



The production of soluble and correctly folded recombinant bovine β -lactoglobulin variants A and B in *Escherichia coli* for NMR studies

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ABSTRACT

The production of soluble and correctly folded eukaryotic proteins in prokaryotic systems has always been hampered by the difference in or lack of cell machinery responsible for folding, post-translation modification and secretion of the proteins involved. In the case of bovine β -lactoglobulin (BLG), a major cow's milk allergen and a protein widely used for protein folding studies, a eukaryotic yeast expression system has been the preferred choice of many researchers, particularly for the production of isotopically labeled protein required for NMR studies. Although this system yields high amounts of recombinant protein, the BLG produced is usually associated with extracellular polysaccharides, which is problematic for NMR analysis. In our study we show that when co-expressed with the signal-sequence-less disulfide bond isomerase (AssDsbC) in the dual expression vector, pETDUET-1, both BLG A and BLG B can be reproducibly produced in a soluble form. Expression was carried out in *Escherichia coli* Origami(DE3), a *trxB/gor* mutant for thioredoxin- and glutathione reductase, which allows for proper formation of disulfide bonds in the cytoplasm. The protein was purified by anion exchange chromatography followed by salting-out at low pH and size exclusion chromatography. Our expression system is able to consistently produce milligram quantities of correctly folded BLG A and B with no additional amino acid residues at the N-terminus, except for a methionine. ¹⁵N-labeled BLG A and B, prepared and purified using this method, produced HSQC spectra typical of native bovine BLG.

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Introduction

β -Lactoglobulin (BLG)¹ is the most abundant protein found in the whey fraction of ruminant milk [1]. It is a small globular protein consisting of 162 amino acid residues with a monomeric molecular mass of 18 kDa and forms a dimer at physiological pH. As a member of the large and diverse lipocalin family, BLG has the common antiparallel β -barrel fold that forms a calyx into which small hydrophobic molecules bind [2,3]. The actual physiological function of BLG is still unknown, but it is thought to bind and transport small hydrophobic ligands [4]. Interest in BLG, at least in the dairy industry, has largely been attributed to the problem of heat-induced aggregation and gelation, which tends to foul heat exchangers during the processing of milk [5]. BLG has also been widely used as a model to study the

folding pathway of proteins due to its small size and refolding processes that involve a molten globule intermediate and non-native α -helical intermediates in the early phase of folding. The importance of such studies cannot be underestimated because of continuing investigations into the folding processes responsible for the conversion of a normally soluble protein into a prion protein. BLG has been used as a model for such transformations due to its propensity to switch between mainly β and mainly α structure in different solvents [6–12].

There are several genetic variants of bovine BLG, with A and B being the more common ones, followed by C. Variants A and B differ at two positions: Asp64Gly and Val118Ala, while B and C differ at one position: Gln59His. These variants have slightly different thermal denaturation and aggregation properties [13], which can be studied by monitoring their dynamics at different temperatures using various techniques including nuclear magnetic resonance (NMR) spectroscopy.

Recombinant BLG was first produced in the *Escherichia coli* system almost two decades ago, but was essentially expressed in an insoluble form [14]. Since then BLG has been expressed in both prokaryotic and various eukaryotic systems (as cited in [15]) as either insoluble or soluble protein. Most notably, expression in

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¹ Abbreviations used: BLG, β -lactoglobulin; BSA, bovine serum albumin; CD, circular dichroism; DsbC, disulfide bond isomerase C; HSQC, heteronuclear single quantum coherence; IPTG, isopropyl- β -D-thiogalactopyranoside; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

the yeast *Pichia pastoris* produces a high yield of soluble bovine BLG, including isotopically labeled BLG necessary for the majority of bio-NMR experiments [9,16–22]. BLG produced in this system usually has a mix of different extraneous residues at the N-terminus. In addition, it also requires the removal of a significant amount of co-purifying carbohydrates that interfere with NMR experiments [17].

Expression in bacteria is considered the most inexpensive and uncomplicated way of producing recombinant proteins. We had previously described the expression of recombinant BLG A in *E. coli* as a soluble protein fused to thioredoxin [15]. Although this system initially worked well for BLG variant A, the integrity of the protein produced was not consistent, and was worse for BLG B. Since one of our original intentions was to use NMR spectroscopy to identify differences in the dynamic behavior of the BLG variants, we continued to investigate the development of (i) a reliable and cost-effective expression system that could be used to isotopically label different recombinant BLG variants and mutants in a bacterial host and (ii) a simple and reproducible purification protocol for the recombinant BLG produced.

Bovine BLG contains two disulfide bonds (Cys106–Cys119, Cys66–Cys160) and one free cysteine (Cys121). Disulfide bonds are known to play a crucial role in the folding and stability of many secretory and membrane proteins. The *E. coli* cytoplasm is a reducing environment, and is therefore unable to support the formation of disulfide bonds, which are formed through pairwise oxidation of the free thiol groups present on cysteine residues. To circumvent this problem, an *E. coli* mutant strain with a cytoplasmic redox potential comparable to that of the mammalian endoplasmic reticulum, was developed by Bessette and co-workers [23] by disrupting the thioredoxin and glutathione reductase genes. This *trxB/gor* double mutant, commercially known as Origami (Novagen), is conducive to the formation of disulfide bonds in the cytoplasm. When proteins with more than two cysteines are oxidized, non-native disulfide bonds inevitably form, which result in a randomly scrambled set of protein isomers [24]. The unscrambling of these non-native disulfide bonds requires a folding catalyst or chaperone, and this step could be critical for the correct folding of BLG.

Periplasmic co-expression of horseradish peroxidase (HRP), a protein with four non-consecutive disulfide bonds, and DsbC, a thiol disulfide bond isomerase and chaperone [25,26] known to reshuffle disulfide bonds during *in vivo* folding, was reported to increase the total yield of active HRP [27]. The same strategy was also used to increase the yield of several active recombinant single-chain fragment variable (scFv) antibodies in the cytoplasm of *trxB/gor* mutants [28–30].

In this present work, we report the use of Origami(DE3) cells to produce correctly folded soluble labeled BLG. This was achieved through co-overexpression with signal-sequence-less DsbC (Δ ssDsbC) in the cytoplasm.

Materials and methods

All chemicals were of analytical grade and supplied by Sigma–Aldrich, while enzymes used in molecular cloning were supplied by Roche Diagnostics. BLG variants A and B, purified from bovine milk, were provided as a lyophilized sample by Fonterra Cooperative Group Ltd. and purchased from Sigma–Aldrich respectively. Labeled ammonium sulfate (^{15}N) and glucose (^{13}C) were purchased from Cambridge Isotope Laboratories Inc., MA, USA.

Bacterial strains and plasmids

Escherichia coli XL-1 Blue was used as the host strain for cloning, while *E. coli* BL21(DE3) (Stratagene) and Origami(DE3) (Novagen) were chosen as the host strains for expression. The

plasmid pETDUET-1 (Novagen), which has two separate multiple cloning sites (MCS) each preceded by a T7 promoter/*lac* operator and a ribosomal binding site (rbs) to facilitate the co-expression of two target genes was used as the expression vector.

Construction of pETDUET-DsbC

A DNA fragment coding for the Δ ssDsbC protein was amplified using the plasmid pET40b (Novagen) as a template with the forward primer 5'-CAGGCC**ATGG**ATGACGCGGCAATTCAACAAAC-3', which has an *NcoI* restriction site (italics) containing an initiation codon (bold), and a reverse primer 5'-CAGCAAGC **TTTCCTTTA**CCGCTGGTCAT-3', which has a *HindIII* restriction site as well as a stop codon (bold). The PCR product obtained was digested with *NcoI* and *HindIII*, purified and ligated into MCS1 of the vector pETDUET-1 restricted with the same enzymes to obtain the intermediate construct pETDUET-DsbC. The sequence and the reading frame of the insert were verified by DNA sequencing.

Construction of pETDUET-DsbC-BLG A and B

A DNA fragment coding for the BLG A protein was amplified from the construct described by Ariyaratne and co-workers [11] with the forward primer 5'-GAATTC**ATATG**CTGATTGTGACCA-GACC-3', which has an *NdeI* restriction site containing an initiation codon (bold), and a reverse primer 5'-CAGCGGTACCAGATCT**TTAT-TAA**TATGGC-3', which has a *KpnI* restriction site (italics) as well as two stop codons (bold). The PCR product obtained was digested with *NdeI* and *KpnI*, and ligated into MCS2 of the intermediate construct pETDUET-DsbC restricted with the same enzymes to obtain the final construct pETDUET-DsbC-BLG A. BLG B, which was previously constructed by mutations Asp64Gly and Val118Ala through PCR (site-directed mutagenesis) was also introduced into this intermediate construct in a manner similar to that described above to generate pETDUET-DsbC-BLG B. The sequences of the inserts and the reading frames were verified by DNA sequencing. A control construct with only BLG A in the pETDUET-1 vector was also made, for comparison of BLG expression in the absence of DsbC. This construct was made by cloning BLG A into the *NdeI/KpnI* site in MCS 2 of pETDUET-1 using the same primers mentioned above.

Small scale expression of recombinant BLG A and B

The pETDUET-DsbC-BLG A (and B) constructs and the control pETDUET-BLG A construct were transformed into *E. coli* Origami(DE3) or BL21(DE3) competent cells and plated on LB plates supplemented with 100 $\mu\text{g}/\text{mL}$ ampicillin, 15 $\mu\text{g}/\text{mL}$ kanamycin and 12.5 $\mu\text{g}/\text{mL}$ tetracycline, or 100 $\mu\text{g}/\text{mL}$ ampicillin, respectively. A single colony was picked and inoculated into LB broth supplemented with the same antibiotics and grown with shaking at 37 °C. When the cultures reached an OD₆₀₀ of 0.4–0.6, expression of the target proteins was induced by adding isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and the cultures incubated at 25 °C overnight. At the end of the induction period, the cells were harvested by centrifugation, resuspended in 20 mM Tris–HCl, pH 7.5 and disrupted by sonication. The samples were centrifuged at 16,000g at 4 °C for 10 min and the supernatant taken as the soluble fraction, while the pellet was taken as the insoluble fraction and resuspended in an equal volume of the same buffer.

Large scale expression and purification of isotopically labeled recombinant BLG A and B

For isotopically labeled proteins, starter cultures were grown with shaking in LB medium supplemented with ampicillin, kana-

mycin and tetracycline at 37 °C overnight. The cells were harvested and washed in M9 minimal salts and a 1% inoculum was used to inoculate 1 L of M9 minimal medium containing no antibiotics nor nitrogen or carbon source (to starve cells) and grown with shaking for 1 h at 37 °C. Ampicillin was then added together with $(^{15}\text{NH}_4)_2\text{SO}_4$ (to a final concentration of 1 g/L) and glucose or $^{13}\text{C}_6\text{H}_{12}\text{O}_6$ (to a final concentration of 4 g/L) and incubation continued with shaking at 37 °C. When the cultures reached an OD_{600} of 0.5–0.7, IPTG was added to a final concentration of 0.5 mM and incubation continued at 25 °C.

Cells were harvested after overnight growth at 25 °C. The cell pellet was resuspended in 20 mL lysis buffer (20 mM Bis-Tris, pH 6.5) and lysed by three passages through a French Press (Aminco) at 6000 psi. Clarified cell lysate was then loaded onto an anion exchange column (HR 30/10, GE Healthcare) packed with Source Q resin with a bed volume of approximately 24 mL at 1.5 mL/min. Proteins were eluted using a stepwise NaCl gradient of 0.05 M, 0.10 M, 0.20 M, 0.25 M and 1.0 M in 20 mM Bis-Tris, pH 6.5 (five column volumes each) at 1.5 mL/min.

Fractions were analyzed by SDS–PAGE (15% acrylamide) and those fractions containing BLG were pooled and dialyzed overnight in 50 mM sodium phosphate, pH 7.5 at 4 °C. The concentration of protein in the dialyzed sample, estimated by absorbance at 280 nm, was first adjusted to ~1 mg/mL before being subjected to NaCl fractionation at low pH [31]. Briefly, 0.1 M HCl was added to the sample dropwise with continuous stirring at room temperature to achieve a pH of 2.6 before the addition of NaCl to a final concentration of 7% (w/v). Contaminating *E. coli* proteins denatured under these conditions were removed by centrifugation at 30,392g and the supernatant containing BLG was recovered. The final concentration of NaCl in the solution was then adjusted to 30% (w/v) to precipitate the BLG and the suspension re-centrifuged at 30,392g. The resulting pellet was resuspended in a small amount of 50 mM potassium phosphate buffer, pH 6.5 and subjected to size exclusion chromatography (Superdex 75 10/300 GL, GE Healthcare) in 50 mM potassium phosphate, pH 2.6 at a flow rate of 0.5 mL/min. Peak fractions of 0.3 mL containing pure monomeric BLG were pooled and concentrated to approximately 18 mg/mL (~1 mM) by ultrafiltration (Vivaspin 20, 10 kDa MWCO, Vivascience). Protein concentrations were determined from the absorbance at 280 nm, assuming the BLG molar extinction coefficient, ϵ_{280} to be $17,600 \text{ M}^{-1} \text{ cm}^{-1}$.

NMR spectroscopy

All NMR experiments were conducted at pH 2.6 in 50 mM potassium phosphate at 34 °C. Recombinant ^{15}N BLG A and ^{15}N BLG B at 1 mM each, as well as native BLG A and native BLG B from bovine milk, at 4 mM each, were used to acquire sensitivity enhanced ^{15}N -HSQC spectra using the echo/anti-echo method on a Bruker Avance 700 MHz NMR spectrometer equipped with a cryoprobe. Spectral widths of 11 kHz and 2.5 kHz, digitized with 2048 and 256 points were used for the ^1H and ^{15}N dimensions respectively. Four scans per increment were used for each of the ^{15}N increments for the ^{15}N -labeled samples whereas 200 scans were used for the unlabeled native protein samples. A recycle delay of 1.6 s was used for all spectra. All spectra were processed using Bruker's Topspin software using standard parameters.

CD spectroscopy

Circular dichroism (CD) spectra of recombinant BLG A and B were recorded on Chirascan™ CD spectrometer (Applied Photophysics, Surrey, United Kingdom) equipped with a TC125 temperature controller (Quantum North West). Near-UV (250–350 nm) spectra were recorded in a 10 mm Quartz Suprasil cell (#100-QX, Hellma, Essex, United Kingdom) at 25 °C. Sample concentrations

were 1 mg/mL in 10 mM phosphate buffer, pH 7.5. An average of 10 scans was recorded at 1 nm intervals at 0.25 s per point. The results were averaged and smoothed with a factor of 2 and corrected for background signals from buffer.

HPLC–ESI-MS

Both the BLG samples were diluted in 0.1% TFA/water to obtain a final concentration of approximately 3.3 mg/mL and filtered through a 0.22 μm spin filter (Ultrafree-MC GV, Millipore, NSW, Australia) before analysis. High-performance liquid chromatography (HPLC) was performed on a Dionex Ultimate 3000 HPLC system controlled by Chromeleon Chromatography software (version 6.3). A Jupiter C₁₈ column (4.6 mm $\times$ 250 mm i.d., 300 Å; Phenomenex, Auckland, New Zealand) with a linear gradient from 0.1% TFA/water to 60% acetonitrile/0.1%TFA/water over 30 min was used at a flow rate of 1 mL/min. at 25 °C. The BLG fractions, which eluted at approximately 54% acetonitrile/0.1%TFA/water, were collected and lyophilized. The resultant lyophilized powder was reconstituted in approximately 200 μL 40% acetonitrile/0.1% TFA for mass spectroscopic analysis on an LTQ-Orbitrap™ mass spectrometer (Thermo Electron Corporation, Waltham, MA).

Results

Protein expression

Expression of the construct pETDUET-DsbC-BLG A in the *E. coli* strain Origami(DE3) in LB medium was analyzed by separating the soluble and insoluble cell extracts on SDS–PAGE (Fig. 1A). Two protein species of approximately 18.4 kDa and 23 kDa, corresponding to BLG and DsbC, respectively, were found to be overexpressed mostly in the soluble fraction. The same was observed for BLG B (Fig. 1B).

To determine the expression pattern of BLG in a prokaryotic system, without the aid of a folding chaperone, the expression patterns of constructs pETDUET-BLG A and pETDUET-DsbC-BLG A were compared in the Origami(DE3) as well as BL21(DE3) cells (Fig. 1A). BLG A was only partially soluble (approximately 40%) without the presence of DsbC. A further comparison was also made between the expression of pETDUET-DsbC-BLG A in Origami(DE3) and BL21(DE3) cells (Fig. 1A). When co-expressed with DsbC in BL21(DE3) cells, only 30% of BLG A was found in the soluble fraction, whereas, when expressed alone in BL21 cells, only about 10% of the protein was soluble (data not shown).

Protein purification

The soluble fractions for both BLG A and B were first purified by anion exchange chromatography. Since both variants behaved in a similar manner during purification, only data for BLG A are shown. Fractions containing reasonable amounts of BLG, which eluted in the 0.1 M and 0.2 M NaCl fractions, were pooled for the subsequent salting-out step (Fig. 2A). Most of the overexpressed chaperone DsbC did not bind to the column and thus was eluted in the flow-through and washes. At low pH (2.6), almost all contaminating *E. coli* proteins precipitated at 7% (w/v) NaCl, while BLG precipitated at 30% (w/v) NaCl (Fig. 2B). When the 30% NaCl pellet was resuspended in 50 mM potassium phosphate buffer pH 6.5, a small amount of the BLG protein remained insoluble and was removed by centrifugation at 30,392g for 30 min. We suspect this salting-out step at low pH removes any BLG that has not attained the correct conformation since native bovine BLG is very acid stable. Size exclusion chromatography was used to check the molecular weight of soluble protein. Both BLG A and B eluted in a single peak at pH

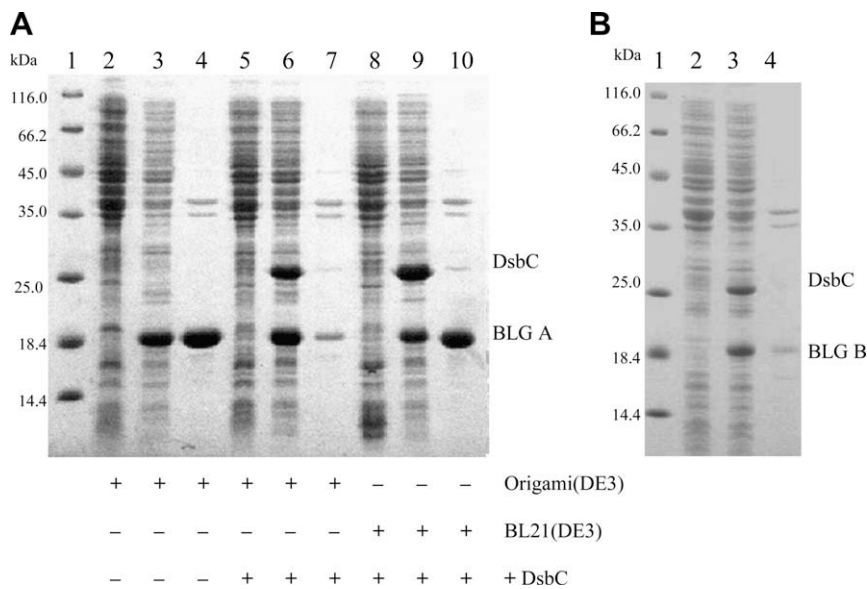


Fig. 1. (A) Coomassie-stained SDS gel of BLG A expressed in *E. coli* cells. Lane 1, molecular weight marker; lanes 2–4, whole cell lysate of uninduced Origami(DE3) cells, soluble fraction and insoluble fraction of cell lysate from induced Origami(DE3) cells containing pETDUET-BLG A; lanes 5–7, whole cell lysate of uninduced Origami(DE3) cells, soluble fraction and insoluble fraction of cell lysate from induced Origami(DE3) cells containing pETDUET-DsbC-BLG A; lanes 8–10, whole cell lysate of uninduced BL21 (DE3) cells, soluble fraction and insoluble fraction of cell lysate from induced BL21 (DE3) cells containing pETDUET-DsbC-BLG A. (B) Coomassie-stained SDS gel of pETDUET-DsbC-BLG B expressed in *E. coli* Origami(DE3) cells. Lane 1, molecular weight marker; lanes 2–4, whole cell lysate of uninduced cells, soluble fraction and insoluble fraction of cell lysate from induced cells.

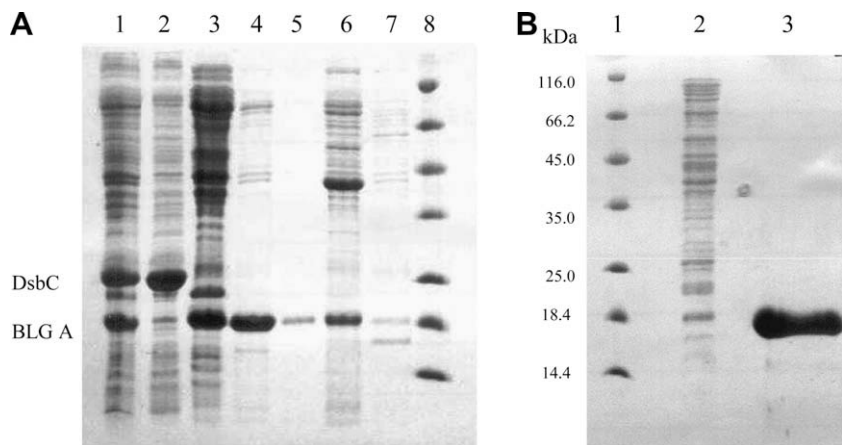


Fig. 2. Purification profile of recombinant BLG A co-expressed with DsbC in *E. coli* Origami(DE3). (A) Coomassie-stained SDS gel of eluted fractions from anion exchange chromatography. Lane 1, crude lysate; lane 2, flowthrough, lanes 3–5, fractions of 0.1 M NaCl eluate; lanes 6–7, fractions of 0.2 M NaCl eluate, lane 8, molecular weight marker. (B) Coomassie-stained SDS gel of NaCl salting out of BLG A at pH 2.6. Lane 1, molecular weight marker; lane 2, insoluble pellet/precipitate obtained from 7% NaCl at pH 2.6; lane 3, soluble pellet/precipitate obtained from 30% NaCl at pH 2.6.

2.6 at an elution volume of approximately 12 mL indicating they were monomers at low pH (data not shown). Table 1 shows the protein yield of BLG A from the purification steps. The yield of BLG B was 5.7 mg/L, which is quite similar to that for BLG A.

NMR spectroscopy

Since the solubility of a recombinantly expressed protein and a purification protocol similar to that used for native BLG are not a proof of its native conformation, we analyzed the recombinant proteins using NMR spectroscopy, which is able to provide information on the purity, conformation and the folded states of a protein.

Growth conditions of Origami(DE3) cells, harboring pETDUET-DsbC-BLG A, in minimal media had to be optimized for glucose concentrations, with the doubling of concentration from 0.2% to 0.4% resulting in a drastic improvement in cell density. The growth

Table 1
Purification of recombinant BLG A.^a

Sample	Total protein (mg)	Yield (%)
Crude lysate	1489 ^a	100
Anion exchange	85 ^a	5.7
Soluble pellet from 30%NaCl precipitate at pH 2.6	8.4 ^b	0.564
Concentrated sample post-size exclusion chromatography at pH 2.6	8.3 ^b	0.557

Values for BLG B were very similar to those obtained for BLG A and are thus not included.

^a These results are obtained from a one liter culture grown in LB medium.
^b The amount of total protein measured by Bradford assay with BSA as standard.
^c The amount of total protein measured by absorbance using $\epsilon_{280} = 17,600 \text{ M}^{-1} \text{ cm}^{-1}$.

requirements, initially optimized in M9 minimal medium, were later applied to the expression of isotopically labeled BLG A and B by supplementing M9 minimal medium with ^{15}N (and ^{13}C) sources. The HSQC spectra of both recombinant BLG A and B are very similar to that of the native protein obtained from bovine milk as shown in Fig. 3. There are a small number of peaks that appear in the spectrum of the recombinant protein that cannot be seen in the spectrum of the native material at the displayed contour threshold. Many of these peaks can be seen if the vertical scaling of the native spectrum is increased sufficiently, but at the expense of producing plots showing significantly more baseline noise. A few peaks show small changes in chemical shift between the native and recombinant protein. Although these could indicate a subtle conformational change between the two forms, they are more likely to be the result of small differences in pH and/or temperature between the samples.

CD spectroscopy

Near-UV CD spectra of both recombinants A and B show a sharp minimum at 293 nm and a strong shoulder at 286 nm (Fig. 4), two features that are typically seen in native bovine BLG and attributed to Trp19 [32]. These troughs are highly diagnostic of the correct tertiary structure of bovine BLG. The far-UV spectra (data not shown) are also identical to those of native bovine BLG, further confirming the retention of the secondary structure in the recombinant proteins.

Mass spectra

The calculated molecular masses of recombinant BLG A and B are 18498.4 and 18412.4 respectively, while the observed masses were 18494.54 and 18408.52. The differences in mass correspond to the loss of four hydrogen atoms due to the formation of two disulfide bonds in each variant.

Discussion

The folding of a polypeptide into its final three-dimensional structure is based on the information contained in its primary sequence. Nevertheless, this process often takes place with the help of a group of proteins and enzymes known collectively as molecular chaperones [33]. Therefore, when expressing a eukaryotic protein in a prokaryotic system, which lacks the specific machinery required for correct folding, it is advantageous to provide the miss-

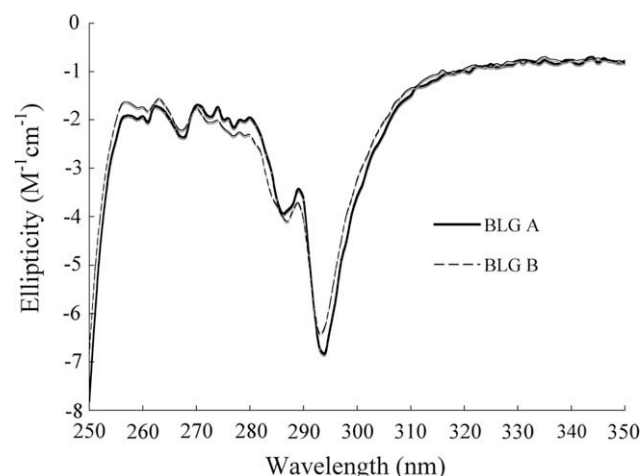


Fig. 4. Near-UV CD spectra of recombinant BLG A (continuous line) and BLG B (dashed line). Each spectrum represents an average of 10 accumulated scans, each sampled at 1 nm intervals, 0.25 s per point with baseline subtracted. Samples were measured at 25 °C using a BLG concentration of 1 mg/mL in 10 mM phosphate buffer, pH 7.5.

ing tools or the required chaperones. In the case of proteins with multiple disulfide bonds, the presence of a disulfide bond isomerase aids in reshuffling incorrect linkages formed during the rapid cysteine oxidation step. This isomerization reaction is slow and is part of the rate-limiting step in the folding of proteins containing many disulfide bonds. DsbC, a bacterial disulfide bond isomerase has been reported to isomerize aberrant disulfide bonds in several proteins that have formed, both *in vitro* and *in vivo* under oxidizing conditions [25,26,34,35], and has been recommended as the bacterial enzyme of choice for enhancing the yield of active proteins with complex disulfide bonding patterns [35].

Recombinant proteins that are soluble are not necessarily properly folded or biologically active. Although our previous system, which produced recombinant BLG as a thioredoxin fusion protein in *E. coli* G1724 cells [15] yielded soluble protein, it proved to be unreliable, especially for variant B. In the course of this work, we have evaluated many different expression constructs and host cells in order to obtain reproducibly soluble recombinant BLG. These included the use of solubility tags, such as maltose binding protein (MBP), thioredoxin and SUMO, as well as co-expression with chaperones, such as GroEL/GroES. None of these was successful (data not shown). We therefore decided to investigate the co-expression of BLG and DsbC in the cytoplasm of Origami cells. The Origami

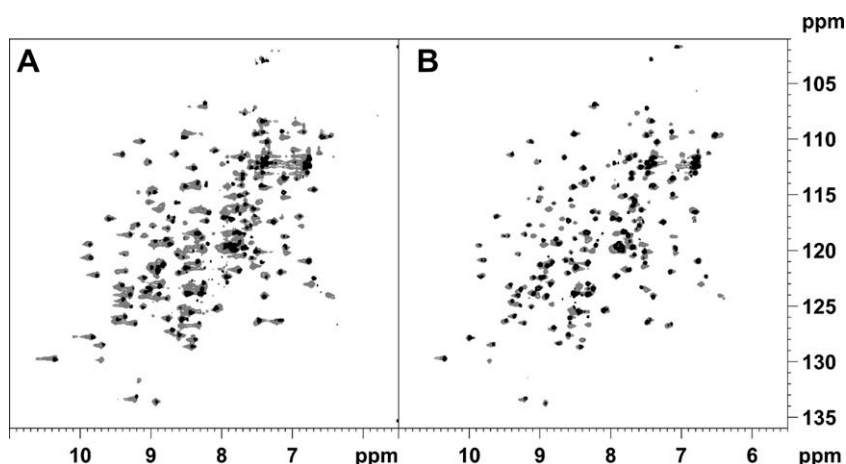


Fig. 3. ^{15}N HSQC spectra of ^{15}N -labelled recombinant and unlabelled native BLG isolated from bovine milk. (A) Comparison of recombinant ^{15}N BLG A (grey contours) with native BLG A (black contours). (B) Comparison of recombinant ^{15}N BLG B (grey contours) with native BLG B (black contours).

strains offer an alternative environment for the production of disulfide-bonded proteins in the normally reducing *E. coli* cytoplasm.

In the presence of DsbC, most of the recombinant BLG produced in Origami(DE3) cells was found to be soluble. The oxidized thioredoxins, glutathione and glutaredoxins present in the cytoplasm of Origami cells, usually kept reduced in wild type cells, are now able to promote the formation of disulfide bonds. In addition, DsbC catalyzes the isomerization of incorrect bonds, which is especially important for proteins with multiple cysteines. As well as its isomerase activity, DsbC is able to catalyze disulfide bond formation *in vivo* [25,36], although it only has about 4% of the oxidase activity of protein disulfide isomerase (PDI) [37]. This is probably why approximately 30% of BLG remained soluble when co-expressed with DsbC in the reducing environment of the cytoplasm of BL21 cells.

Interestingly, some BLG was still soluble when produced in Origami cells in the absence of DsbC, although it consistently co-purified with other proteins, as seen on SDS–PAGE. Thus, although the recombinant BLG was soluble in the oxidative conditions of the Origami cell cytoplasm, it most likely contained a mixture of incorrect disulfide linkages, as well as exposed cysteines because of the absence of DsbC, which would result in the formation of intermolecular disulfide linkages with other *E. coli* host proteins.

BLG A (inserted into a modified pET32a vector carrying the OmpA signal peptide) was also co-expressed with four periplasmic chaperones (DsbA, FkpA, SurA as well as DsbC) that are encoded on the helper plasmid, pTUM4, a strategy which worked for retinol binding protein (RBP), a fellow lipocalin [38]. Compared to the yield of the four chaperones, the amount of soluble BLG recovered from the periplasmic fraction was very low, with most of the protein found in the insoluble fraction (data not shown). Our observation is in agreement with that of Bessette [23] who found that higher yields of biologically active disulfide-bonded proteins were produced in the cytoplasm of a *trxB/gor* mutant compared to the periplasm of a wild type strain. Thus, the oxidizing environment of Origami cells is better able to support the formation of disulfide bonds. Moreover, Zhang [39] reported that, while the cytoplasmic co-expression of DsbC produced 90% soluble fibrolase, the periplasmic chaperones, Skp and FkpA, did not have any effect on solubility.

Recently Invernizzi and co-workers reported the expression of soluble BLG in the *E. coli* Rosetta-Gami strain, which supplies rare tRNA codons in a *trxB/gor* background [40]. Interestingly, a Cys121–Ser mutation was not soluble when expressed in this system, leading them to suggest that while oxidative folding in a *trxB/gor* mutant background is important, Cys121 also plays a role in achieving the correct fold [40]. In contrast, when expressed in *P. pastoris*, Cys121 mutants had structures similar to those of wild type BLG including the native disulfide bonds even though they appeared to be less stable [20,41]. In addition, the Cys121Ala mutation, introduced to avoid disulfide shuffling, was able to significantly improve the yield of refolded denatured BLG compared to that obtained using wild type BLG [20]. A study by Hattori and co-workers showed that to achieve complete refolding of denatured BLG, the gradual removal of reducing agent was crucial to prevent the formation of incorrect disulfide bonds [8].

Taken together, these findings suggest that the correct formation of disulfide bonds in BLG is essential for achieving a native conformation. Furthermore, our findings suggest that in an *E. coli* expression system, DsbC is necessary to achieve the proper conformation of the one non-consecutive disulfide bond in BLG [42,43]. While the NMR and CD spectra further verify the conformation of both the recombinant BLG expressed in this system as identical to the native protein, the MS data confirm the correct amino acid sequences as well as the presence of two disulfide bonds in both variants.

Several advantages can be obtained from the use of this expression and purification system. The pETDUET-1 plasmid used in this study allows the co-overexpression of two genes under the control of the T7 promoter. Since a folding catalyst is being co-expressed rather than being fused to the target, the additional steps needed for removal of a fusion partner by protease cleavage and its subsequent separation are avoided. Very recently, New England Biolabs has made available a new expression strain (SHuffle) that both overexpresses cytoplasmic DsbC and contains the *trxB/gor* mutation [44]. This is very similar to our strategy, and is expected to improve the correct folding of disulfide-bonded proteins in the *E. coli* cytoplasm. As native BLG is acid resistant, incorporating a low-pH denaturation step in the purification protocol eliminated other contaminating bacterial proteins to produce a homogeneous preparation. Moreover, the salting-out at low pH acts as a culling step for BLG species that are not properly folded. Our group has also successfully employed this method on BLG variant C and other BLG mutants (data not shown).

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