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Abstracts

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(N, Note; RP, Rapid Paper; SC, Short Communication)

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Expression of Recombinant Bovine β-Lactoglobulin in *Escherichia coli*

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Bovine β-lactoglobulin A was expressed in *Escherichia coli* in its mature form. The gene was constructed using a cDNA clone which coded for amino acid residues Leu-11 to Ile-162 and a synthetic oligonucleotide coding for the initial 10 amino acids preceded by a translational start. The met-β-lactoglobulin was expressed using a tac promoter vector, pTTQ18, and accounted for approximately 15% of the total cellular protein. The recombinant met-β-lactoglobulin migrated with the same molecular weight as native β-lactoglobulin A on SDS-PAGE. The majority of the met-β-lactoglobulin produced was found in an insoluble form but could be solubilized using guanidine–HCl. The renatured preparation was >80% pure and migrated similarly to purified β-lactoglobulin A under nondenaturing conditions.

β-Lactoglobulin is a whey protein, found in the milk of animals including bovine species, sheep, deer, dogs, and pigs, but not in humans.[1] It occurs as several different variants due to genetic differences in each species. It is a single polypeptide of 18 kDa which corresponds to 162 amino acid residues. The complete amino acid sequence of bovine β-lactoglobulin has been reported and genetic variations in the amino acid sequence identified.[2]

The function of β-lactoglobulin is not clear, especially since the milk of certain mammalian species appear to be devoid of it. β-Lactoglobulin has a significant degree of amino acid sequence homology with retinol binding protein,[1] which functions *in vivo* to bind and transport the nonpolar retinol molecule through the aqueous medium of the blood serum from the liver to the target cells. Crystallographic studies of both proteins have demonstrated that they share the same conformational structure as well; both structures consist of 8 strands of antiparallel beta sheets twisted into a calyx-shaped beta-barrel, with the interior cavity of the calyx being predominantly hydrophobic.[3] Both proteins contain, at the bottom of the interior cavity, a tryptophan residue which has been identified as important for the binding of retinol to retinol binding protein. β-Lactoglobulin has been shown to bind retinol more strongly than retinol binding protein,[4] as well as binding other nonpolar molecules insoluble in an aqueous medium, such as nonanone. The binding coefficient for retinol is approximately 2×10^{-8} M.[5] Furthermore, it has been demonstrated that retinol bound in this manner is protected from chemical oxidation while being transported.

The gene coding for ovine β-lactoglobulin was first isolated from a cDNA library by Gaye *et al.*[6] Subsequently we isolated a gene coding for the bovine β-lactoglobulin A from a cDNA bank constructed from poly(A^+)

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Abbreviations: [ ], denotes plasmid carring state; IPTG, isopropyl-β-D-thiogalactopyranoside.

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mRNA isolated from bovine mammary gland.[7] The gene was isolated using rabbit polyclonal antibodies raised against bovine β-lactoglobulin. Its nucleotide sequence codes for β-lactoglobulin A, from amino acid residues Ile-12 to Ile-162, as based on the amino acid sequence reported by Braunitzer *et al.*[8] In addition to the 455-bp coding sequence, our clone, pBβL4-10, contains a 3′ nontranslated region of approximately 270 bp. High level expression of β-lactoglobulin was required as a prelude to studying the structure/function relationships through the use of site-directed mutagenesis. This study describes the expression and purification of a met-β-lactoglobulin in *E. coli*. It also represents the first report to our knowledge of the expression of a milk protein in its mature form using *E. coli*.

Materials and Methods

Bacterial strains and plasmids. *E. coli* TG1 was used as a host strain for all experiments. pTTQ18 was purchased from Amersham Co., it is a *tac* promoter vector with a *lac*I^q gene.[9] The cDNA β-lactoglobulin gene was carried on pBβlac4-10.[7] It is a pBR322 derivative carrying a 700-bp G-C tailed fragment in the *Pst*I site of the β-lactamase gene and includes the 3′ nontranslated region with the polyA$^+$ tail.

DNA manipulations. Restriction enzymes were purchased from either Amersham Co. or Boerhringer-Mannheim and used according to their instructions. Plasmid DNA was isolated using the large scale procedure of Maniatis *et al.*[10] For small scale isolation the alkaline lysis method of Maniatis *et al.*[10] was used. The oligonucleotides were synthesized using an Applied Biosystems Model 70 synthesizer, and were not purified before use.

Recombinant met-β-lactoglobulin production. *E. coli* TG1 [pTTQ18βlac.7.7.1]was grown in LB medium at 37°C with shaking to an OD_{600} of 0.5. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 2 mM and the culture incubated for an additional 4 hr at 37°C. The cells were harvested by centrifugation and lysed by the method of Schein and Noteborn[11] or by passage through a French pressure cell. The cell debris and inclusion bodies containing the met-β-lactoglobulin were collected by centrifugation.

Purification of recombinant met-β-lactoglobulin. The insoluble form of β-lactoglobulin contained in inclusion bodies was solubilized by treatment with 8 M guanidine-HCl with 1 mM dithiothreitol. The solution was held at 4°C for 30 min and any insoluble material removed by centrifugation. The 8 M guanidine-HCl was diluted 1:10 in 10 mM potassium phosphate buffer (pH 6.2), 1 mM DTT, and 1 mM PMSF and then dialyzed against 100X volume of 10 mM phosphate buffer with 1 mM DTT, overnight at 4°C. Any insoluble material remaining after dialysis was removed by centrifugation and the supernatant stored at −20°C.

PAGE and Western analysis. Western analysis was done using a 12.5% SDS PAGE gel and electroblotted onto nitrocellulose membranes. For non-denaturing PAGE the samples were prepared without the addition of SDS in the sample buffer and SDS was omitted from both the stacking and resolving gels as well as from the tank buffer. β-Lactoglobulin forms A and B could be clearly separated on a non-denaturing PAGE under these conditions (data not shown). Immunologically reacting protein was detected using polyclonal antibodies raised in Flemish Giant rabbits.[7] Primary antibody/antigen complexes were detected using goat anti-rabbit antiserum conjugated to horseradish peroxidase and developed using the BioRad HRP substrate.

Results

Construction of met-β-lactoglobulin gene

The cDNA coding for the bovine β-lactoglobulin gene, pBβlac4-10, was missing the first ten amino acids from the mature protein.[7] Curiously, the initial cDNA clone for the ovine β-lactoglobulin gene was also truncated for the first 10 amino acids from the amino terminus of the mature protein. Attempts to identify additional cDNA clones containing these missing amino acids using pBβlac4-10 as a probe from the same bovine mammary cDNA bank were not successful. Therefore an oligonucleotide coding for the initial ten amino acids of mature β-lactoglobulin, an ATG translation start codon and a ribosome binding site (RBS) spaced 10 bp upstream from the translational start was synthesized (Fig. 1). The codons used for the first ten amino acids were chosen based upon an *E. coli* codon bias using a reverse translation program which specifically selects codons using a prescribed codon bias.[12] Both the top and bottom strands were synthesized. Other salient features of this oligonucleotide were the addition of a *Hpa*I site between the

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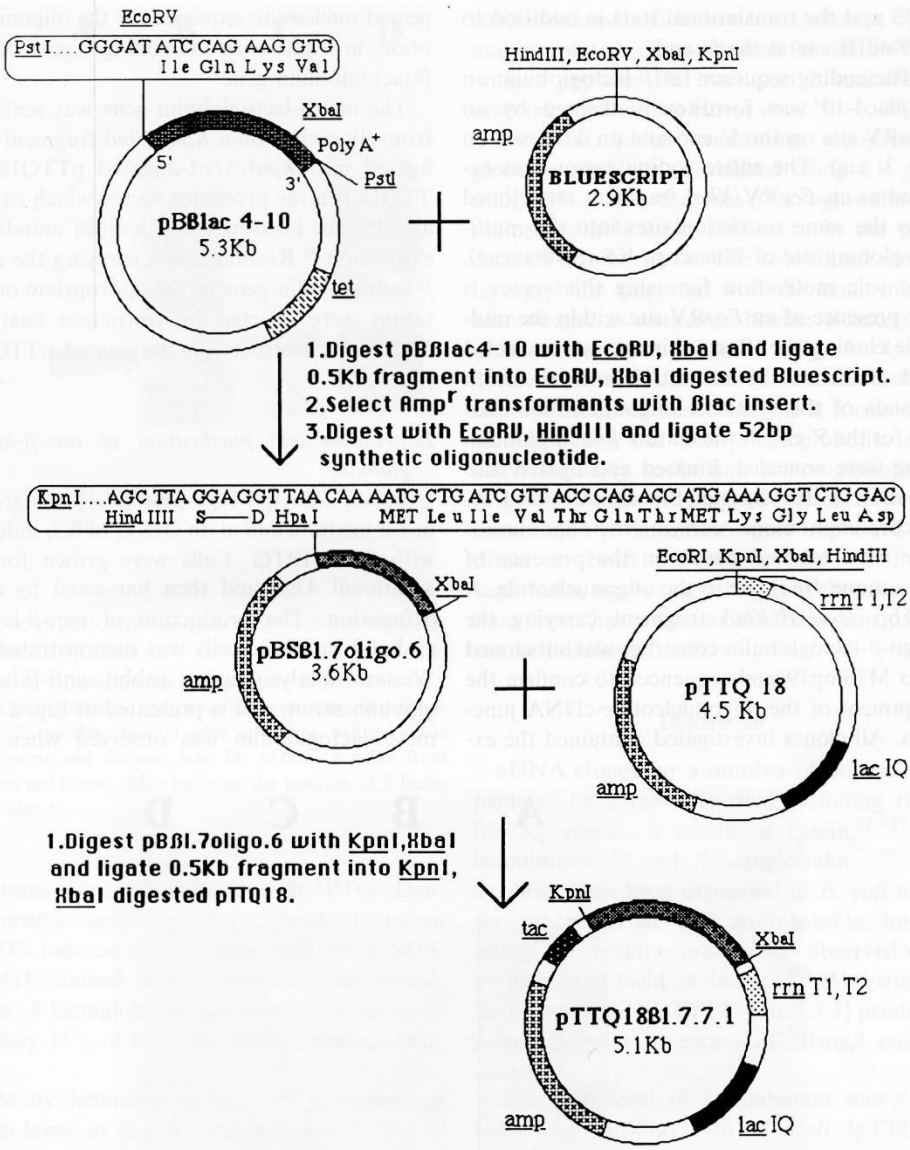

Fig. 1. Schematic for Construction of the Met-r-β-lactoglobulin and Its Expression in pTTQ18.

pBβlac4-10 is a pBR322 derivative carrying the 0.7 kb cDNA coding for bovine β-lactoglobulin from Ile-12 to Ile-162 flanked by an *Eco*RV and an *Xba*I site. This fragment was cloned into the complimentary sites of the Bluescript and Amp^r transformants selected. A transformant carrying the appropriate insert was selected by restriction analysis and a 52-bp oligonucleotide inserted into the *Hin*dIII/*Eco*RV sites 5′ to the cDNA. Amp^r transformants were selected and screened for the presence of the oligonucleotide using a unique *Hpa*I site designed into the oligonucleotide. Finally a 525 bp *Kpn*I/*Xba*I fragment was removed from Bluescript and inserted into the complimentary sites of pTTQ18 for expression *via* the *tac* promoter. Amp^r transformants were screened for the r-met-β-lactoglobulin insert by restriction analysis.

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RBS and the translational start in addition to a *Hin*dIII site at the 5′ end.

The coding sequence for β-lactoglobulin on pBβlac4-10 was fortuitously defined by an *Eco*RV site on the 5′ end and an *Xba*I site on the 3′ end. The entire coding region was excised as an *Eco*RV/*Xba*I fragment and cloned into the same restriction sites into the multiple cloning site of Bluescript KS (Stratagene). The sole motivation for using this vector is the presence of an *Eco*RV site within the multiple cloning site. Recombinants were selected and confirmed by restriction analysis. Both strands of the synthetic oligonucleotide coding for the 5′ end of the mature β-lactoglobulin gene were annealed, kinased and ligated into *Hin*dIII/*Eco*RV digested Bluescript/Bβlac4-10. Recombinant clones carrying the oligonucleotide were selected based on the presence of the unique *Hpa*I site in the oligonucleotide. A 510 bp *Hin*dIII/*Xba*I fragment carrying the oligo-β-lactoglobulin construct was subcloned into M13mp19 and sequenced to confirm the alignment of the oligonucleotide-cDNA junction. All clones investigated contained the expected nucleotide sequence for the oligonucleotide, in frame with the coding region of the β-lactoglobulin gene.

The met-β-lactoglobulin gene was removed from Bluescript on a *Kpn*I/*Xba*I fragment and ligated into *Kpn*I/*Xba*I-digested pTTQ18. pTTQ18 is a *tac* promoter vector which carries a *lac*I^q gene insuring a low level of uninduced expression.[9] Recombinants carrying the met-β-lactoglobulin gene in the appropriate orientation were selected by restriction analysis. The final construct was designated pTTQ18-βlac.7.7.1.

Expression and purification of met-β-lactoglobulin

E. coli TG1 [pTTQ18βlac.7.7.1] was grown in LB medium and at an OD_{600} of 0.5, induced with 2 mM IPTG. Cells were grown for an additional 4 hrs and then harvested by centrifugation. The production of met-β-lactoglobulin in whole cells was demonstrated by Western analysis using rabbit anti-β-lactoglobulin serum and is presented in Fig. 2. No met-β-lactoglobulin was observed when the

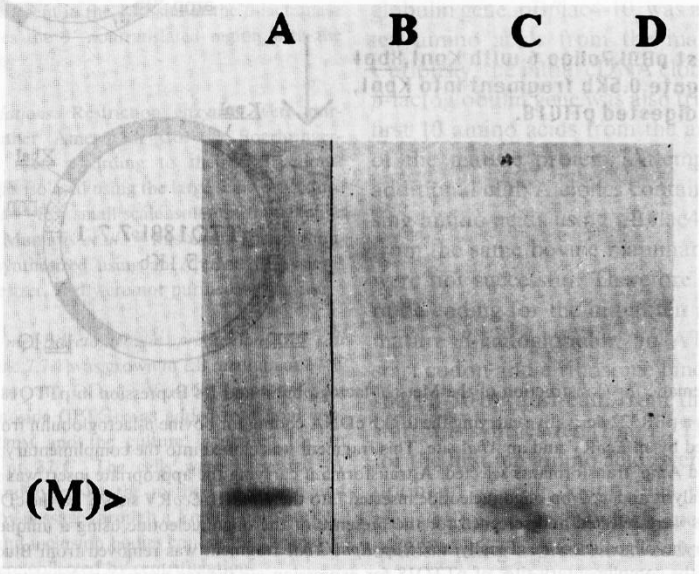

Fig. 2. Western Analysis of *E. coli* TG-1 [pTTQ18βlac.7.7.1] Lysates Separated on a 12.5% SDS-PAGE. Lane A, β-lactoglobulin (Sigma); lane B, no IPTG added; lane C, induced with 2 mM IPTG; lane D, 12,000 × *g* pellet from culture induced with IPTG. M > indicates the position of β-lactoglobulin A.

Motif Exhibit 1137, Page 8 of 11

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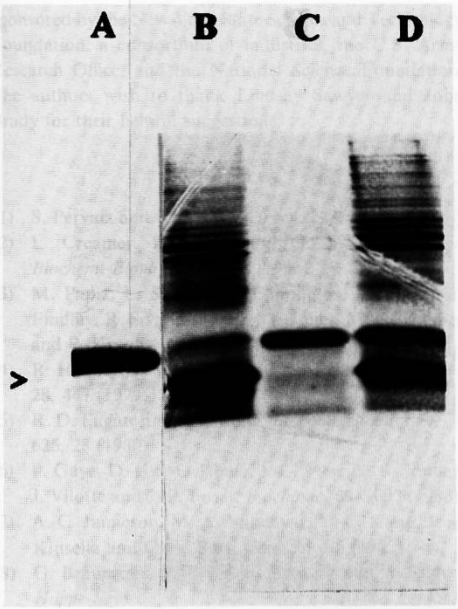

Fig. 3. SDS-PAGE Analysis of *E. coli* TG-1 [pTTQ-18β1ac.7.7.1]Lysates.

Lane A, β-lactoglobulin (Sigma); lane B, Insoluble fraction after guanidine hydrochloride treatment and dialysis; lane C, soluble fraction after guanidine hydrochloride treatment and dialysis; lane D, 12,000 × *g* pellet from whole cell lysate. (M) > indicates the position of β-lactoglobulin A.

cultures were not induced with IPTG. Densitometric scanning of the lysate from an IPTG induced culture separated on a SDS-PAGE stained with Coomassie blue reveals that β-lactoglobulin accounts for approximately 15% of the total cellular protein (Fig. 3).

Many heterologous proteins expressed at high levels in *E. coli* form inclusion bodies.[12] Met-β-lactoglobulin is no exception and insoluble complexes of it were observed in cell lysates. Attempts to produce a soluble form of met-β-lactoglobulin by growing IPTG induced cultures at 30°C[11] (data not shown) were not successful. The majority of the met-β-lactoglobulin formed was insoluble, with the overall production at 30°C less than that observed at 37°C (data not shown). Furthermore the insoluble met-β-lactoglobulin could not be solubilized using fast-flow ion exchange chromatography as demonstrated for an SV40 T antigen peptide, Th, interlukin 2, and the v-myb oncoprotein by Hoess *et al.*[14]

Inclusion bodies containing met-β-lactoglobulin could be solubilized using 8 M guanidine–HCl and the met-β-lactoglobulin remained soluble after dialysis to in the presence of dithiothreitol. The solubilized met-β-lactoglobulin migrated with the same molecular weight as authentic bovine β-lactoglobulin on denaturing PAGE (Fig. 3). Under nondenaturing PAGE conditions which allow the separation of the A and the B forms of β-lactoglobulin, the met-β-lactoglobulin migrated similarly to bovine β-lactoglobulin A (Fig. 4). A trace of a higher molecular weight band, barely detectable, may represent the dimer in both the bovine and met-β-lactoglobulin, again suggesting that the recombinant form has a similar conformation to the native β-lactoglobulin.

Discussion

cDNA clones for a number of bovine milk proteins have been reported including those for α_{s1} casein, β casein, κ casein,[15–17] α-lactalbumin,[18] and β-lactoglobulin.[7,19] The α_{s1} casein has been expressed in *E. coli* using the *tac* promoter and an insoluble, immunologically reactive protein was observed with an estimated yield of 16 mg/l.[20] We estimate that *E. coli* TG1 [pTTQ18βlac.7.7.1] produces β-lactoglobulin in excess of 50 mg/l culture medium.

The high level of recombinant met-β-lactoglobulin produced in *E. coli* [pTTQ18-βlac.7.7.1] could be the result of a variety of features in our construct. First, the proper spatial alignment of the RBS and the translational start of the protein was provided in the design of the oligonucleotide. The effect of varying the distance between the RBS and translational start have been elegantly demonstrated, for example with the expression of chymosin.[21] A change in the spacing between the RBS and translational start from 8 bp to

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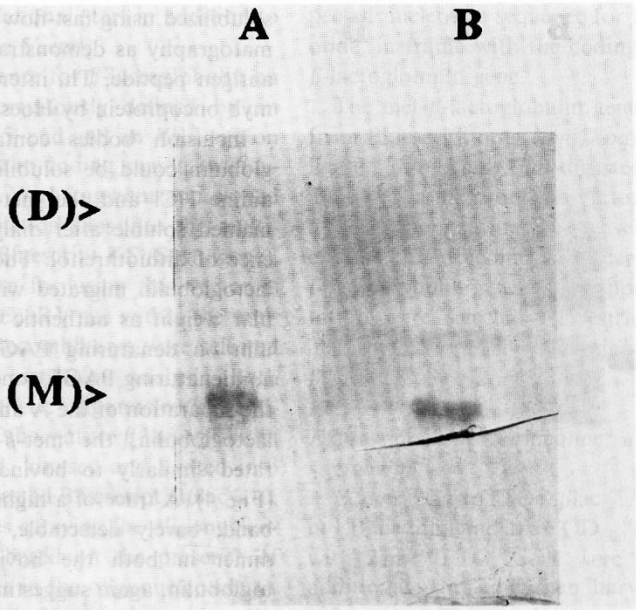

Fig. 4. Western Analysis of Purified and Recombinant β-Lactoglobulin Separated on a 12.5% Non-denaturing-PAGE.

Lane A, β-lactoglobulin A (Sigma); lane B, soluble fraction after guanidine hydrochloride treatment and dialysis from lysate of *E. coli* TG-1 [pTTQ18βlac.7.7.1] induced with IPTG. (M) > indicates position of monomer; (D) > indicates position of dimer.

6 bp resulted in a 20-fold decrease in the level of chymosin expressed. In our construct, the spacing between the RBS and the translational start was 8 bp, although we have not conducted an exhaustive study to demonstrate that this distance is optimal for expression. Second, we used an *E. coli* codon bias for the first ten amino acids of met-β-lactoglobulin, to insure efficient translation.[22] Last, although the physiological role of β-lactoglobulin is not known, it has no demonstrated enzymatic activity and does not bind divalent cations. It should therefore not present any toxicological problems to *E. coli* even at high intracellular concentrations. pTTQ18 is tightly regulated by virtue of a *lac*I^q on the plasmid; expression is only obtained upon the addition of IPTG or another suitable inducer. Therefore the potential deleterious effects (if any) of β-lactoglobulin on growth are minimized until the culture is induced by the addition of IPTG.

Our current interest in β-lactoglobulin is focused on elucidating and manipulating its structure and hence its function. It has recently been suggested by Sawyer that both molecules belong to a larger class of evolutionarily related proteins, including others such as bilin-binding proteins from various insects, which function to transport insoluble or chemically fragile molecules, and he has pointed out the possibility of using this basic biological architecture to design artificial transport proteins for industrial applications.[23] Continued work will serve as a model for the rational design of proteins to serve as receptors for small molecules and in general examine the molecular recognition of small molecules by proteins and designing proteins for specific food and industrial applications.

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