGE analysis showed that both mild extraction methods, the method proposed by Bessa *et al.* [1] using arginine and the method using urea as described in the main manuscript, were leading to similar results (Figs. S1 und S2). Both mild extractions methods lead to a mixture of monomeric, dimeric and oligomeric rhBMP-2 (Figure 1S). The Heparin-affinity purified rhBMP-2 preparations obtained either by arginine or urea extraction were

tested regarding bioactivity using the sensitive rhBMP-2 (BRE-Luc) bioactivity assay. As a positive control conventionally refolded and also commercially available rhBMP-2 were used (details given in the main manuscript). The results of these analyses clearly revealed that neither mildly extracted rhBMP-2 by L-arginine nor rhBMP-2 mildly extracted by urea possess bioactivity. Moreover, also 40-fold higher concentrated samples of these two different mildly extracted rhBMP-2 preparations did not exhibit any bioactivity (data not shown). These results corroborate the conclusion presented in the main manuscript that complex disulfide-bonded proteins, e.g. those containing the complex cystine-knot scaffold might not reach their proper structure within *E. coli* derived IBs suggesting also that mild extraction procedures might be limited to less complex proteins.

References

[1] 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.

[2] I. El Bialy, W. Jiskoot, N.M. Reza, Formulation, delivery and stability of bone morphogenetic proteins for effective bone regeneration, Pharm. Res. 34 (2017) 1152-1170.

Supplementary Figures

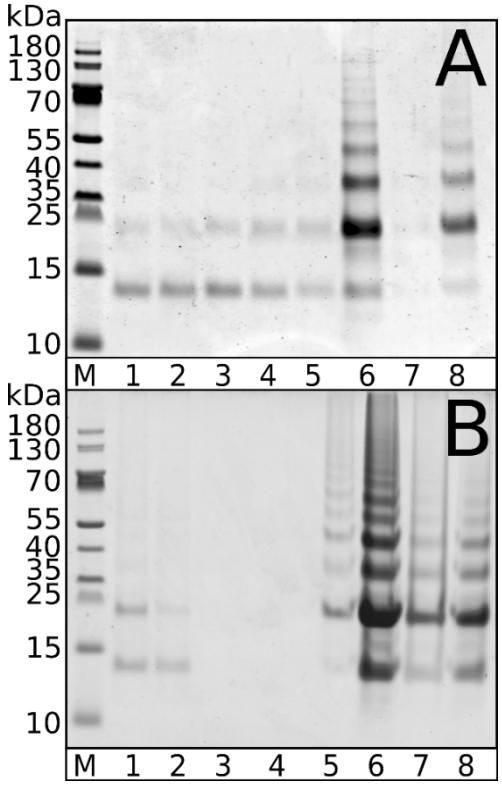

Figure 1S: Heparin-affinity chromatography of mildly extracted rhBMP-2 using either (A) arginine or (B) urea. Non-reducing SDS-PAGE analysis of samples taken during Heparin-affinity chromatography of mildly extracted rhBMP-2 using **(A)** arginine as proposed by Bessa *et al.* [1] or using **(B)** urea as described in the main manuscript. M: Molecular mass marker, 1: mildly extracted rhBMP-2 prior to Heparin-affinity chromatography, 2: Flow through, 2-4: Washing fractions, 5-7: Elution fractions, 8: Combined and dialyzed elution fractions.

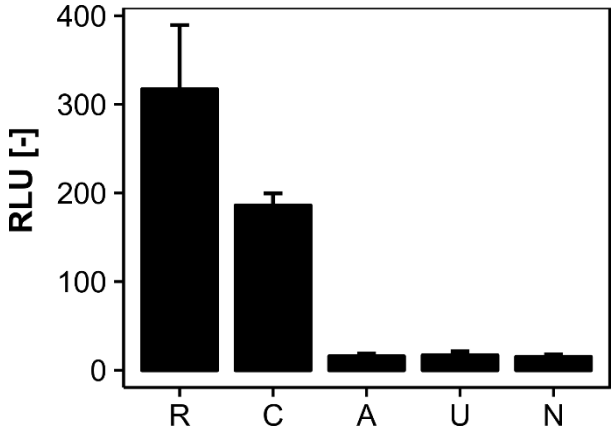

Figure 2S: Bioactivity determined by BRE-Luc assay of mildly extracted rhBMP-2 (after Heparin-affinity chromatography and dialysis) using either arginine or urea. R: Refolded rhBMP-2, C: commercial rhBMP-2, A: mildly extracted rhBMP-2 using L-arginine as proposed by Bessa *et al*. [1], U:mildly extracted rhBMP-2 using urea as described in the main manuscript. N: negative control ddH_2O. All samples contained 100 ng/ml rhBMP-2 and activities are given as relative light units (RLU).