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RESEARCH

Exploring Functionality Gain for (Recombinant) β-Lactoglobulin Through Production and Processing Interventions

Loes J. G. Hoppenreijs[1] · Sarah E. Brune[2,3,4,5] · Rebekka Biedendieck[3,4] · Rainer Krull[2,4,5] · Remko M. Boom[1] · Julia K. Keppler[1]

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Abstract

Interventions in the upstream production and further processing of recombinant food proteins affect its properties when used for food application. Often the efficiency of particular interventions is evaluated based on molecular purity and yield rather than final functional properties. Yet, the formulation of foods, including the amount of protein required, can be affected when the functional properties have changed. In this explorative study, we exemplify how far we can extend the functionality range of the major whey protein β-lactoglobulin (BLG), in terms of foaming and (heat-set) gelling, through various interventions. Slight changes in the amino acid sequence of BLG affected its functional properties significantly. Foams were up to ten times more stable, when selecting different natural isoforms of BLG (isoform A instead of B) or when inducing targeted cysteine mutations. The isoform B yielded stronger thermally induced gels (+40%) compared to isoform A. During downstream processing of recombinantly secreted BLG, limited purification of up to ~ 67 wt% enabled reasonable foaming properties and superior gelation, while a lower purity of ~ 22 wt% resulted in poor performance in both cases. Post-processing allowed conversion of native whey protein into soluble amyloid-like aggregates. These aggregates resulted in better foam stability (i.e., approximately four times longer than non-aggregated protein), but did not improve gelation. The presented study demonstrates that one should consider not only protein yield and purity, but also functional properties when developing recombinant proteins for food application. In turn, these functional properties are a result of the complete upstream and downstream chain.

Keywords Cellular agriculture · Heterologous proteins · Protein engineering · Purification

Abbreviations

BLG	β-Lactoglobulin
rBLG	BLG recombinantly produced by *Escherichia coli*
yBLG	BLG recombinantly produced by *Pichia pastoris*
bBLG	BLG from bovine milk
DSP	Downstream processing
HMP	Hexametaphosphate
G”	Loss modulus
G’	Storage modulus
WPI	Whey protein isolate

✉ Julia K. Keppler
Julia.keppler@wur.nl

[1] Laboratory of Food Process Engineering, Wageningen University, P.O. Box 17, 6700 AA Wageningen, the Netherlands

[2] Institute of Biochemical Engineering, Technische Universität Braunschweig, Rebenring 56, 38106 Braunschweig, Germany

[3] Institute of Microbiology, Technische Universität Braunschweig, Rebenring 56, 38106 Braunschweig, Germany

[4] Braunschweig Integrated Centre of Systems Biology (BRICS), Technische Universität Braunschweig, Rebenring 56, 38106 Braunschweig, Germany

[5] Center of Pharmaceutical Engineering (PVZ), Technische Universität Braunschweig, Franz-Liszt-Straße 35a, 38106 Braunschweig, Germany

Introduction

Livestock-based production of proteins for food formulations requires at least partial replacement, due to sustainability and animal welfare issues. Precision fermentation has gained attention as an alternative production route for those proteins, as it allows production of the same high-quality proteins without requiring livestock. Precision fermentation has been used for decades to produce specific proteins, such as enzymes and pharmaceuticals

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(Dupuis et al., 2023), but not yet for bulk-scale production of relatively inexpensive food proteins of animal origin. For these, the overall production costs have to be reduced substantially. For this purpose, interventions can be made in both the upstream production and downstream processes (DSP). The consequences of such interventions are often evaluated in terms of relative changes in protein yield or purity. Yet, the molecular design, the expression, and the processes applied during DSP can alter the protein structure, its stability, and the association behavior (Brune et al., 2023; Hoppenreijs et al., 2023; Matthiesen et al., 1996). Such molecular changes could affect the techno-functional properties of the final protein ingredient. Positive effects may allow efficient application in food formulations, thus necessitating less protein. In contrast, negative effects above a certain degree may be unacceptability for food application. It is currently unclear how strongly the functionality range of one protein can be modulated based on such interventions.

The aim of this study is to demonstrate how the choice on production and DSP may influence the technical functionalities for food formulations. Rather than aspiring to a full coverage of the effects, we aim to exemplify how the functionality range of recombinant proteins can be altered, by implementing a range of interventions in both upstream production and DSP (Fig. 1). The functionality was evaluated based on foaming and gelling properties, screening for the ability to stabilize interfaces, and the ability to form space-spanning networks.

The major whey protein β-lactoglobulin (BLG) was used as an example, being a common animal-based protein ingredient in food application. Besides, its recombinant production has already been demonstrated in previous studies for research purposes (for example, Denton et al., 1998; Keppler et al., 2021; Kim et al., 1997). To capture a broad range of functional properties that can be achieved, we intentionally selected (recombinant) BLG from several sources (i.e., bovine, yeast, bacteria). As a result, BLG ingredients differ in the protein amino acid sequence (with structural consequences) and purities, as summarized in Table 1.

Intervention A: Choices in Upstream Production

A first intervention includes the selection of a specific primary protein structure during upstream production (intervention A). Here, the two major natural isoforms BLG A and B (isolated from milk) were investigated. BLG B was recombinantly produced by *Escherichia coli* (rBLG), which has a slightly modified N-terminus compared to the bovine protein (Keppler et al., 2021; Table 1). In addition to the production of the natural isoform, it has already been shown that additional stepwise substitution of different cysteines in rBLG by alanine (rBLG-SH, rBLG-SS, rBLG-C) can alter the structure of BLG from secondary to quaternary level (Brune et al., 2023). We further explore the consequences for their functional properties in this study. All these BLG variants still show a sequence homology of > 96% and have comparable purity (> 90 wt%; Brune et al., 2023). *E. coli* was used for producing recombinant variants, due to the ease of genetic manipulation, relatively low cost, and rapid growth (Francis & Page, 2010). However, *E. coli* does not secrete the proteins into the extracellular medium. Downstream processing is then more challenging, as the cells have to be broken prior to purification and cellular impurities are released (including the modified DNA). This is a major drawback for industrial application (Kleiner-Grote et al., 2018). Nevertheless, the recombinant production of BLG by *E. coli* still serves our purpose here, as it demonstrates the influence of the primary structure on the functional properties.

Interventions B: Mild Purification

A second intervention focused on the purity of the BLG ingredients that is achieved through DSP. Any DSP configuration typically needs to find a balance between purity and yield (intervention B). Extensive purification usually comes at the expense of yield, but the functional properties are probably not linearly dependent on the protein purity; lower purity may even result in better functionality. Therefore, we investigate the impacts of impurities on the functional

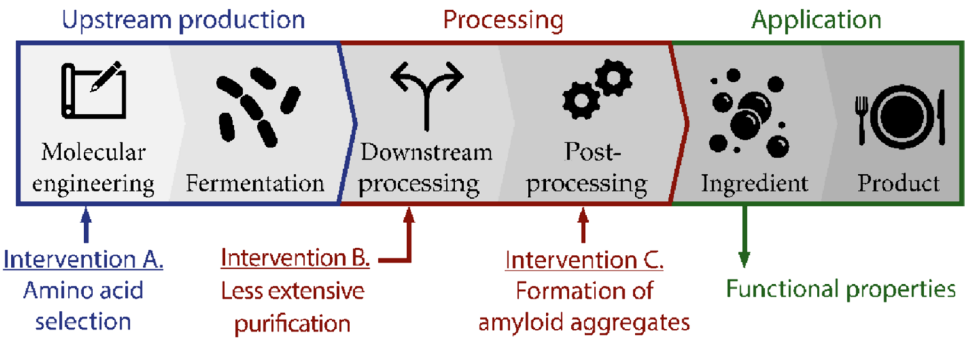

Fig. 1 Overview of the upstream production and downstream processing (DSP) of recombinant proteins for food application. Interventions in several stages of the production process are highlighted underneath

Table 1 BLG ingredients that are investigated in this study to evaluate the impact of particular interventions during upstream production, downstream processing (DSP), or post-processing

Intervention			Protein variant	Abbreviation
Upstream production	A) Amino acid selection	Natural isoforms	Bovine BLG isoform A (bBLG A; Asp64 and Val118)	bBLG A
			Bovine BLG isoform B (bBLG B; Gly64 and Ala118)	bBLG B
		Cysteine mutations (*E. coli*)	Recombinant BLG B (Leu1Ser/Ile2Ala)	rBLG
			rBLG B lacking free thiol (Cys121Ala)	rBLG-SH
			rBLG B lacking exposed disulfide (Cys66Ala/Cys160Ala)	rBLG-SS
			rBLG B lacking all cysteines (Cys66Ala/Cys106Ala/Cys119Ala/Cys121Ala/Cys160Ala)	rBLG-C
DSP	B) Mild purification (*P. pastoris*)		~26 wt% recombinant BLG[a]	Crude yBLG
			~72 wt% recombinant BLG[a] + 1 wt% hexametaphosphate	Purified yBLG
Post-processing	C) Transformation		Whey protein isolate (WPI; 73 ± 1 wt% BLG)	WPI (control)
			Aggregates prepared by heating WPI at pH 3.5 for 5 h	WPI amyloid-like aggregates
			Aggregates prepared by heating WPI at pH 7.0 for 5 h	WPI amyloid fibrils

[a]Isoform A with N-terminal modification AKEEGVSLEKR and truncated forms (Hoppenreijs et al., 2024)

properties of recombinant BLG. In this case, *Pichia pastoris* was used for the production of recombinant BLG (yBLG). *P. pastoris* is an attractive host for food application, as it has GRAS (generally regarded as safe) status, can secrete the recombinant protein in the growth medium, and is able to grow to relatively high cell densities (Dupuis et al., 2023). The yBLG purity is about 26 wt% (based on dry matter) after removal of the yeast cells and low molecular weight impurities by ultrafiltration (cut-off value of 10 kDa); we refer to this material as "crude yBLG" (Hoppenreijs et al., 2024). Further purification of up to ~ 72 wt% can be achieved by selective and reversible precipitation of protein by the food-grade salt hexametaphosphate (HMP; Hoppenreijs et al., 2024). In this procedure, excess HMP is removed from the purified material ("purified yBLG") but a residual amount of ~ 1 wt% remains, probably ionically associated with the protein. This relatively simple purification process could potentially replace large-scale expensive chromatographic processes, if the resulting less pure ingredients still exhibit functional properties.

Intervention C: Transformation Through Post-Processing

In particular, amyloid(-like) aggregates have gained interest to improve functional properties of BLG (reference). Thereby, native BLG could be transformed into functional amyloid(-like) aggregates for efficient application through post-processing (intervention C). More efficient application is of particular interest for the valuable recombinant BLG, but is also relevant for conventional BLG ingredients. WPI and BLG are demonstrated to behave similar in terms of their functionality (Ye, 2010; Phillips et al., 1990; Mahmoudi et al., 2007), while their amyloid-like aggregates also have similar microstructure (Mahmoudi et al., 2007). The resemblance between BLG and WPI suggest that WPI is suitable to evaluate the overall effects of post-processing on the functionality of (recombinant) BLG, even though absolute values may deviate.

Experimental Section

Preparation Protein Ingredients

Intervention A: Variation in the Amino Acid Sequence

To obtain pure forms of BLG with natural mutations, bovine BLG natural isoforms A and B (bBLG A and B, respectively) were purified from milk of cows that was homozygous for the corresponding variants, according to Brune et al. (2023). The nitrogen content was determined by Dumas (Elementar Rapid N exceed, Germany), using L-aspartic acid (Sigma-Aldrich, USA) as a reference, and converted

into the protein content using a conversion factor of 6.33 (Kosters et al., 2010). Recombinant BLG (rBLG; natural isoform B) was produced in *E. coli*. Recombinant cysteine variants were produced through substitution of cysteine residues with alanine residues including the BLG variant without free thiol (Cys121; rBLG-SH), the outer disulfide bond (Cys66, Cys160; rBLG-SS), or any cysteines (Cys66, Cys106, Cys119, Cys121, Cys160; rBLG-C). After disruption of the *E. coli* cells, the protein variants rBLG, rBLG-SH, and rBLG-SS were purified up to > 90 wt% through ion exchange chromatography, ammonium sulfate precipitation, and desalting (via size exclusion chromatography). Purification of rBLG-C (> 90 wt%) was relatively simple as this variant formed inclusion bodies during production. After cell disruption, rBLG-C was resolubilized with urea, and refolded and purified with a single ion exchange step. Details about the production and purification can be found in Brune et al. (2023).

Intervention B: Less Extensive Purification

Recombinant BLG was secreted from *Pichia pastoris* (yBLG; natural isoform A). Production and purification were performed as described in Hoppenreijs et al. (2024). In short, production, filtration (> 10 kDa), and spray drying were performed to obtain "crude yBLG" (obtained from Formo; Germany). Crude yBLG was further purified by selective precipitation of the protein fraction by sodium hexametaphosphate (HMP) at pH 4.0. After centrifugation, the protein-rich pellet was solubilized in ultrapure water through pH neutralization. Excess HMP was precipitated through the addition of calcium chloride. Furthermore, dialysis was performed to desalt the sample. Finally, purified yBLG was obtained after freeze-drying (Beta 2-8 LSCplus, Martin Christ, Germany). The BCA assay kit (Pierce™, ThermoFisher Scientific, USA) was used to determine the protein content of the samples.

Intervention C: Transformation into Amyloid Aggregates

Whey protein isolate (WPI; BiPro, USA) was used to prepare two types of amyloid aggregates: (1) amyloid fibrils and (2) amyloid-like aggregates. About 300 mL of 1 wt% WPI was prepared in a 500-mL volume Schott bottle, and the pH was adjusted to pH 2.0 (for amyloid fibrils) or pH 3.5 (for amyloid-like aggregates) using 1 M hydrochloric acid (HCl). The solution was heated in a water bath at 90 °C, while stirred at 350 rpm using a heat- and water-resistant magnetic stirring plate (MIXdrive 15 HT, 2Mag AG, Germany). After incubation for 5 h, the solution was cooled on ice and the pH was quickly adjusted to 7.0 using 1 M sodium hydroxide (NaOH). The protein solution was dialyzed for 3 days, using a dialysis membrane with 8 kDa cut-off value (BioDesign Inc., USA), in a cooled room (4 °C). For dialysis, demineralized-water was used in a volume that was approximately 100 times larger than the protein solution and was refreshed twice a day. Finally, the solutions were frozen and lyophilized to obtain dried amyloid-like aggregates. Analysis with transmission electron microscopy (TEM) was performed throughout processing of the aggregates to check whether the morphology was maintained, according to Hoppenreijs et al. (2023).

Functionality Experiments

Foaming

The foaming properties of protein solutions were determined at different concentrations by analysis with the Foamscan™ (Teclis, France). Protein solutions were prepared in a concentration of 1 mg mL^{-1} in a 10 mM phosphate buffer (pH 7.0), a day before the measurement in independent triplicates. Subsequent dilution with the buffer was performed to obtain solutions with different protein concentrations. All solutions were analyzed at 1, 0.5, 0.25, and 0.1 mg mL^{-1}, except for WPI amyloid-like aggregates (1, 0.5, 0.15, 0.1, 0.05 mg mL^{-1}). For the recombinant cysteine variants, the solution at 1 mg mL^{-1} was re-used after analysis to prepare the subsequent solutions. To check whether the protein content decreased due to the analysis, the absorbance was measured at 280 nm (DR6000; Hach, USA) before and after analysis (diluted to 0.5 mg mL^{-1}) in a quartz cuvette.

To analyze the foaming properties, the protein solution (31 mL) was stepwise injected into the Foamscan to calibrate the vertical electrodes: first 16 mL (i.e., approximately 15 mL detected and 1 mL to fill the dead space in the injection tube) and then three subsequent steps of 5 mL. Foaming was induced through sparging air through a glass frit (P3; porosity of 16–20 µm; Teclis, France) at 250 mL min^{-1}, until a total foam volume of 160 mL was reached.

To evaluate the foamability, the time to reach 160 mL of foam was recorded by a camera. The foam stability was derived as the half-life time ($t_{1/2}$) of the foam collapse. A second camera captured the foam structure for 1 mg mL^{-1} solutions. Pictures were taken directly after sparging ended and analyzed using Matlab (R2022B; Version 9.13.0.2166757) and the toolbox DIPimage (Luengo Hendriks & van Vliet, 2000; Version 2.8). The script is available upon request. The minimum size of bubbles included in the analysis was set to 50 $pixels^2$ to avoid artifacts to be included, such as reflections. Finally, the Sauter mean diameter ($d_{3,2}$) of the bubbles in the foam and air fraction of the foam were determined.

Gelation

Protein samples were solubilized in ultrapure water (1.5 mL) in a concentration of 15 wt% protein. The pH was adjusted to 7.0 when necessary, using 1 M NaOH or HCl, and the solution was stored overnight the fridge to ensure complete hydration. The next day, the solution was degassed: it was placed in a desiccator (used as vacuum chamber) connected to a vacuum pump. While slowly stirring with a magnetic stirrer, a vacuum (1/4 of total power) was applied to the chamber of the desiccator for 20 min. Afterwards, the sample was analyzed for its gelation. A rheometer (MCR502; Anton Paar GmbH, Austria) was equipped with a small volume concentric cylinder (CC10/T200/SS; Anton Paar GmbH). The cup was filled with 900 µL of sample. After lowering the cylinder into the sample, 100 µL of paraffin oil (Supelco; Merck, Germany) was transferred onto the sample to prevent evaporation during heating. First, the temperature was stabilized at 20 °C and the sample was equilibrated for 5 min. To induce gelation, a heating cycle was applied: first, the temperature was increased from 20 to 90 °C in 20 min, then held at 90 °C for 30 min, then cooled back to 20 °C in 20 min, and another 5 min kept at 20 °C. During the heating cycle, the shear strain and shear frequency were kept constant at 1% and 1 Hz, respectively. The gel point was determined as the point at which the storage modulus (G') became higher than the loss modulus (G") and the torque was above the limit of detection (i.e., 0.0005 mNm; Anton Paar GmbH).

After gel formation, deformation was applied by increasing the shear strain amplitude from 0.01 to 1010% at a constant frequency of 1 Hz. The initial strengths of the gels were determined as the average storage modulus at low shear strain (0–1%; linear viscoelastic region), i.e., when the G' was independent of the shear strain. The critical strain was determined as the strain at which the G' decreased by 5%, as compared to the initial gel strength. Lissajous plots were prepared by plotting the intracycle shear stress and shear strain response at a shear strain amplitude of 2.4, 23.8, 101, 318, and 1010%.

Results and Discussion

First, the impact of slight changes in BLG amino acid sequence (intervention A) on the functional properties is discussed in "Intervention A: Variations in the Amino Acid Sequence" section, considering both natural isoforms and the cysteine mutations. "Intervention B: Less Extensive Purification" section then describes to what extent simplified purification of expressed BLG (intervention B) affects the functionality of the protein. In "Intervention C: Transformation into Amyloid Aggregates" section, additional post-processing to transform WPI into amyloid aggregates (intervention C) is considered to improve functional properties. Finally, a summary and comparison of the performance of all BLG ingredients is given in "Comparison BLG Ingredients" section.

Intervention A: Variations in the Amino Acid Sequence

Bovine milk containing only bBLG isoforms A or B was collected, and these proteins were purified. After purification, highly pure protein powders were obtained containing 94.6 ± 1.2 and 94.0 ± 1.1 wt% bBLG A and B, respectively. Besides, production of recombinant BLG B and cysteine variants in *E. coli* and subsequent purification also resulted in high purity (> 90 wt%; Brune et al., 2023). The complete structural characterization of these variants, as well as the bovine control, can be found in Brune et al. (2023). The main structural elements are summarized in Table 2. Even though bBLG A was not included in that same study, others noted the structural resemblance between isoforms A and B (Dong et al., 1996, Qin et al., 1999).

Impact of Intervention A on Foaming Properties

The formation of foam, the structure of the foam, and foam collapse of bBLG A and B solutions are depicted in Fig. 2. The foams from bBLG A were formed more quickly (Fig. 2A), and thus contained relatively less air and more liquid, as compared to bBLG B (Fig. 2C). In addition, smaller

Table 2 Overview of the structure of the cysteine mutants, according to Brune et al. (2023)

Variant	Secondary structure [%]				Tertiary structure	Quaternary structure[a]
	α-helix	β-sheet	Random coil	β-turn		
bBLG B	16 ± 0	47 ± 1	26 ± 0	11 ± 1	Globular	Mostly dimers
rBLG	16 ± 1	51 ± 1	21 ± 1	12 ± 1	Globular	Mostly dimers
rBLG-SH	22 ± 1	51 ± 1	18 ± 0	10 ± 2	Globular	Mostly dimers
rBLG-SS	21 ± 1	**35 ± 1**	**36 ± 1**	8 ± 1	**Opened**	**Mostly monomers**
rBLG-C	29 ± 2	**9 ± 3**	**57 ± 2**	6 ± 2	**Disordered**	**"Dimers"**

Main differences are in boldface

[a]Determined at a protein concentration of about 1 mg mL−1 phosphate buffer (10 mM; pH 7.0) and ambient temperature

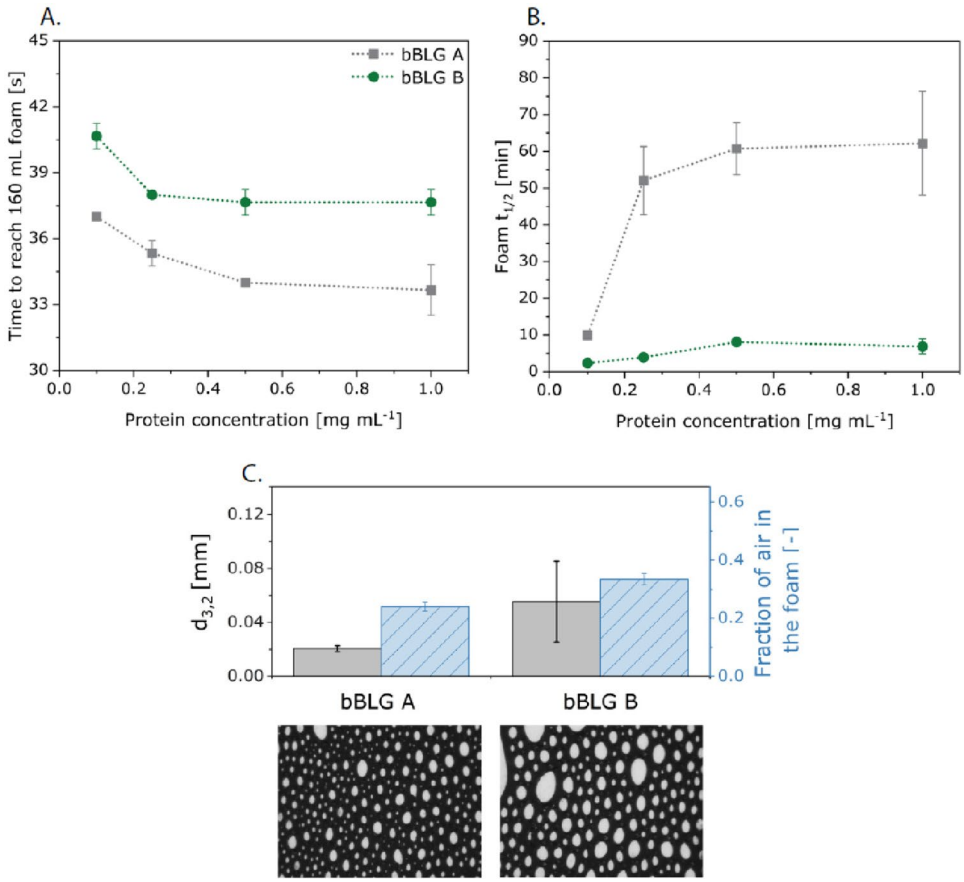

Fig. 2 (**A**) Time to reach 160 mL of foam volume when sparging (250 mL min^{-1}) solutions of bovine BLG natural isoforms A and B, and (**B**) the corresponding foam half-life time ($t_{1/2}$). (**C**) Immediately after formation of the foam from 1 mg mL^{-1} solutions, the Sauter mean diameter ($d_{3,2}$) of the bubbles and the fraction of air in the foam is shown, with corresponding pictures

average bubble sizes were noted (Fig. 2C). Smaller bubbles can lift liquid more efficiently into the foam during foaming (with less drainage). This increased the relative liquid fraction in the foam and thus necessitates shorter foaming times to get to the required foam volume. Euston et al. (1999) observed also smaller droplet size in emulsions stabilized by bBLG A, as compared to bBLG B. Furthermore, foams from bBLG A were stable for almost 10 times as long, as from bBLG B. Delahaije and Wierenga (2022) reported that the foam stability largely depends on the initial foam structure, while the surface hydrophobicity did not affect foam stability. Besides, Ipsen et al. (2001) demonstrated that partial hydrolysis of bBLG A results in similar interfacial properties, but the foam stability was improved due to faster adsorption and smaller average bubble size. It is therefore likely that the higher stability of the bBLG A foam, as compared to the bBLG B foam, resulted from the relatively smaller bubbles in the initial foam (Fig. 2).

The better foamability of bBLG A is probably not directly related to its higher surface charge (surface charge given in Table S1), as this is generally expected to increase the energy barrier for adsorption to the interface (Foegeding et al., 2006). Instead, structural differences may explain the different foamabilities. About 48% of bBLG A is expected to assume a dimeric configuration under the applied conditions, while 63% of bBLG B is dimeric (calculated with the association constant in a 50 mM phosphate buffer at pH 6.9, Zimmerman et al., 1970; calculation in Supporting Information A). The dominating monomeric configuration and structural flexibility of bBLG A may enhance its foaming properties, as compared to the dominating dimeric configuration and tighter structure of bBLG B. Perriman et al. (2007) reported a preferential adsorption of BLG monomers over dimers. They describe that the hydrophobic residues involved in the adsorption to the air-water interface are also involved in the dimer interface. Thereby, dimers have to dissociate into monomers before being able to interact with the interface.

Besides to the different monomer-dimer equilibrium, BLG A exhibits a higher conformational flexibility: it has also been reported to be more sensitive to unfolding upon pressurizing (Olsen et al., 2022), to tryptic hydrolysis (Creamer et al., 2004), and to exchange of the hydrogen atoms within its structure (Dong et al., 1996). This makes it likely that bBLG A anchors more easily to the air-water interface, and/or that structural rearrangements occur to a larger extent, as compared to bBLG B. This is in line with Ipsen and Otte (2004), who demonstrate faster adsorption of bBLG A to the air-water interface (< 0.1 mg mL^{-1} BLG in a 20 mM imidazole buffer; pH 7.0). However, the same

authors also found that bBLG B yielded a more stable foam (10 mg mL^{-1} BLG in a 20 mM imidazole buffer), when the foam was prepared under high-shear whipping conditions. Mackie et al. (1999) studied the adsorption of the separate isoforms to the air-water interface (surface layers and not foams), measuring changes in surface tension and the resulting surface rheology. They found faster adsorption of bBLG B to the air-water interface and attribute this to the higher surface hydrophobicity in its native state (< 0.02 mg mL^{-1} BLG in a 10 mM phosphate buffer; pH 7.0). This opposite conclusion regarding the relative performance of these natural isoforms might be due to different conditions: the applied protein concentration and/or method. A low protein concentration was applied in the study of Mackie et al. (1999), which could alter the monomer-dimer equilibrium (Euston et al., 1999). In case of Ipsen and Otte (2004), the high shear might have facilitated destabilization of the variants. This reduces the impact of the stability of the native structures on the foamability and resulting foam stability.

Figure 3 summarizes the formation of foam, the structure of the foam, and foam collapse for the produced recombinant BLG variants: the wild-type (rBLG), the variant lacking free thiol (Cys121; rBLG-SH), the variant lacking the outer disulfide bond (Cys66–Cys160; rBLG-SS), and the variant lacking all cysteines (rBLG-C). The variant bBLG B is depicted as a control. Since only low amounts of recombinant protein material were available, the sample at 1 mg mL^{-1} after measuring was re-used to prepare the further dilutions. Re-use of the sample was not expected to affect the results, as conformation changes upon foaming are expected to be reversible (Phillips et al., 1995). The measurement resulted in a slight dilution caused by residual water in the device after cleaning. This dilution was in the range of 5.0 to 8.8 wt% and was similar for the different samples ($p > 0.05$). The protein concentrations depicted for these samples (Fig. 3) were corrected for this dilution.

The foaming behavior was different between bovine BLG B and the wild-type rBLG. At concentrations < 1 mg mL^{-1}, bBLG B demonstrated slightly better foamability and stability (Fig. 3C). The wild-type rBLG contains two changes at the N-terminus: Leu1Ala and Ile2Ser. Both Leu and Ile in bovine BLG are more hydrophobic than Ala and Ser in the wild-type bBLG B (Wilce et al., 1995). Especially in the protein-poor regime, increased hydrophobicity can accelerate protein adsorption (Delahaije & Wierenga, 2022). The faster adsorption of bBLG B to the interface might also explain the smaller bubble size, as compared to rBLG (Fig. 3C). When increasing the protein concentration from 0.5 to 1 mg mL^{-1}, the foam stability of bBLG B was not improved. Surprisingly, the foam of rBLG had a higher stability at 1 mg mL^{-1} than that of bBLG B (Fig. 3B), even though its bubbles were larger (Fig. 3C). To explain these differences, further investigation of the interfacial properties is recommended.

Removal of the free thiol (Cys121Ala) slightly increased the foamability of rBLG (Fig. 3A). In addition to the improved foamability of rBLG-SH, as compared to rBLG,

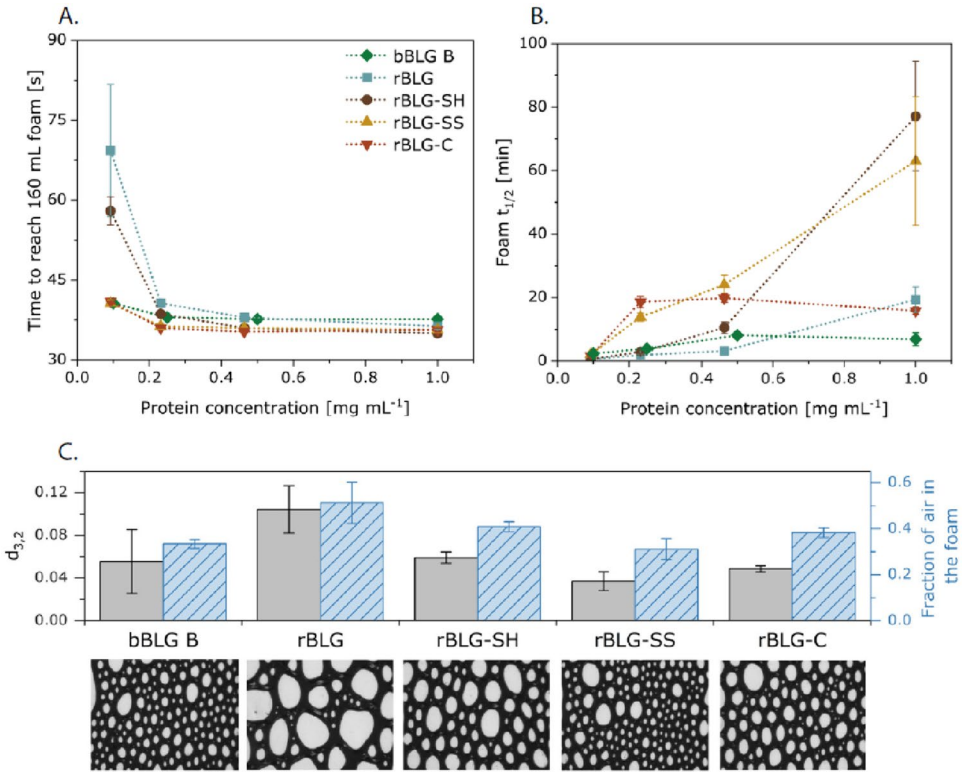

Fig. 3 (**A**) Time to reach 160 mL of foam volume when sparging (250 mL min^{-1}) solutions of recombinant BLG variants rBLG, rBLG-SH, rBLG-SS, and rBLG-C, and (**B**) the foam half-life time ($t_{1/2}$). (**C**) Immediately after formation of the foam from 1 mg mL^{-1} solutions, the Sauter mean diameter ($d_{3,2}$) of the bubbles and the fraction of air in the foam is shown, with corresponding pictures

the foam stability was also enhanced (Fig. 3C). Croguennec et al. (2006) reported improved foaming properties of BLG when blocking the free thiol, which was attributed to the destabilizing impact of the modification. Similarly, our induced removal of the free thiol also destabilized BLG: the onset temperature for denaturation was decreased by approximately 5 °C (Brune et al., 2023). Destabilization could facilitate rearrangements at the interface, potentially improving the surface coverage. If so, this could in turn explain the formation smaller bubbles (Fig. 3C). Finally, some studies suggest that intermolecular disulfide bonds might form upon adsorption to the interface, which in turn would contribute to the foam stability (Bos & Van Vliet, 2001; Martin et al., 2002; Nicorescu et al., 2009). This hypothesis does not align with our results, as removal of the free thiol improved and not suppressed the foam stability after bubbling (Fig. 3). However, disulfide bond formation may be more relevant in foams formed under conditions that stimulate protein unfolding, such as heating.

Removal of the intramolecular disulfide bond in rBLG-SS and rBLG-C also improved the foamability, especially in the protein-depleted regime (< 0.5 mg mL^{-1}; Fig. 3A). Kella et al. (1989) observed increased adsorption of whey proteins when 50% of the disulfide bonds were cleaved, due the increased unfolding ability. Recombinant removal of the intramolecular disulfide(s) is expected to increase the tendency to unfold at the air-water interface, but also the extent of this unfolding. Interestingly, rBLG-SS and rBLG-C demonstrated similar foamability (Fig. 3A). This indicates that the improved behavior is related to removal of the outer SS bond (Cys66–Cys160), and not necessarily by the removal of the inner disulfide bond (Cys106–Cys119).

Removal of all cysteines in rBLG-C led to a similar foam stability at concentrations < 1 mg mL^{-1}, but lower foam stability at a concentration of 1 mg mL^{-1}, as compared to rBLG-SS (Fig. 3B). At higher concentrations, the denser packing at the interface could allow a thicker interface for the partially destabilized rBLG-SS, as compared to the more disordered rBLG-C (Table 2). Similarly, Ipsen et al. (2001) reported a lower viscoelastic modulus of interfaces stabilized by completely hydrolysed bBLG A, compared to those stabilized by partially hydrolysed bBLG A. Interfaces stabilized by disordered caseinate exhibit a lower viscoelastic modulus, compared to globular proteins (Lajnaf et al., 2022). Thereby, we hypothesize that while completely destabilizing BLG by substituting all cysteines leads to better foam formation, partial preservation of the structure allows the formation of a stronger interface.

We earlier hypothesized that under the conditions applied, the better foaming functionality of bBLG A is mostly related to its destabilized and monomeric configuration, as compared to bBLG B. This is consistent with our hypothesis that destabilization improves foam formation. Removal of the outer disulfide bond in recombinant BLG B (variant rBLG-SS) transforms the genetic B variant into a monomeric configuration that is partially destabilized (Table 2). This variant demonstrated similar foaming properties at 1 mg mL^{-1}, as compared to BLG A, while the foaming properties at lower concentrations were inferior to those of bBLG A (Figs. 2 and 3). This indicates that the improved properties of bBLG A in the protein-depleted regime under the applied conditions are not only due to structural destabilization and/or its monomeric configuration, but are (also) related to other physicochemical properties that are changed due to one or both mutation(s).

Overall, it is clear that the foaming properties can be highly influenced by the substitution of only a few amino acids, as shown for the natural isoforms of induced cysteine mutations. We hypothesize that foam formation is mostly facilitated by structural destabilization (on secondary to quaternary level), while the residual structure (stabilized by the inner disulfide bond) contributes to foam stability. In addition, other changes in the physicochemical properties can also play a role.

Impact of Intervention A on Heat-Set Gelation

The heat-induced gelation of solutions of bBLG A and bBLG B is shown in Fig. 4, while the main parameters are listed in Table 3. For both variants, gelation occurred when 15 wt% protein solutions were heated for approximately half an hour (Table 3). Variant bBLG A gelled slightly earlier (~ 0.4 min) and showed a steeper increase in the storage modulus (G'; Fig. 4), as compared to bBLG B. Huang et al. (1994) and McSwiney et al. (1994) also observed earlier gelation and more rapid initial gelation of bBLG A, as compared to bBLG B solutions, and attributed this to its lower heat stability. Similarly, we found earlier unfolding of bBLG A under the applied conditions (onset temperature for unfolding is listed in Table S3). Thus, we hypothesize that the free thiol is exposed more rapidly and then formed intermolecular disulfide bonds more easily. This is in line with Elofsson et al. (1996), who also reported earlier disulfide bond formation for bBLG A, as compared to bBLG B (determined at ~ 48 mg mL^{-1}). Disulfide bond formation in BLG B could also be slightly delayed due to the necessity to first dissociate the dimeric configuration. In contrast, Manderson (1998) reported a slower aggregation of bBLG A, as compared to bBLG B, in dilute systems (2–5 mg protein per mL). They argued that the opposite might be true at high protein concentrations (> 50 mg mL^{-1}) or at temperatures < 95 °C, as also suggested by Nielsen et al. (1996).

During longer heating times, bBLG B showed a more gradual increase in G', but eventually yielded a stronger gel than bBLG A (Fig. 4, Table 3): the final G' for bBLG B was about 40% higher as compared to bBLG A. The

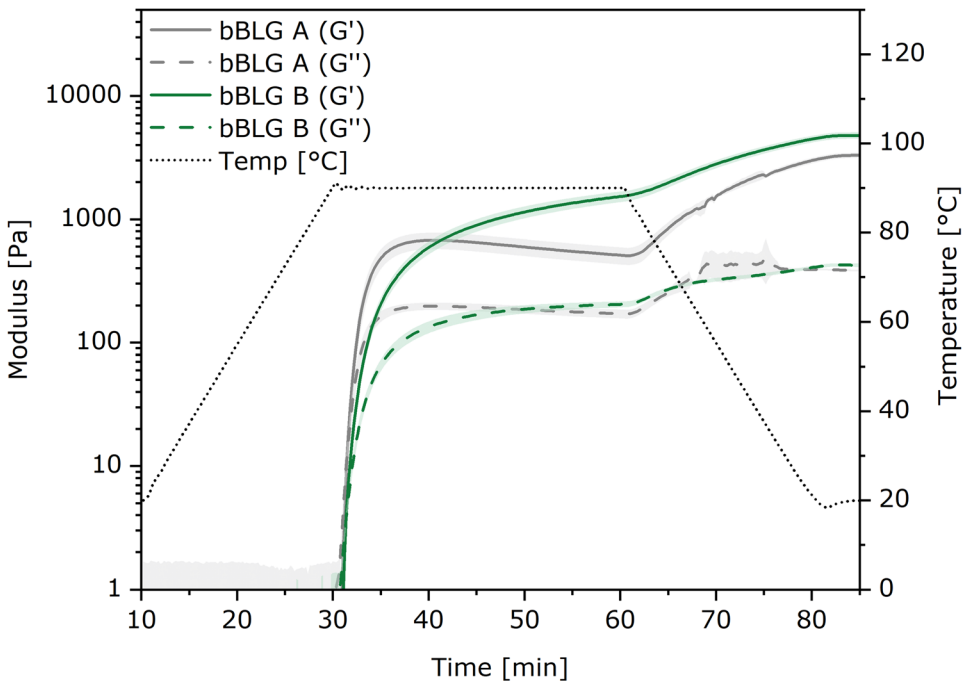

Fig. 4 (**A**) Storage (G') and loss (G") moduli upon heating 15 wt% protein solutions of bovine BLG natural isoforms A and B

subsequent deformation showed that the bBLG A gel was less elastic upon small deformation, as it showed a lower critical strain (Table 3). The behavior upon large deformation is depicted in the Lissajous plots, shown in Fig. 5. Only the Lissajous plots for the bBLG B gels exhibit inverted sigmoidal shape at 101% and 318%. This so-called strain stiffening behavior indicates a high resistance of the gel against larger deformation (i.e., when the strain increases). This may indicate a higher cross-link density within the gel (Groot et al., 1996). Besides, Fig. 5 confirms stronger gels for isoform B, as compared to isoform A; the shear stresses obtained are relatively higher and irreversible deformation (i.e., opening of the Lissajous plot) is delayed.

The stronger and more ductile gel that is obtained with bBLG B, as compared to bBLG A, has not been previously reported. Huang et al. (1994) and McSwiney et al. (1994) obtained a higher G' for bBLG A, instead of bBLG B, when heating BLG in a buffer at 80 to 85 °C. Yet, Huang et al. (1994) noted that the G' values became more similar for the two variants during longer heating times. This is in line with our results, indicating different aggregation mechanisms with bBLG A and bBLG B, when comparing the initial and the extended gelation phases. If the variants are heated for a longer period of time (> 40 min) at sufficiently high temperature (90 °C), complete destabilization of the monomeric BLG is expected. Under these conditions, disulfide bond formation can be maximized and the extent of disulfide bond formation in bBLG B gels may exceed that in bBLG A gels. This increased density of disulfide cross-links could explain the higher G' (Fig. 4), as well as the strain stiffening behavior of the gel (Fig. 5). Qin et al. (1999) reported higher accessibility (and resulting reactivity) of Cys121 in bBLG B, due to a greater mobility of this region. Manderson (1998) mentioned that the different amino acid at position 64 between bBLG A and B is close to the disulfide bonds Cys66–Cys160. The charged Asp64 in bBLG A could have caused increased electrostatic repulsion, as compared to the neutral Gly64 in bBLG B, and subsequent decreased tendency to participate in thiol-disulfide interchange reactions. This electrostatic repulsion might be suppressed in both the studies of Huang et al. (1994) and McSwiney et al. (1994), as they use buffers. In those cases, the reactivity of Cys66–Cys160 towards disulfide bond formation can be more similar for BLG A and B. The structural flexibility of BLG A could then result in more interactions to form stronger gels. Thus, specific isoforms can be selected to improve gelation, while the preferred isoform can differ when the gelation conditions are changed.

Table 3 Gelation properties of various protein solutions (15 wt% total protein)

	Gel point (min)	Final G' (Pa)	Critical strain (%)
bBLG A	31.7 ± 0.1	3,442 ± 146	5.2 ± 0.8
bBLG B	32.1 ± 0.1	4,831 ± 336	8.4 ± 1.5

Intervention B: Less Extensive Purification

Recombinant BLG (natural isoform A) was produced with *P. pastoris*. Crude yBLG is the material that we refer to after production and secretion of recombinant BLG and

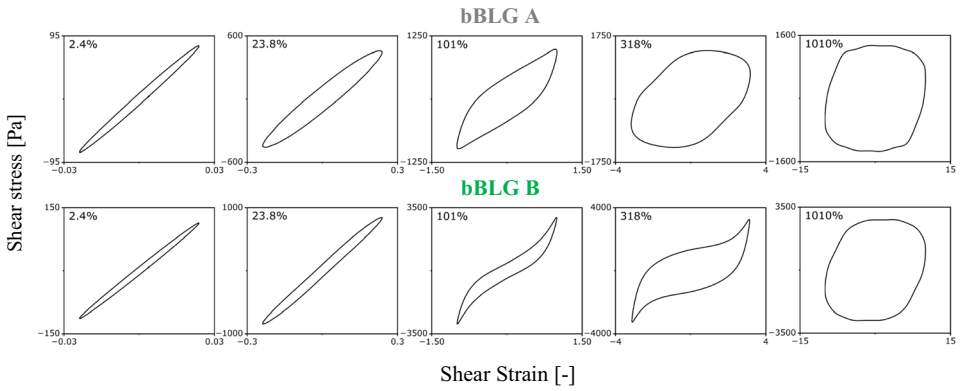

Fig. 5 Lissajous plots of heat-set bBLG A and bBLG B gels (15 wt% protein; average of triplicate), plotting the shear stress and shear strain (for sinusoidal strain). The maximum strain amplitude is depicted in the upper left corner of the graphs

removal of insoluble, as well as small molecular weight (< 8 kDa) material (e.g., salts, nutrients and released small cellular impurities). When repeating the purification as performed in Hoppenreijs et al. (2024), we obtained the material we refer to as "purified yBLG." This material had a comparable protein content to that reported in Hoppenreijs et al. (2024; Table 4). The same study reports that yBLG was the only protein in the sample. We assume that the carbohydrate content (i.e., mostly mannan impurities) and HMP content were also in comparable range, while a fraction of ~ 20 wt% of impurities remained unidentified. Finally, we do not have a suitable control that has the same sequence as yBLG (i.e., with modified N-terminus; Table 1) without any impurities. Thereby, we depict the results of bBLG A, being the bovine isomer that resembles yBLG most (Table 1), for comparison.

Impact of Intervention B on Foaming Properties

The formation of foam, the structure of the foam, and foam collapse of solutions containing crude and purified yBLG are shown in Fig. 6. At the highest concentration of 1 mg mL^{-1}, an instable foam was formed for crude yBLG. Therefore, no further dilutions were tested. The foam already collapsed during its formation (i.e., 43–164% of the sparged gas was released). Therefore, the time to reach 160 mL of foam was almost twice as long as for other BLG variants (Fig. 6A). Due to the early collapse of the bigger bubbles, the mean bubble size in the final foam and the fraction of air in the foam were relatively small (Fig. 6C). The poor foaming properties of the crude yBLG seem to be related to specific impurities, and not necessarily the N-terminal modification, as the foaming properties improved significantly after purification (Fig. 6). Since both crude and purified yBLG contain a considerable amount of mannan impurities (Table 4), mannan is not expected to cause the poor foamability of crude yBLG. Besides, a model system of pure bBLG A (1 mg mL^{-1}) with and without added mannan (from Saccharomyces cerevisiae; equal to the content in crude yBLG) resulted in similar foaming time and foam stability ($p > 0.05$; data not shown). Further identification of the unknown fraction in crude yBLG is suggested to determine the cause of the poor functionality of crude yBLG.

The purified yBLG demonstrated better foaming properties than crude yBLG, but inferior than bBLG A (Fig. 6). This is not expected to be due to the presence of HMP; a model system of pure bBLG A (1 mg mL^{-1}) with and without added HMP (equal content to the HMP content in purified yBLG) resulted in similar foaming time and foam stability ($p > 0.05$; data not shown). Purified yBLG formed larger bubbles and the foam was about 10 times less stable, as compared to bBLG A. Similar to crude yBLG, but to a lesser extent, the inferior foaming could have been caused by unknown impurities. Furthermore, the extended N-terminus could have also influenced the foaming properties of the purified yBLG, as compared to bBLG A, for example, by enhancing electrostatic repulsion.

Impact of Intervention B on Heat-Set Gelation

The heat-set gelation of crude and purified yBLG is depicted in Fig. 7, while the main parameters are listed in Table 5.

Table 4 Expected composition of crude yBLG and purified yBLG, as reported in Hoppenreijs et al. (2024)

Material	Content (wt%)			
	Protein	Carbohydrate	Hexametaphosphate	Unidentified
Crude yBLG	26 ± 0 **22.4 ± 0.2**	53 ± 1	n.a	21 ± 1
Purified yBLG	72 ± 0 **67.1 ± 0.7**	9 ± 1	1 ± 0	18 ± 1

The protein content in this current research is given in bold

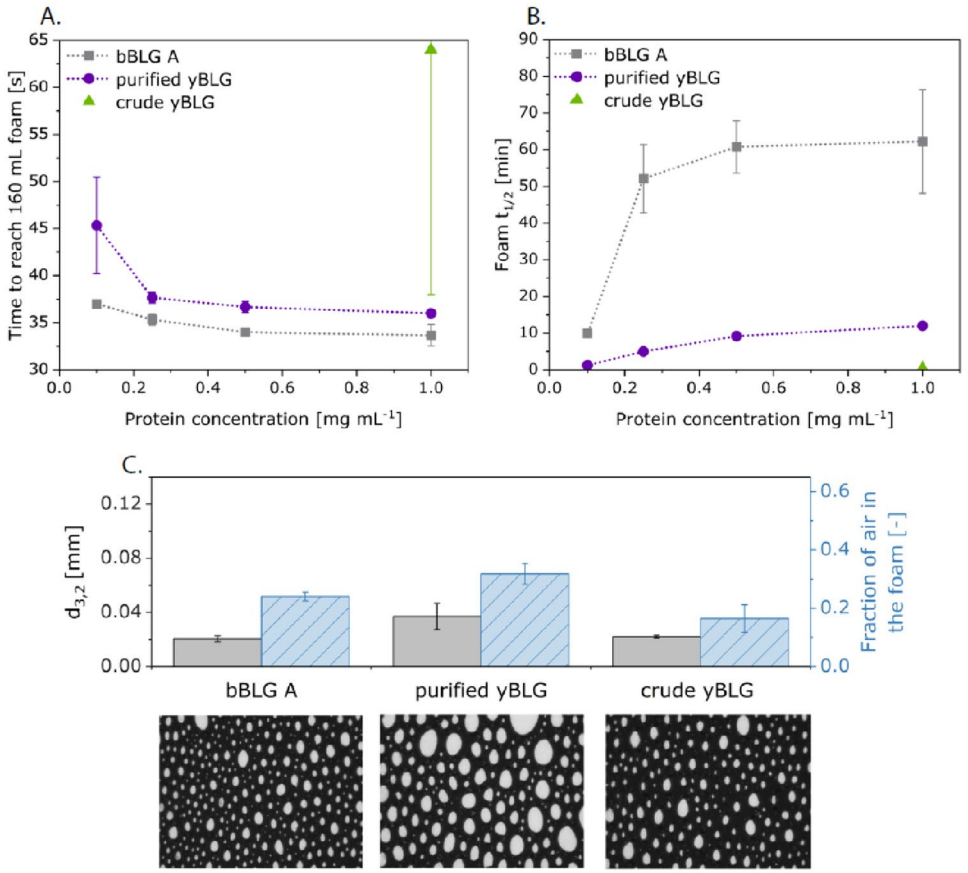

Fig. 6 (**A**) Time to reach 160 mL of foam volume when sparging (250 mL min^{-1}) solutions of recombinant yBLG before and after purification, and bovine BLG A for comparison, and (**B**) the foam half-life time ($t_{1/2}$). (**C**) Immediately after formation of the foam from 1 mg mL^{-1} solutions, the Sauter mean diameter ($d_{3,2}$) of the bubbles and the fraction of air in the foam is shown, with corresponding pictures

The crude yBLG (~ 22 wt% protein) showed extremely poor gelling properties. Compared to pure bBLG A at the same protein concentration, twice as long heating time was required to reach gelation, while the formed gel was very weak (Table 5). Although crude yBLG was slightly more thermostable, it is expected to unfold and subsequently aggregate at 90 °C (having an onset temperature of 72 °C; Table S1). The gelation properties of yBLG clearly improved after purification (purified yBLG; Fig. 7), and even exceeded those of bBLG A. This indicates that impurities prior to purification had counteracted the gelation, while the intrinsic functionality of purified yBLG is very good.

Gelation of purified yBLG occurred earlier and resulted in a much stronger gel, as compared to pure bovine BLG

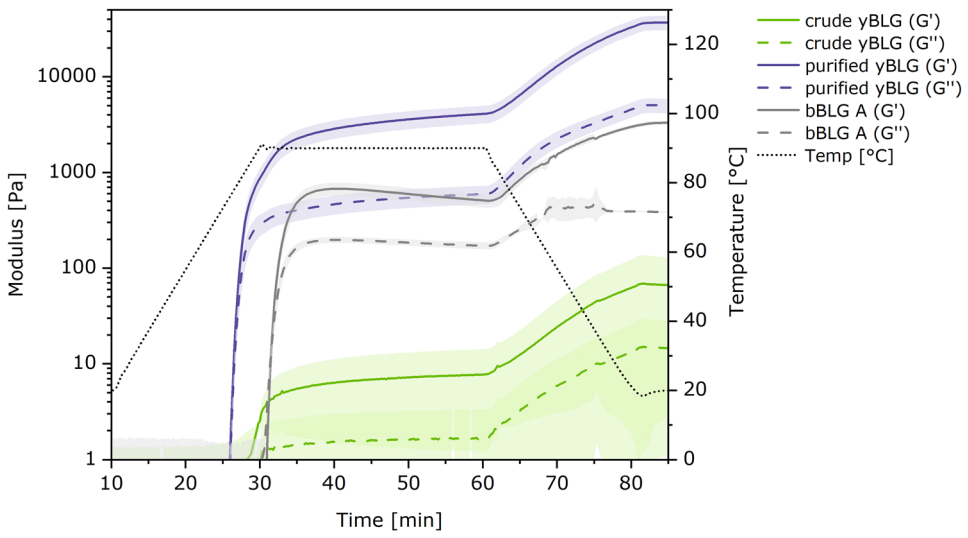

Fig. 7 Storage (G') and loss (G") moduli upon heating 15 wt% solutions of recombinant BLG from yeast before ("crude yBLG") and after purification ("purified yBLG")

Table 5 Gelation properties of various protein solutions (15 wt% total protein)

	Gel point (min)	Final G' (Pa)	Critical strain (%)
bBLG A	31.7 ± 0.1	3,442 ± 146	5.2 ± 0.8
Crude yBLG	65.8 ± 5.4	88 ± 63	1.4 ± 0.4
Purified yBLG	26.7 ± 0.1	37,361 ± 6209	2.9 ± 0.5

A, with an approximate tenfold higher final G' (Table 5). During both heating and cooling, the increase in the moduli was much more pronounced for purified yBLG, as compared to bBLG A (Fig. 7). Although the gel of purified yBLG was stronger as compared to bBLG A, it was also more brittle during small deformation (i.e., lower critical strain; Table 5). Furthermore, the gel formed from purified yBLG was white, indicating a particulate network (Langton & Hermansson, 1992). In contrast, both isoforms of pure bBLG formed a transparent gel at pH 7.0, which is associated with a fine-stranded network (Langton & Hermansson, 1992). During large deformation, the purified yBLG gel exhibited strong strain stiffening at a shear strain of 101% (Fig. 8). In contrast, bBLG A already started to yield at that strain (Fig. 8).

Some additional tests were performed with model systems to evaluate the impact of impurities that we know are similar to those present in purified yBLG. We combined pure bBLG A with mannan (from *Saccharomyces cerevisiae*) or hexametaphosphate (HMP). The quantities of mannan or HMP corresponded to their content in purified yBLG. The results are depicted in Supplementary Information C. Both compounds increased the final G' of bBLG A by a factor of 4 and 15 in the presence of mannan or HMP, respectively. In addition, the gels were more ductile upon small deformation, and the critical strains increased in the presence of either mannan or HMP (Table S3). Lastly, bBLG A with mannan added demonstrated stronger strain stiffening behavior upon large deformation, but this was not observed in the presence of HMP (Fig. S2).

The poor gelation of crude yBLG is unlikely to be related to the presence of mannan, as a model system with pure bBLG A and yeast mannan improved gelation (Fig. S1). Instead, the remaining unidentified impurities in crude yBLG could have caused its poor gelation properties. Purified yBLG contains HMP, which accelerates gelation and increases the final G' (Table S3). Nevertheless, gelation of purified yBLG resulted in a lower G', as compared to the model system bBLG + HMP (difference of 8,344 Pa; Tables 5 and S3). Therefore, either the residual impurities in purified yBLG and/or the modified N-terminus interfered with the gelation. Furthermore, purified yBLG formed a more particulate gel network, as compared to the fine-stranded bBLG A (both with and without mannan or HMP). Reducing the electrostatic repulsion by adding ions or adjusting pH is reported to form particulate instead of fine-stranded networks (Langton & Hermansson, 1992). Although the purified yBLG is not expected to contain many monovalent or divalent salts (due to dialysis), NaOH was added to neutralize the pH prior to gelation. The final concentration of Na^+ in the protein solutions was approximately 10 mM. This is relatively low and not expected to cause the white appearance. For example, it was reported that this shift in appearance of heat-set whey protein isolate was evident above NaCl additions of approximately 100 mM (Urbonaite et al., 2016). Alternatively, the N-terminal modification may have played a role, even though the additional charged amino acids would be expected to increase rather than decrease the overall electrostatic repulsion.

Intervention C: Transformation into Amyloid Aggregates

Post-processing was performed to prepare anisotropic aggregates at pH 2.0 (amyloid fibrils) or pH 3.5 (amyloid-like aggregates) and to obtain an easy-to-handle food ingredient. The pH was adjusted to 7.0, excess ions were removed, and the material was dried. The aggregate morphology throughout this processing was visualized using TEM. The aggregates before and after processing are

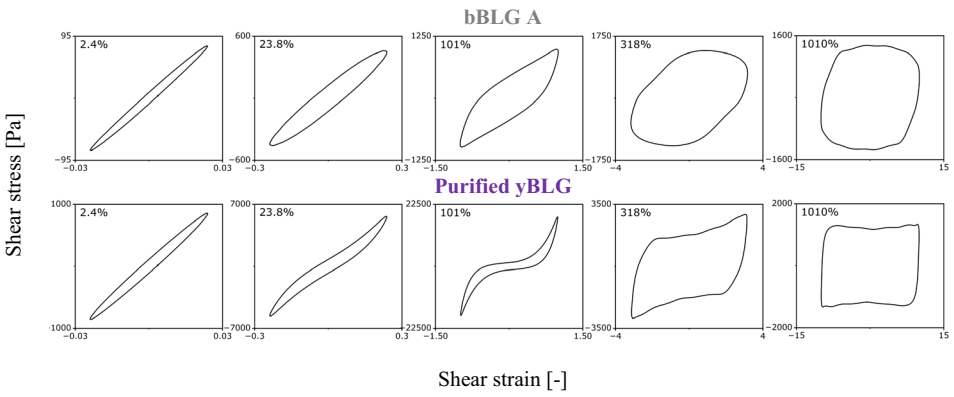

Fig. 8 Lissajous plots of heat-set bBLG A and purified yBLG gels (15 wt% protein; average of triplicate), plotting the shear stress and shear strain (for sinusoidal strain). The maximum strain amplitude is depicted in the upper left corner of the graphs

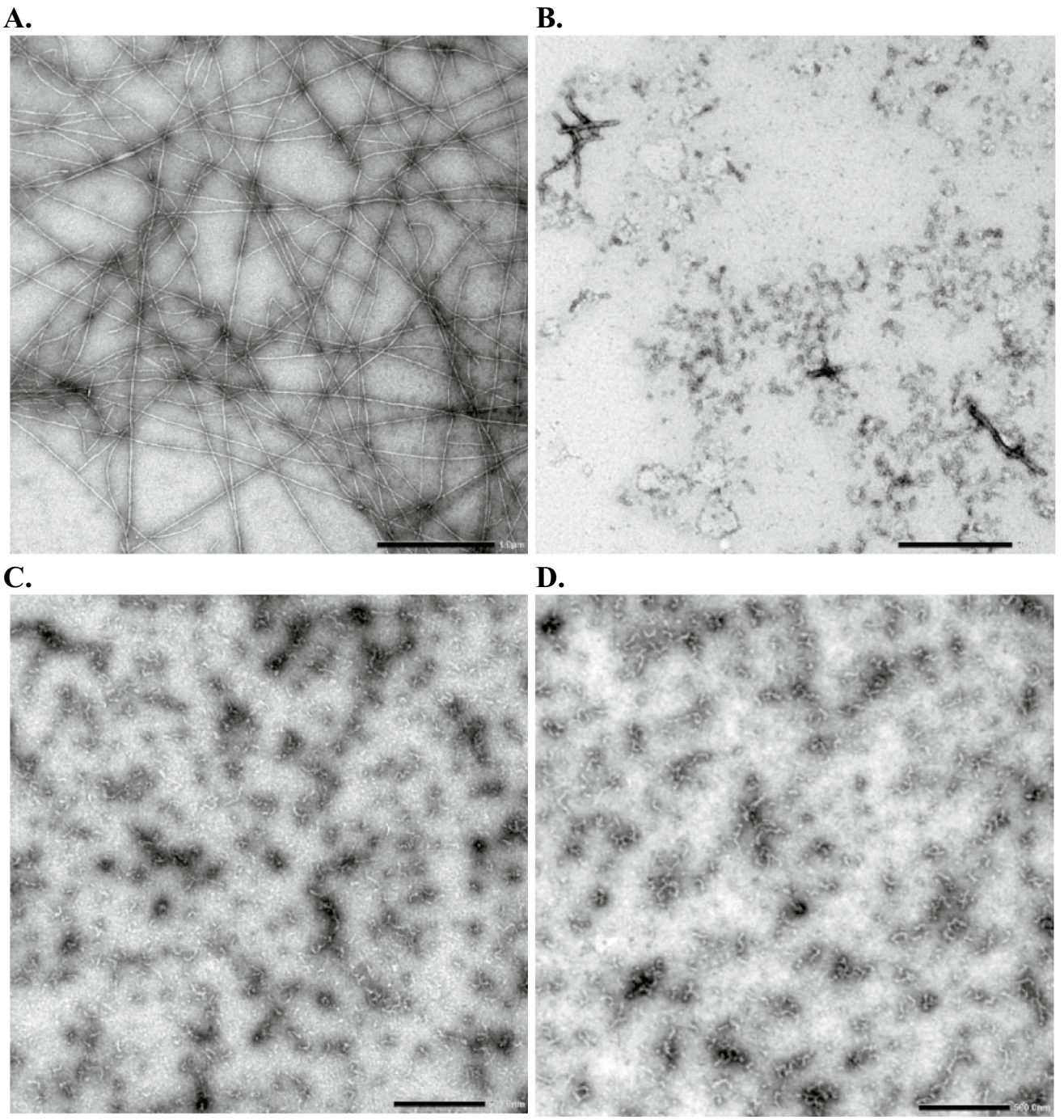

Fig. 9 Amyloid fibril (**A**) before and (**B**) after processing, and amyloid-like aggregates (**C**) before and (**D**) after processing (pH neutralization, desalting, and freeze-drying). Protein concentration of the visualized samples were 5 and 10 mg mL^{-1} for amyloid-like aggregates and amyloid fibrils, respectively

depicted in Fig. 9, while the morphology for intermediate processing steps can be found in Fig. S3. Amyloid-like aggregates mostly retained their shape and solubility (i.e., solutions remained clear; data not shown), while amyloid fibrils fragmented and aggregated (i.e., turbid sample and precipitation observed; data not shown). Both aggregation and fragmentation of amyloid fibrils have been previously reported (Mantovani et al., 2016). Precipitation appeared to have occurred primarily after the freezing step (data not shown), possibly due to further aggregation during concentration of the fibrils. Since the amyloid fibrils were mostly destroyed and insoluble after processing, we only

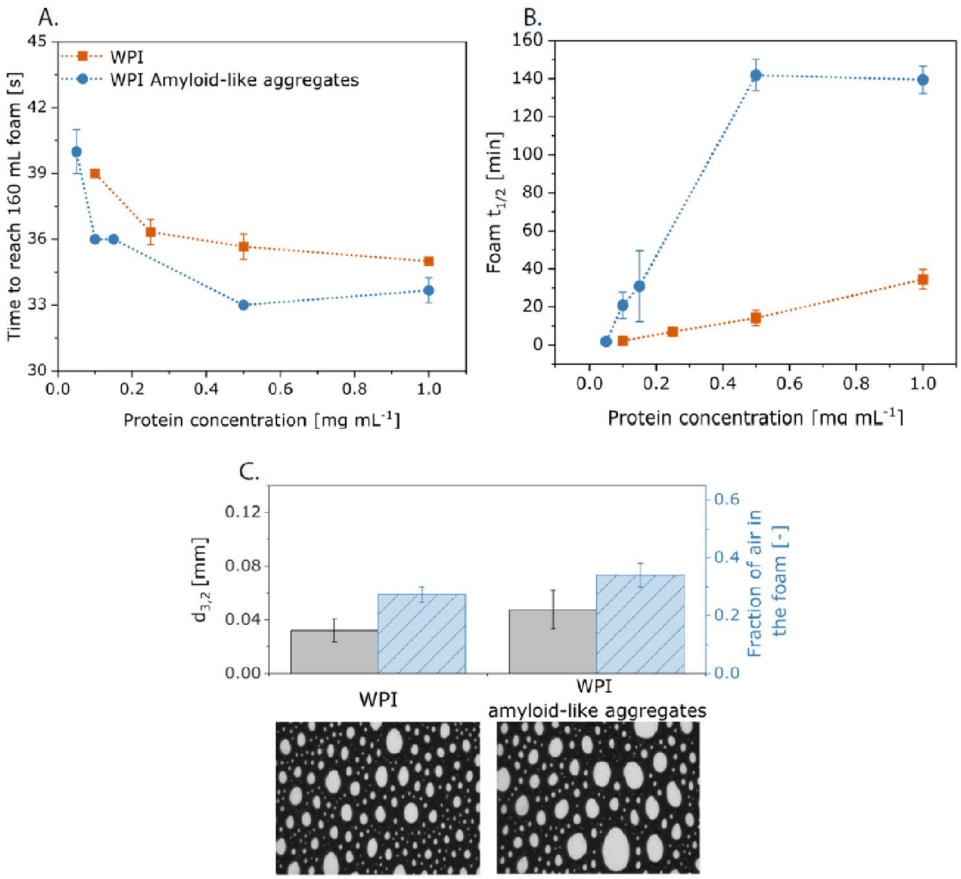

Fig. 10 **A** Time to reach 160 mL of foam volume when sparging (250 mL min^{-1}) solutions of WPI and WPI amyloid-like aggregates (WPI AA), and **B** the foam half-life time ($t_{1/2}$). Immediately after formation. **C** Immediately after formation of the foam from 1 mg mL – 1 solutions, the Sauter mean diameter ($d_{3,2}$) of the bubbles and the fraction of air in the foam is shown, with corresponding pictures

considered the amyloid-like aggregates for further evaluation of the functional properties.

Impact of Intervention C on Foaming Properties

The formation of foam, the structure of the foam, and foam collapse of solutions containing native WPI or its amyloid-like aggregates is shown in Fig. 10. The formation of foam was slightly faster and resulted in larger bubbles in the case of WPI amyloid-like aggregates, as compared to native WPI. This could be related to less drainage. The somewhat larger bubbles in the foam stabilized by WPI amyloid-like aggregates could be due to slower diffusion of some larger aggregates to the interface (Davis & Foegeding, 2004; Rullier et al., 2008). Although small differences were noted, the structure of the foam was more or less similar. In contrast, the foam stability improved drastically when using amyloid-like aggregates instead of native WPI: a similar foam stability could be achieved with WPI amyloid-like aggregates using 4–10 times less protein (Fig. 10B).

Dombrowski et al. (2016) demonstrated that the size of soluble (spherical) BLG aggregates positively correlated with their ability to stabilize foams. Schmitt et al. (2007) suggested that increased elongation of the WPI aggregate morphology could contribute to a thicker interfacial network. In addition, the non-aggregated material is also expected to improve foam properties, as reported for both BLG aggregates and amyloid fibrils (Lux et al., 2023; Rullier et al., 2008). Lux et al. (2023) suggested that this non-aggregated material acts as space fillers between aggregates. The shape and size of the WPI amyloid-like aggregates in the sample are not homogeneous (Fig. 5C). It might very well be that the faster-diffusing small aggregates in the sample primarily stabilize the interface, while larger aggregates mostly counteract drainage in the thin liquid layer. Thus, WPI amyloid-like aggregates are superior foaming agents. As aggregates from WPI and BLG have similar microstructure (Mahmoudi et al., 2007), this may also be the case for recombinant BLG. Further investigations are necessary to confirm this, as well as whether the aggregate morphology contributes to the foam stability.

Impact of Intervention C on Heat-Set Gelation

High concentration protein solutions (15 wt% protein) were prepared to evaluate the heat-set gelation. However, the solution containing WPI amyloid-like aggregates was highly viscous at this concentration (data not shown). This observation demonstrates the ability of these aggregates to increase the viscosity. Loveday et al. (2012) reported the formation of

highly flexible amyloid aggregates (formed upon heating at pH 2.0 with $CaCl_2$) that appear morphologically similar to our amyloid-like aggregates, and report similar enhanced viscosity. As it was challenging to obtain a homogenous and completely solubilized sample, we decided to add amyloid-like aggregates to a solution of native WPI (1:9, w/w; 15 wt% total protein) and investigate whether this improved its heat-set gelation. The main gelation properties are listed in Table 6, while the other results (moduli during gelling, LAOS) are depicted in Supplementary Information E.

The addition of amyloid-like aggregates prior to heating had a negative impact of heat-set gelation: it resulted in slightly later gelation (~2 min), lower gel strength (−37%), and less ductile gel structure (Table 6; Fig. S5). WPI gelation is reported to occur in two phases: first the formation of primary aggregates, and subsequently association of these aggregates into larger clusters (Mercade-Prieto & Gunasekaran, 2016). We suggest that WPI amyloid-like aggregates differ structurally from the primary aggregates formed by heating WPI at pH 7.0. Subsequently, the WPI amyloid-like aggregates are unable to cross-link and participate in the gel network, while primary aggregates from (initially native) WPI are able to do so. Thus, WPI amyloid-like aggregates are unsuitable as heat-set gelling agents. As aggregates from WPI and BLG have similar microstructure (Mahmoudi et al., 2007), the post-processing does not show potential to improve the functionality recombinant BLG in terms of gelation.

Comparison BLG Ingredients

Consumers primarily observe the foaming and gelling properties of the protein ingredients in terms of foam stability (min) and gel strength (Pa), respectively. We therefore use these two product characterizing parameters to summarize the impact of the interventions on the functionality range of BLG. For the foam stability, the performance at the highest protein concentration (1 mg mL^{-1}) was compared, since the natural isoform B showed relatively low foam stability at this concentration, which should be further improved for application. An overview of the performances of the BLG ingredients is given in Fig. 11, and is briefly discussed below.

Table 6 Gelation properties of various protein solutions (15 wt% total protein)

	Gel point (min)	Gel strength (Pa)	Critical strain (%)
WPI	32.6 ± 0.2	2,993 ± 327	6.3 ± 1.1
WPI + 10% amyloid-like aggregates	34.5 ± 1.6	1,890 ± 935	6.9 ± 1.1

When using BLG as a foaming agent, minor differences in the amino acid sequence had major consequences for the final foam stability. By choosing natural isoform A over B, the foam stability can be improved by almost a factor of 10 (Fig. 11). Furthermore, improvements were observed when inducing cysteine mutations: in particular, substitutions of the free thiol (rBLG-SH; Cys121) and outer disulfide bond (rBLG-SS; Cys66–Cys160) improved the foam stability of bBLG B approximately by a factor of 10 (Fig. 11). Whether these mutations can also further improve foam stability of the natural isoform A remains to be determined. The N-terminal modification of yBLG most probably caused the inferior foam stability of purified yBLG, as compared to bBLG A (Fig. 11). Nevertheless, the foam obtained with purified yBLG was almost twice as stable, as with native bBLG B (Fig. 11). Therefore, purified yBLG can still be considered a functional foaming agent, even though a considerate amount of impurities is present (~33 wt% impurities). In contrast, crude yBLG was unable to stabilize foams (Fig. 11), demonstrating the need for further purification (> 22 wt% protein). Finally, post-processing of WPI into amyloid-like aggregates resulted in superior foam stability, leading to almost four times more stable foam, as compared to native WPI (Fig. 11). Future research could demonstrate whether such transformation on meso-scale outweighs the impact of minor molecular modifications (i.e., natural isoforms or cysteine mutations).

For the use of BLG as a gelling agent, also major differences between the natural isoforms were observed. About 40% higher gel strength was achieved with bBLG B, as compared to bBLG A (Fig. 11). Because different heat-set

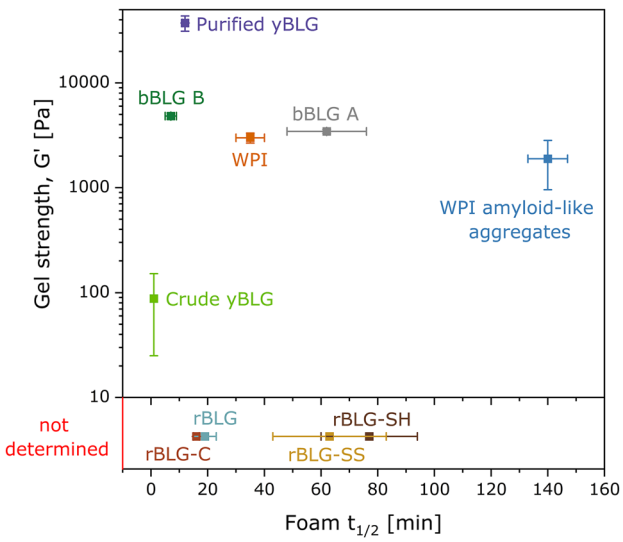

Fig. 11 Overview of the gelation (i.e., final gel strength, Pa) and foaming (i.e., foam $t_{1/2}$, min) properties of BLG ingredients used in this study. The gelation properties of recombinant BLG variants produced in *E. coli* (rBLG, rBLG-SH, rBLG-SS, and rBLG-C) were not investigated, and thereby indicated as "not determined"

gelation mechanisms were at play, a different isoform can be preferred when the gelation conditions are changed. For example, when salt is part of the formulation or a particular heating profile is used. In addition to the influence of molecular variation, impurities had major impact on gelation. Slight purification of up to ~ 22 wt% was not sufficient to allow proper gelation. After purification of up to ~ 67 wt%, the final G' upon heating was 10 times higher as compared to bBLG A. Thus, purified yBLG is a suitable gelling agent. Finally, the addition of WPI amyloid-like aggregates to native WPI was not a suitable route to improve heat-induced gel strength, as it decreased by about a third (Fig. 11). However, since high viscosity was observed prior to gelation and the WPI amyloid-like aggregates did not actively participate in the gel network, they can be used as thermostable viscosity enhancers.

Conclusions

Three interventions in the upstream production, DSP, and post-processing of BLG were considered as routes towards affordable recombinant BLG. Their potential was further evaluated based on the final functional properties of the BLG ingredient.

Intervention A included the selection of specific amino acid sequences for improved functional properties of recombinant BLG. Natural isoform A showed superior foaming (i.e., foam was 10 times more stable), while natural isoform B yielded stronger heat-set gels under the conditions applied (G' + 40% stronger). The substitution of cysteine by alanine in isoform B allowed a more effective stabilization of foams, in particular the substitution of only Cys121, or Cys66 and Cys160 (being up to 10 times more stable).

Intervention B simplified DSP in terms of purification. The removal of cellular material and low MW impurities (< 10 kDa) by centrifugation and filtration was not sufficient to obtain a functional ingredient. This material contained ~ 22 wt% protein and demonstrated poor gelling and foaming. Further purification of up to ~ 67 wt% protein was achieved by reversible precipitation using hexametaphosphate (HMP), while residual HMP remained (~ 1 wt%). The higher purity of yBLG and the introduction of HMP led to a stronger heat-set gel, with a G' that was 10 times higher as compared to bovine BLG, although the gel network seemed to be different. The foaming properties were in a similar range as compared to BLG B, but inferior to BLG A. The mild purification method thus has potential for future application, although identification of residual impurities is required.

Intervention C added post-processing to transform native whey protein (WPI) into functional aggregates. While amyloid fibrils are reported to have superior functionality, we observed them to be instable upon processing at food-grade conditions and drying. WPI amyloid-like aggregates were stable during processing and heating. These aggregates were excellent foaming agents, being able to stabilize foams up to four times longer as compared to the native WPI. Thereby, amyloid-like aggregation is promising for efficient application of recombinant proteins. Future research should weigh such gains in functionality against the additional costs of the further processing required.

This explorative study indicates the strong dependence of protein functionality on the different interventions. Thus, for designing production processes for recombinant food proteins, one should consider gains in protein functionality (i.e., for efficient application), just as much as gains in protein purity and yield (i.e., for efficient production).

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Author Contributions Loes Hoppenreijs did the experimental work and prepared the initial draft of the manuscript. Sarah Brune, Rebekka Biedendieck and Rainer Krull worked on the production of the recombinant BLG variants (from *E. coli*) and reviewed the manuscript. Remko Boom and Julia Keppler supervised Loes Hoppenreijs and reviewed the manuscript.

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Data Availability The data is available upon request.

Declarations

Conflict of Interest The authors declare no competing interests.

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