Recombinant production of β-Lactoglobulin variants with lacking disulfide bonds

S. Brune[1,2,3,4], G. Günther[1,3], E. Kempf[2,4], D. Jahn[1,3], R. Biedendieck[1,3]*, R. Krull[2,3,4]*

[1]Institute for Microbiology, [2]Institute of Biochemical Engineering, [3]Braunschweig Integrated Centre of Systems Biology (BRICS), [4]Centre of Pharmaceutical Engineering (PVZ), Technische Universität Braunschweig, Germany

s.brune@tu-braunschweig.de

Introduction

- β-Lactoglobulin (Blg) is the main component of whey protein in the milk of many mammalian species where it functions as a globular transport protein.
- Blg contains five cysteines forming two disulfide bonds (an inner and an outer one) and one not involved (Fig. 1).
- Due to its versatile properties, the protein is currently used in both food technology and medical research.
- 2010 correctly folded and soluble Blg was recombinantly produced in *Escherichia coli* for the first time [1].
- This system was modified and purification steps were established to recombinantly produce Blg variants that do not differ to native bovine Blg [2,3].
- This new production and purification system provides the possibility to recombinantly produce different variants of Blg based on amino acid exchanges.
- The focus is particularly on the variants with lacking disulfide bonds in order to investigate their influence on the protein. For that a different purification process based on inclusion bodies have to be established due to insolubility of variants II and III.

BlgB-variants with lacking disulfide bonds

- Currently the focus is on three variants of BlgB (Tab. 1): variant I with missing „outer“ disulfid bond and free cysteine residue, variant II with missing „inner“ disulfid bond and free cysteine residue and variant III without any cysteine residue.
- For variants I and III two different purification processes could be established (Fig. 2).

Table 1: Current variants of recombinant BlgB with lacking disulfid bonds.

#	Protein	Characteristics	Solubility
I	BlgB_C66A_C160A_C121A	Missing outer disulfid bond + free cysteine residue	Soluble
II	BlgB_C106A_C119A_C121A	Missing inner disulfid bond + free cysteine residue	Insoluble
III	BlgB_C66A_C106A_C119A_C121A_C160A	Missing all cysteine residues	Insoluble

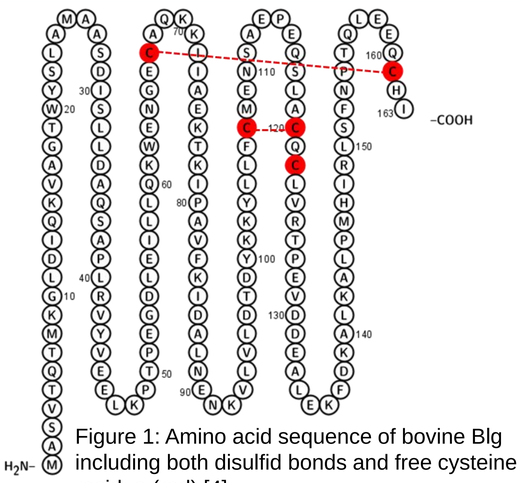

Figure 1: Amino acid sequence of bovine Blg including both disulfid bonds and free cysteine residue (red) [4].

Purification process

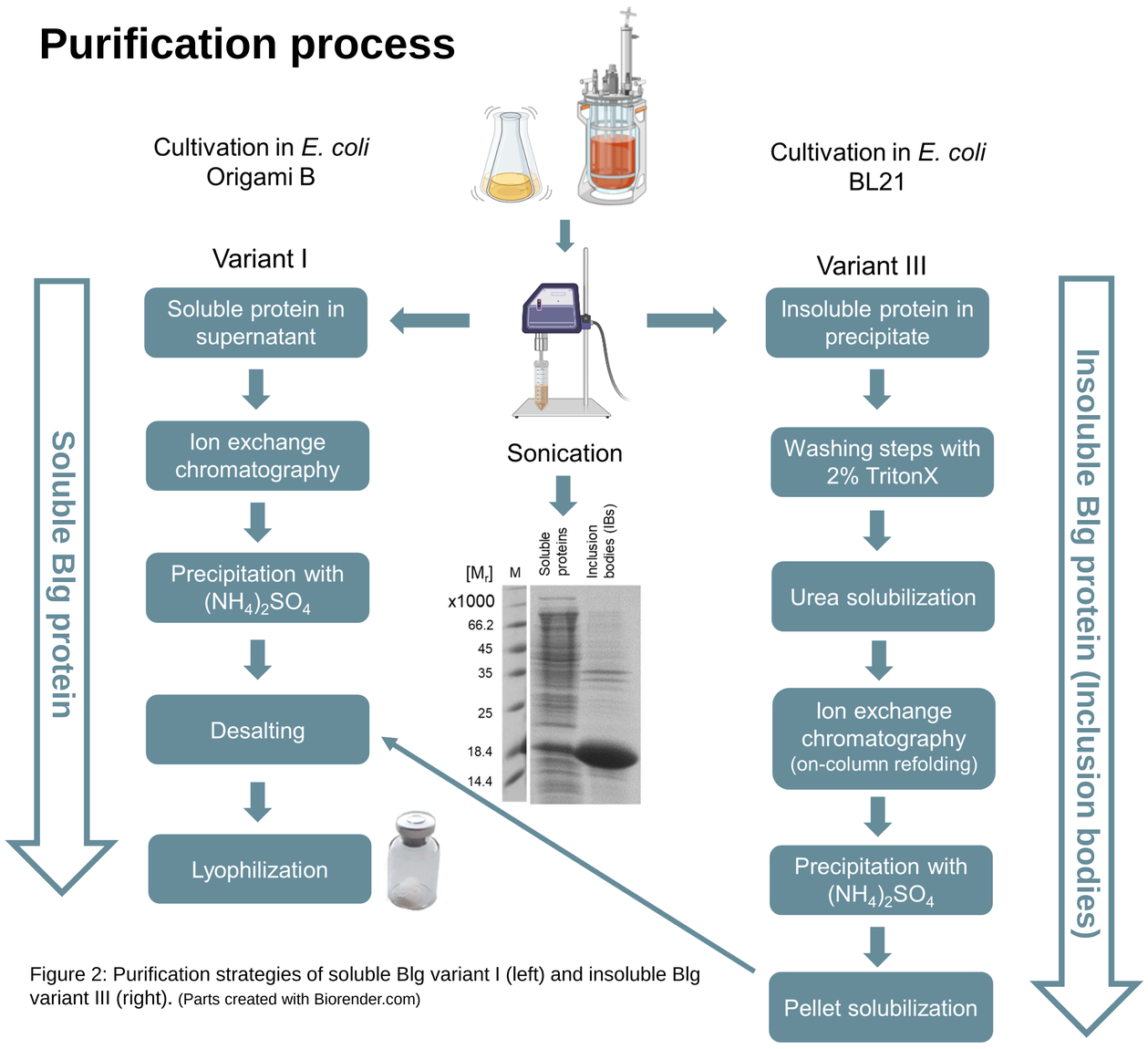

Figure 2: Purification strategies of soluble Blg variant I (left) and insoluble Blg variant III (right). (Parts created with Biorender.com)

- In contrast to Blg variant I, variant III is produced in insoluble protein aggregates (inclusion bodies) which require a different purification method (Fig. 2).
- Isolation, solubilization and refolding of these Blg inclusion bodies were optimized.
- While the purification process of variant III has already been established, an inclusion body purification process of variant II needs to be determined, in which the remaining outer disulfide is properly formed.

Validation of refolding

Native PAGE

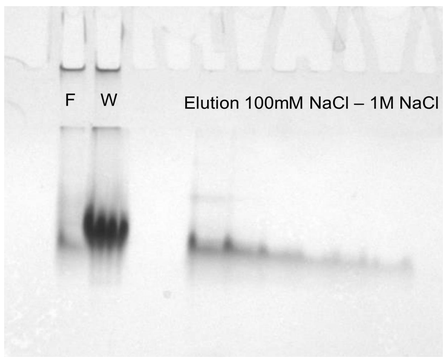

Figure 3: Native PAGE (15%) of Q-Sepharose ion exchange chromatography fractions of Blg variant III in 8 M Urea.

- Validation of the refolding after ion exchange chromatography of Blg variant III took place by native gel analysis and intrinsic fluorescence measurments.
- While large aggregates of Blg are observed in the flow-through (F) and wash fractions (W) of native PAGE, monomeric Blg elutes with NaCl (Fig.3).
  - ➢ This implies a uniform monomeric conformation after on-column refolding.

Intrinsic fluorescence

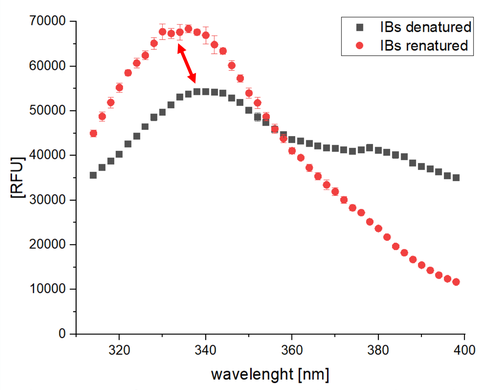

Figure 4: Fluorescence emission maxima of denatured Blg variant III (black) and after on-column refolding (red). $\lambda_{Extinction}$= 270 nm.

- Emission maxima of eluted Blg protein after on-column refolding has a shift compared to denatured Blg inclusion bodies in 8 M Urea (Fig. 4).
- Refolded protein showed emission maxima of 332 nm while unfolded protein undergoes a red shift up to 340 nm.
  - ➢ This observation suggests that the Blg protein has a different conformation after on-column refolding in contrast to the denatured protein in 8 M urea.

Acknowledgement

The authors gratefully acknowledge financial support from the German Research Foundation (DFG) in the Priority Programme 1934 *DiSPBiotech* (project number 315460011).

References

[1] Ponniah et al. (2010). *Protein Expr. and Purif.* 70 (2): 283-289.
[2] Loch et al. (2016). *Mol. Biotechnol.* 58 (10): 205-618.
[3] Keppler et al. (2021). *Food Hydrocoll.* 110: 106-132.
[4] Omasits et al. (2014). *Bioinformatics*. 30(6): 884-886.

BRICS Braunschweig Integrated Centre of Systems Biology