

Expression of a Lipocalin in Prokaryote and Eukaryote Cells: Quantification and Structural Characterization of Recombinant Bovine β -Lactoglobulin

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In this paper we quantify and characterize the expression of recombinant β -lactoglobulin (rBLG) in prokaryote and eukaryote cells. In *Escherichia coli* we used the pET26 vector, which permits the secretion of rBLG in periplasm. We studied the expression of rBLG in COS-7 cells and *in vivo* in mouse tibialis muscle. The expression of rBLG was measured by two immunoassays specific, respectively, for BLG in its native and denatured conformation. We observed that rBLG was essentially expressed in a denatured form in *E. coli* even in the periplasm, whereas rBLG in eukaryote cells was found in its native conformation. © 1999

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β -Lactoglobulin (BLG)² is the most abundant component of the whey fraction of milk and is regarded as a dominant allergen. The molecular weight of bovine BLG is 18 kDa, which corresponds to 162 amino acid residues. It contains two disulfide bridges and one free cysteine. Significant structural analogies between BLG and retinol-binding protein suggest a possible physiological role for BLG in binding and transport of retinol (1). There are two main variants due to point mutations, BLG A and B (2). Bovine BLG A has been cloned and over-expressed in *Escherichia coli* (3,4) and in yeast (5). More recently, Kim *et al.* (6) produced more

than 1.5 g/liter of bovine recombinant BLG A in *Pichia pastoris*. Recombinant BLG (rBLG) was used to study thermostable variants (7), allergenic structures (4), and to probe the retinol-binding site (8,9).

In this paper our aim is to determine the ability of different prokaryote and eukaryote expression systems to produce rBLG in a native conformation. We quantified the expression and characterized the structure of rBLG in *E. coli* using the pET26 vector, which permits the secretion of rBLG in periplasm, in COS-7 cells, and *in vivo* by injection of plasmid in mouse tibialis muscle. We quantified and analyzed the structure of rBLG using two immunoassays, one specific for BLG in its native conformation and the other specific for reduced and carboxymethylated BLG (RCM-BLG) (10). In *E. coli* even in the periplasm, irrespective of the conditions, rBLG was essentially expressed in a denatured form, close to RCM-BLG. In eukaryote cells, and especially *in vivo*, rBLG was found only in its native conformation.

MATERIALS AND METHODS

Purification of BLG from cow's milk. Natural BLG (nBLG) was purified from the milk of one single cow homozygous for the variant A of BLG as described in Wal *et al.* (11). RCM-BLG was prepared as described in Negroni *et al.* (10) by a method slightly modified from McKenzie *et al.* (12).

Two-site enzyme immunometric assay (EIA) for native and RCM-BLG. The two-site enzyme immunometric assays (EIA) for native and RCM-BLG are described in Negroni *et al.* (10). Briefly, assays were performed in 96-well microtiter plates coated with a first monoclonal antibody (mAb) (capture antibody) specific for either native or RCM-BLG. Then 50 μ l of standard (nBLG or RCM-BLG), or 50 μ l of samples, and 50 μ l of tracer consisting of a second mAb labeled

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² Abbreviations used: BLG, β -lactoglobulin; rBLG, recombinant BLG; RCM-BLG, reduced and carboxymethylated BLG; nBLG, natural BLG; EIA, enzyme immunometric assay; mAb, monoclonal antibody; AChE, acetylcholinesterase; PAGE, polyacrylamide gel electrophoresis; PE, periplasmic extract; S, soluble protein fraction; I, insoluble protein fraction; TA, tibialis anterior; rBLGn, rBLG in the native conformation; rBLGd, rBLG in a denatured conformation; PDI, protein disulfide isomerase; PPIase, peptidyl prolyl isomerase.

with acetylcholinesterase (AChE), a conjugate recognizing either nBLG or RCM-BLG, were added. The capture and tracer antibodies were directed against different complementary epitopes. After an 18-h reaction at 4°C, the plates were washed and solid phase-bound AChE activity was measured using Ellman's method (13). Detection limits of 30 and 200 pg/ml were obtained for nBLG and RCM-BLG, respectively, with very low or negligible cross-reactivity with the other milk proteins and tryptic fragments of BLG.

SDS-PAGE and Immunoblot. SDS-PAGE analysis was performed using a tricine buffer as described by Schagger and von Jagow (14). For immunoblot analysis, proteins were separated by 12% SDS-PAGE and electroblotted (15) onto polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blotting, nonspecific protein binding sites were blocked with 1% BSA in 50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% Tween 20. The nylon membranes were incubated overnight with a 1/200,000 dilution of monoclonal antibody specific for RCM-BLG. After washing, the membranes were incubated for 1 h with alkaline phosphatase-conjugated anti-mouse antibody (1/7000) (Promega, Madison, WI). Color development was achieved according to the supplier's instructions.

Expression and extraction of recombinant BLG produced by pET26-BLG. pET26-BLG was constructed by inserting the sequence of BLG in a pET26b expression vector (Novagen, Madison, WI). The sequence of BLG was amplified from pTTQ18 β lac.7.7.1 (3) using the two different oligonucleotides PET N BLG and PET C BLG adding, respectively, a *Bam*HI site at the N-terminal of BLG and a *Xho*I site at the C-terminal of BLG. The amplified sequence was then digested by *Bam*HI and *Xho*I. In parallel, pET26b was also digested by the same enzyme. After digestion, the product of amplification and the vector were ligated and electroporated in *E. coli* strain BL21(DE3) (Novagen, Madison, WI).

E. coli BL21(DE3) transformed by pET26-BLG was grown at 37°C to an OD₆₀₀ of 0.5 and induced overnight with 1 mM IPTG at different temperatures (37, 30, and 20°C). After induction, the cells were pelleted by centrifugation for 15 min at 5000g, 4°C. The proteins in the bacterial periplasm, PE, were extracted as described by the supplier. The soluble cytoplasmic protein was then extracted by sonicating the cells resuspended in 50 mM Tris-HCl, pH 7.4. The extract was centrifuged for 15 min, at 10,000g, 4°C. The supernatant was called S and the pellet was resuspended in 50 mM Tris-HCl, pH 7.4, 8 M urea, and 2 mM DTT. After centrifugation for 15 min at 10,000g, 4°C, the supernatant containing resolubilized proteins was called I. Native and denatured BLG were assayed in PE, S, and

I. The amounts of expressed rBLG were referred to the quantity of total protein.

Transfection of mammalian cells. pcDNA3-BLG5 was derived from the eukaryotic expression vector pcDNA3 (Invitrogen, Leek, Netherlands). The sequence of BLG was amplified from the vector pTTQ18 β lac.7.7.1 using the two different oligonucleotides H3KSP BLGN and XBA BLGC adding, respectively, at the N-terminal of BLG a *Hind*III site for further cloning, the Kozak sequence, and the signal peptide of BLG, and at the C-terminal of BLG a *Xba*I site. The amplified sequence and the pcDNA3 vector were digested in parallel by *Xba*I and *Hind*III. After digestion the two sequences were ligated and electroporated in *E. coli* strain DH5. Clones containing the BLG insert were selected, sequenced, and one clone, pcDNA3-BLG5, was amplified and purified with Endotoxin-Free Megaprep (Qiagen, Hilden, Germany).

Expression of rBLG in COS-7 cells was performed by transfection using LipofectAMINE PLUS Reagent (Life Technologies, Paisley, UK). Briefly, 50–80% confluent cells cultured in DMEM, 10% FCS, 2 mM glutamine, 100 U penicillin, and 100 μ g streptomycin were transfected with pcDNA3-BLG5 previously complexed with lipofectamine, following the Life Technologies protocol. At days 1, 2, and 3 posttransfection, cells were harvested, centrifuged in PBS, counted, and sonicated. Soluble and insoluble proteins were extracted as previously described. Native and denatured BLG were assayed in extracts. The amounts of expressed rBLG were referred to the number of cells.

Gene immunization. Four-week-old Balb/c female mice were from CERJ (Centre d'élevage René Janvier, France). Immunizations were performed at the age of 6 weeks under pentobarbital anesthesia (75 mg/kg, ip). Hindlimbs were shaved, and a first injection of 50 μ l 25% sucrose was given in the left tibialis anterior (TA) muscle with a 27-gauge needle. One hundred micrograms of pcDNA3-BLG5 dissolved in a volume of 50 μ l sterile PBS were injected 30 min later. A control group of mice were injected with sucrose and PBS under the same experimental conditions. Injection of pcDNA3 without the BLG gene was previously shown not to induce production of rBLG. Three mice injected with pcDNA3-BLG5 and one mouse injected with PBS were killed at days 3, 7, 14, 21, 28, and 40 postinjection. Left TA muscle was removed from each treated mouse and right TA muscle from control mice. Muscles were weighed and placed in 20 mM Tris-HCl, pH 7.4. Soluble and insoluble proteins were extracted as previously described, except that muscle tissue suspensions were prepared using an Ultra-Turrax grinder (Janke & Kunkel, IKA Labortechnik, Germany), and Triton X-100 was added to a final concentration of 0.1% in the insoluble fraction. Native and reduced BLG assays

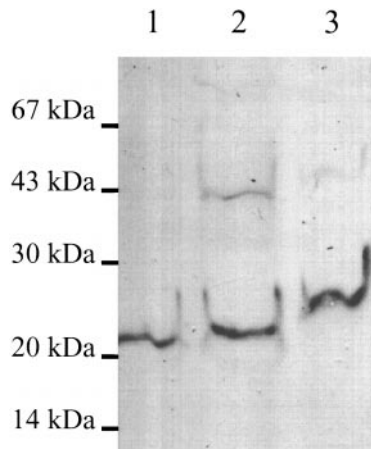


FIG. 1. Western blotting experiment: Lane 1, periplasmic extract; lane 2, soluble cytoplasmic protein fraction; lane 3, insoluble protein fraction.

were performed as previously described. Standard BLG was diluted in the right TA muscle extract of the control mice. The amounts of expressed rBLG were referred to the weight of muscle.

RESULTS

Characterization of rBLG Produced by pET26-BLG in E. coli

pET26-BLG expresses an rBLG, which carries an N-terminal pelB signal sequence for potential periplasmic localization and a C-terminal His-tag sequence for purification or detection. In Western blot we detected rBLG in periplasm, cytoplasm, and in aggregates (Fig. 1). To achieve the same staining intensity for each band, we loaded 100 times more periplasmic extract than insoluble fraction. The rBLG detected in periplas-

mic and cytoplasmic extract had a lower molecular weight than the rBLG in the insoluble fraction. In the cytoplasmic extract we noted a very faint band of dimeric rBLG.

Total rBLG in each fraction was calculated by adding native and denatured rBLG as measured with the two immunometric assays. When referred to total protein, the total rBLG did not vary between 37 and 30°C, but decreased from 670 to 400 ng rBLG/ μ g protein at 20°C (Fig. 2). The proportion of soluble rBLG was 6% at 37°C and 4% at 30°C, equally distributed between periplasmic extract and cytoplasm. At 20°C the amount of soluble rBLG reached 29% of total rBLG, 1/3 in periplasmic extract and 2/3 in cytoplasm.

rBLG in its native conformation (rBLGn) as seen by the nBLG assay represented 60% of rBLG in PE at 37°C, and 20% at 30 or 20°C (Fig. 3). In cytoplasm, rBLGn corresponded to 25% of rBLG at 37°C, 15% at 30°C, and 2% at 20°C.

Expression in COS-7 Cells

To express rBLG in eukaryote cells we added the signal peptide and the Kozak consensus to the coding sequence of BLG from pTTQ18 (3). The sequences were taken from the complete sequence of bovine BLG cDNA reported by Alexander *et al.* (16).

We quantified and characterized rBLG from 1 day after transfection (D1) to D3. At D2 cells were confluent and began to die at D3. Total rBLG was 1.3 μ g/ 10^6 cells at D1, peaked at 2.4 μ g rBLG/ 10^6 cells at D2, and decreased to 1.6 μ g rBLG/ 10^6 cells at D3. Insoluble rBLG doubled between D1 and D3 representing 26% of total rBLG at D1, 44% at D2, and 56% at D3. The proportion of soluble rBLGn corresponded to 60% of total rBLG at D1, 48% at D2, and 36% at D3.

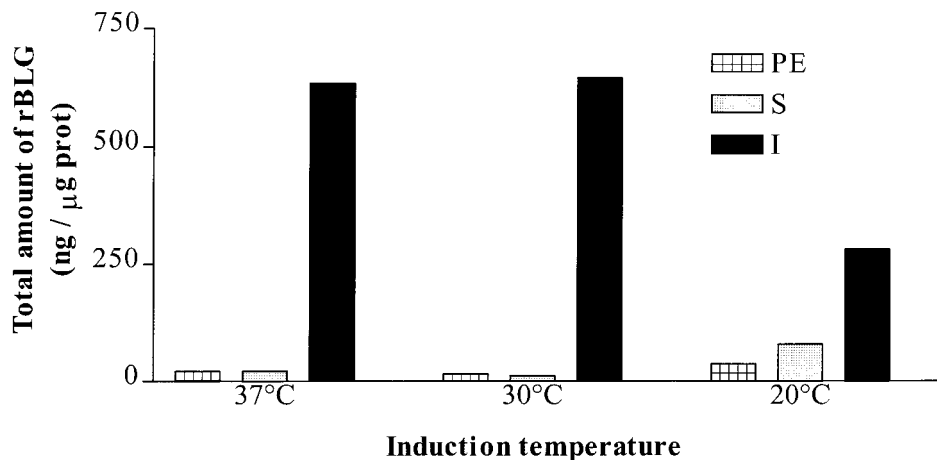


FIG. 2. Production of rBLG in *E. coli* BL21(DE3) using pET26 vector. Measurement of total amount of rBLG in periplasmic extract (PE), soluble cytoplasmic protein fraction (S), and insoluble protein fraction (I) as a function of induction temperature.

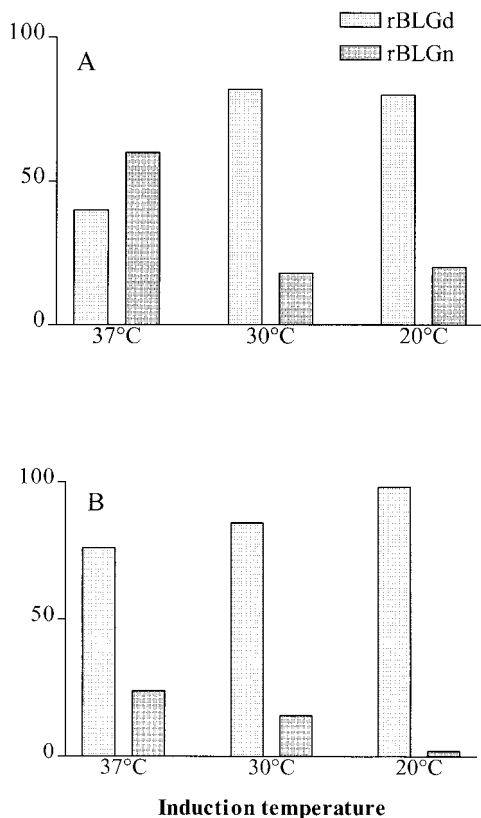


FIG. 3. Measurement of rBLG in the native (rBLGn) and denatured (rBLGd) conformations expressed in BL21(DE3). (A) Periplasmic extract; (B) soluble cytoplasmic protein fraction. Results are given as a percentage of total rBLG.

Expression in Mouse Tibialis Anterior Muscle

pcDNA3-BLG5 was injected directly into the tibialis anterior muscle of the mouse and the expression of rBLG was followed at days 3, 7, 14, and 21 after injection. No trace of rBLG was detected in mouse muscle after injection of pcDNA3 alone. The rBLG was expressed only in the soluble and native conformations. rBLG production dropped markedly from 754 ng rBLG/g muscle at D3 to 82 ng rBLG/g muscle at D7, 14 ng rBLG/g muscle at D14, and 5 ng rBLG/g muscle at D21. rBLG could be detected until 7 weeks after injection. These are the mean values for three mice. The amount of rBLG produced varied greatly between the mice. For example, at D3 it ranged from 40 to 2000 ng rBLG/g muscle. rBLG could be detected in serum from the highest responding mouse.

DISCUSSION

In this paper we compare three expression systems in prokaryotes and eukaryotes by characterizing the biochemical and immunological properties of rBLG. Our aim was to determine the expression system best

able to produce rBLG in a native conformation. The three-dimensional structure of rBLG was analyzed by using monoclonal antibodies through two different sandwich immunoassays specifically measuring BLG in its native or denatured conformation (10). The native BLG assay cross-reacted only slightly with RCM-BLG (0.018%), while the RCM-BLG assay appeared less specific with 0.4% cross-reaction with native BLG. The RCM-BLG assay cannot be considered suitable for quantitatively measuring "denatured BLG" since the "denatured protein" is not a homogeneous entity. This assay provides a relative index allowing semi-quantitative monitoring of the "denatured forms" of BLG. In all experiments we distinguish between the soluble and insoluble fractions. We were able to measure the rBLG in its native or denatured conformation in the soluble fraction. Since rBLG in the insoluble fraction could only be solubilized using concentrated urea and reducing agent, we considered that it is essentially in the denatured conformation.

One way to avoid the formation of aggregates in cytoplasm is to direct the secretion of the protein into the periplasm of *E. coli* where folding catalysts like PDI and PPIase have been identified (17). The pET26b vector produces recombinant protein with signal peptide pelB at the N-terminal for periplasmic secretion and a His-tag at the C-terminal for detection and purification. The difference in electrophoretic migration between rBLG recovered from the periplasm and cytoplasm and rBLG in the insoluble fraction can be explained by cleavage of the signal peptide. If the protein directed to the periplasm is not well folded or is not associated with chaperons, the signal peptide can be cleaved while the protein is not translocated. This could explain why all the cytoplasmic rBLG is processed but recovered in a denatured form. rBLG was always obtained mostly in aggregated form. This is probably due to overproduction of rBLG leading to the formation of aggregates. When the expression temperature was lowered to 20°C, the soluble form reached 30% of the total rBLG, but the proportion of rBLGn remained very low (20% in periplasm and 2% in cytoplasm).

Other allergens of the lipocalin family were also expressed in prokaryotes. The major horse allergen, Equ c1, was produced in *E. coli* BL21(DE3), using a pET 28 vector (Novagen), which adds a C-terminal His-tag to the recombinant protein (18). In complete contrast to our observations, rEqu c1 produced at 37°C represented 30% of total protein and was essentially recovered in the supernatant of the bacterial extracts. This contradiction is possibly linked to the fact that Equ c1 possesses only one disulfide bridge, which is very well conserved in the lipocalin family, and no free cysteine. In 1997, Konieczny *et al.* expressed the major dog allergens, can f1 and can f2, which are salivary lipocalin

proteins (19). They used the pET11d vector which adds a His tag and BL21(DE3) as hosts. Both recombinant proteins were purified using NTA Ni chelating resin and eluted in 8 M urea. This suggests that recombinant proteins were extracted with urea because they were principally in the form of aggregates. Bla g4, the major allergen of *Blattella germanica*, and Bda 20, the major allergen of bovine dander, were expressed in fusion with glutathione S-transferase (20,21). Both proteins were purified by chromatography over glutathione-agarose, which implies a native conformation of the glutathione S-transferase and probably therefore of the recombinant allergen. No other indications were found for the existence of fusion protein in a denatured form.

We also checked the production of rBLG in a eukaryote system to see if we could obtain a better proportion of rBLGn. We therefore constructed a vector including BLG with its proper signal peptide and Kozak consensus. The sequences were taken from the complete bovine BLG cDNA sequence (16). After insertion in a mammalian expression vector, pcDNA3, the rBLG was transfected and expressed in COS-7 cells. One day after transfection, soluble rBLG represents 75% of total rBLG essentially in the native conformation (60% of total rBLG). In this system, the production of total rBLG and the proportion of rBLGn follow the metabolism of the cells.

Production of recombinant protein in eukaryotes is possible in many systems. Yeast and insect cells are the best systems for expressing high quantities of recombinant protein. In 1990, Totsuka *et al.* described the secretion of bovine BLG in *Saccharomyces cerevisiae* growth medium (5). Using a sandwich EIA, they found no trace of rBLGd. Expression and secretion of large amounts of ovine BLG (40–50 mg/liter of culture supernatant) were also described in *Kluyveromyces lactis* (22). More recently, abundant expression and secretion (>1 g/liter) of bovine BLG was realized in *P. pastoris* (6). Other lipocalins, mouse major urinary protein and Bla g4, were also expressed and secreted in *P. pastoris* (23,24). In all cases, the recombinant proteins secreted were indistinguishable in terms of binding activity, biophysical properties, and immunological recognition (cf. natural protein).

Intramuscular injection of a plasmid encoding a protein results in synthesis of the protein by the muscular cells. We injected the pcDNA3-BLG5 vector into mouse tibialis muscle and measured the quantity of rBLG present in the muscle after the injection. rBLGd could not be detected at any time in any extract. We detected rBLGn up to 7 weeks after immunization. Most authors follow the response of the immune system and not the production of the recombinant protein. In allergy, this immunization technique is very interesting because gene immunization preferentially induces a

Th1 response (25). The first demonstration was made with β -galactosidase, which is not known as an allergen. But Hsu *et al.* have proven that this technique can be applied to an allergen, the house dust mite allergen Der p5 (26,27). The data demonstrate that gene immunization induces a Th1 response that dominates an ongoing protein-induced Th2 response in an antigen-specific manner. Gene immunization may thus provide a novel therapeutic approach (28).

Our results and literature data suggest that the folding of bovine BLG in a native conformation is possible only in eukaryotes. It has been shown by site-directed mutagenesis that the secretion of rBLG in *S. cerevisiae* depends upon the correct formation of the two disulfide bonds (5). A disulfide bond between cysteine residues 106 and 119 is required both for secretion and for correct folding in the native conformation. It is worth noting that rEuc1, which possesses just one disulfide bond, is a unique example of an allergen of the lipocalin family which is expressed in soluble form in *E. coli*. The formation of appropriate disulfide bonds, especially C106-C119 in BLG, could be a critical step requiring the presence of folding catalyst.

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