Expression and secretion of recombinant ovine β-lactoglobulin in *Saccharomyces cerevisiae* and *Kluyveromyces lactis*

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High expression and secretion of recombinant ovine β-lactoglobulin has been achieved in the yeast *Kluyveromyces lactis*. The yield of β-lactogobulin is 40–50 mg per litre of culture supernatant and accounts for approx. 72% of the total secreted protein. Constitutive expression is under the control of the *Saccharomyces cerevisiae* phosphoglycerate kinase promoter from an intronless version of the β-lactoglobulin gene. Secretion is specified by the ovine protein's own signal sequence. This system, coupled to an efficient and novel recovery protocol, allows 30 mg of pure protein to be isolated from a typical 1 litre culture. The protein is virtually indistinguishable from β-lactoglobulin conventionally purified from sheep milk by its behaviour in native PAGE and SDS/PAGE, reactivity to antibodies, CD, fluorescence spectroscopy and N-terminal sequencing. Attempts to achieve a similar expression and secretion system in the yeast *S. cerevisiae* met with only limited success, although it was found that heat-shock treatment modestly increased the yield up to approx. 3–4 mg per litre of culture supernatant. Site-directed mutagenesis showed that secretion in *S. cerevisiae* depended upon correct formation of the two disulphide bonds present in β-lactoglobulin.

INTRODUCTION

β-Lactoglobulin is of considerable scientific and commercial interest for a variety of reasons ranging from its relatively small size, ease of preparation in large quantities and stability at acid pH, through to its significant involvement in the dairy industry. The protein itself belongs to the lipocalin family of hydrophobic-molecule carrier proteins which includes retinol-binding protein, insecticyanin, apolipoprotein D and α-1-acid glycoprotein [1,2]. The structure is a cone-shaped eight-stranded β-barrel with a single helix on the outer surface which, in the case of β-lactoglobulin, is close to the molecular 2-fold axis of the dimer. β-Lactoglobulin can exist in both monomeric and dimeric forms, and the latter is the predominant form of the ruminant protein at neutral pH [3]. The protein is notably abundant in the milk of ruminants, and is also present in the milk of other species, generally as a monomer [4]. By contrast, human, mouse and rabbit milks apparently do not contain β-lactoglobulin. The bovine protein exists in several genetic variants as do the β-lactoglobulins of many other species, including sheep [5].

The exact function of β-lactoglobulin is uncertain and, apart from having a role in the nourishment of the neonate, it is possible that a facilitatory role in fatty acid or vitamin uptake may also be important [2,6] as β-lactoglobulin has been shown to bind a wide range of small molecules like fatty acids, retinol and alkanes [7]. Reviews of the general properties of the protein have appeared regularly [4,8–10], while the more specific fatty acid-binding [6] and denaturation properties have been more recently reviewed (L. K. Creamer and L. Sawyer, unpublished work).

β-Lactoglobulin is currently the subject of protein-engineering studies in a number of laboratories, with a view to applications in the food and pharmaceutical industries. There is a consequential requirement for a high-yielding and convenient expression system for the recombinant protein. We report here the development of expression and secretion systems in the two yeasts *Kluyveromyces lactis* and *Saccharomyces cerevisiae*. The *K. lactis* system provides generous quantities of soluble protein and avoids renaturation from inclusion bodies such as is required in the previously reported *Escherichia coli* expression system [11].

EXPERIMENTAL

Bacterial and yeast strains

E. coli strains

Strain TG1 [*F′traD36 lacI*^{q}Δ(*lacZ*)*M15 proA*$^+$*B*$^+$*/supE* Δ(*hsdM-mcrB*)*5* (r_K-m_K-*McrB*-) *thi* Δ(*lac-proAB*)] was used for amplification of plasmids and phage M13. Strain BW313 {*HfrKL16 PO/45* [*lysA*(*61-62*)] *dut ung thi relA1*} was used for site-directed mutagenesis [12], and strain DH5α [*endA1 hsdR17* (r_K-m_K+) *supE44 thi-1 recA1 gyrA*(*Nal*^{r}) *relA1*, Δ(*lacIZYA-argF*)*U169 deoR* (*ø80dlac*Δ(*lacZ*)*M15*)] was used for propagation of the shuttle plasmids pMABLG, pCXJ-Kan1 and pDRBLG.

S. cerevisiae strains

Strain JRY188 (*MATα leu2-3,112 ura3-52 trp1 his4 sir3 rme GAL*) and strain BJ5464 (*MATα ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL*) were used. Strain AH22 (*leu2-3 2-12 his4-519 can1*) was obtained from the National Collection of Yeast Cultures.

K. lactis strain

Strain MW988C (*α uraA argA lysA K*$^+$ *pKD1*^{0}) was used.

Abbreviation used: ECL, enhanced chemiluminescence.

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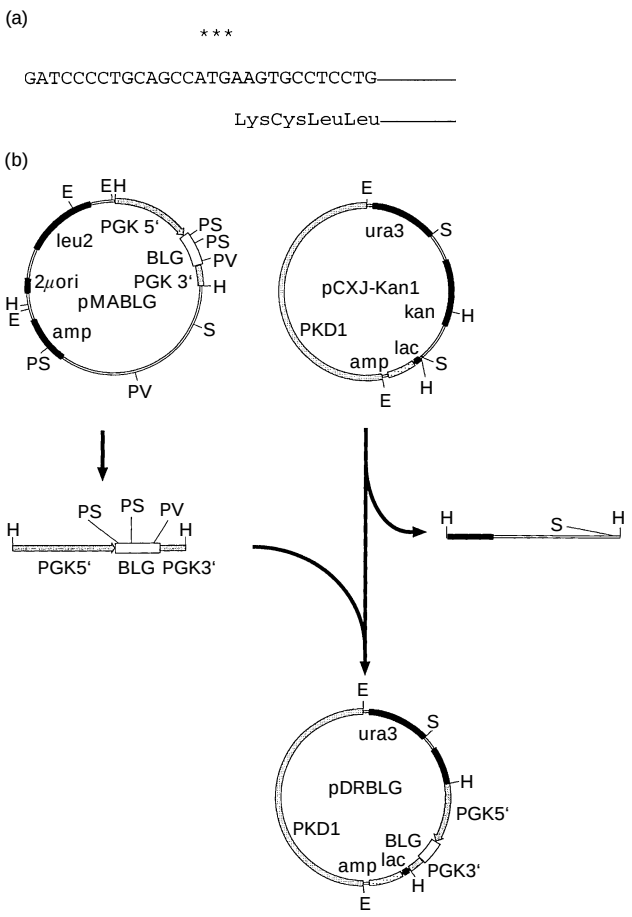

Figure 1 Construction of expression plasmids

(**a**) The upstream terminal sequence of the *Bam*H1 restriction fragment containing the β-lactoglobulin coding sequence that was inserted into plasmid pMA91 to yield the *S. cerevisiae* expression plasmid pMABLG. The start codon is indicated by asterisks. (**b**) The *K. lactis* expression plasmid pDRBLG was formed from the β-lactoglobulin/phosphoglycerate kinase expression cassette located on the *Hin*dIII restriction fragment derived from pMABLG and from pCXJ-Kan. Abbreviations used: E, *Eco*RI; H, *Hin*dIII; PS, *Pst*I; PV, *Pvu*II; S, *Sal*I.

Plasmids

pMA91 is an *E. coli*/*S. cerevisiae* shuttle vector containing the *S. cerevisiae* phosphoglycerate kinase gene [13], and was a kind gift from Dr. S. Kingsman. pMABLG is derived from pMA91 and contains the intronless gene encoding ovine β-lactoglobulin in place of the phosphoglycerate kinase coding sequence. pCXJ-Kan1 is an *E. coli*/*K. lactis* shuttle vector derived from the 1.6 μm circular plasmid pKD1 [14]. pDRBLG is derived from pCXJ-Kan1 and the phosphoglycerate kinase/β-lactoglobulin expression cassette from pMABLG.

Chemicals

Restriction endonucleases were supplied by Northumbria Biologicals, Cramlington, Northumberland. T4 DNA polymerase, T4 DNA ligase and RNase A were from Boehringer Mannheim. The site-directed mutagenesis kit, Sequenase Version 2.0 DNA sequencing kit, [α-^{35}S]dATP and enhanced chemiluminescence (ECL) kit were supplied by Amersham, and the Immobilon-P transfer membrane was from Millipore. Yeast extract, bactopeptone, bactotryptone and bactoagar were from Oxoid, Basingstoke, U.K. Yeast nitrogen base was from Difco and the amino acids were obtained from Sigma. Glass beads were from BDH and all other chemicals were supplied by Sigma.

Growth conditions and transformation

E. coli cultures were grown at 37 °C in L-broth supplemented with ampicillin or kanamycin as required (50 μg/ml) and transformations were performed by the calcium chloride method [15]. *S. cerevisiae* cultures were grown at 30 °C in YPD non-selective medium [1 % (w/v) yeast extract, 2 % (w/v) bactopeptone, 2 % (w/v) glucose] or in YOM Leu$^-$-selective medium [0.67 % (w/v) yeast nitrogen base without amino acids, 2 % (w/v) glucose and supplemented with tryptophan (40 μg/ml), uracil (64 μg/ml) and histidine (40 μg/ml)]. Heat-shock experiments with *S. cerevisiae* were done by growing cultures for 24 h at 30 °C, then 40 min at 39 °C, followed by a further 24 h period at 30 °C. *K. lactis* cultures were grown at 28 °C in YPD or YOM Ura$^-$ [YOM supplemented with arginine (30 μg/ml) and lysine (20 μg/ml)]. Transformations of both species of yeast were done by an improved lithium acetate procedure [16]. Transformants were analysed by plasmid rescue, restriction mapping and double-stranded DNA sequencing (Sequenase version 2.0 manufacturer's instructions).

Plasmid construction and site-directed mutagenesis

All plasmid constructs were generated using standard procedures. An intronless version of the ovine β-lactoglobulin gene derived from plasmid pUC19BLG (a kind gift from Dr. S. Harris, Glaxo, Greenford, Middlesex, U.K.) was inserted into the shuttle plasmid pMA91 to yield the *S. cerevisiae* expression vector pMABLG (Figure 1). The *K. lactis* expression plasmid pDRBLG was constructed from the phosphoglycerate kinase/β-lactoglobulin expression cassette and the shuttle vector pCXJ-Kan1 as shown in Figure 1. The methods of Kunkel [12] and Taylor et al. [17] were both used for site-directed mutagenesis, and both gave mutations with high efficiency.

Protein concentration determination

Protein concentration in solution was measured by a Coomassie Brilliant Blue G250 method [18] using bovine β-lactoglobulin as the standard. Estimation of protein concentration in electrophoresis gels was done by scanning densitometry of an SDS/12 %-PAGE gel using a Joyce-Loebl Chromoscan 3 densitometer.

Protein purification

All steps were done at room temperature (18–22 °C). One litre of a liquid culture of *K. lactis* was centrifuged at 8000 rev./min in the JA14 rotor of a J2-21 Beckman centrifuge for 45 min, and the supernatant containing β-lactoglobulin was collected. Ammonium sulphate was added to 70 % satn. and the precipitated proteins collected by centrifugation at 12000 rev./min as before. Larger scale experiments with culture volumes of 2 litres or more were concentrated to 300 ml using a Bio 2000 MkII cross-flow filtration unit with a 10 kDa cut-off cartridge (BioFlo Ltd) prior to the ammonium sulphate precipitation. The precipitate was suspended in 5 ml of 50 mM sodium phosphate buffer, pH 6.2, and applied to a Sephadex G-75 gel-filtration column (1.5 cm × 90 cm) (Pharmacia Ltd) pre-equilibrated and eluted with the same buffer. Fractions containing homogeneous

β-lactoglobulin, as judged by SDS/PAGE, were pooled, concentrated by ammonium sulphate precipitation and stored at 4 °C.

Electrophoresis and immunoblotting

SDS/PAGE was done on a 12% polyacrylamide gel by the method of Laemmli [19]. After electrophoresis, the separated proteins were either stained using Coomassie Brilliant Blue or transferred on to Immobilon-P membrane by a semi-dry procedure using a Pharmacia-LKB Multiphor II transfer system. β-Lactoglobulin was detected by polyclonal anti-(ovine β-lactoglobulin) antibodies raised in female New Zealand White rabbits. Primary antibody–antigen complexes were detected using donkey anti-(rabbit IgG) serum conjugated to horseradish peroxidase and developed by the Amersham ECL procedure.

Ouchterlony double diffusion

A pattern of five wells was cut into a 1% agar gel that had been poured on to a 6 cm × 6 cm glass slide. The gel was prepared in 50 mM phosphate buffer, pH 6.2. Solutions of antibody and antigen were pipetted into the wells, and left in a moist environment for 24 h for precipitin lines to develop.

CD and fluorescence spectra

CD measurements were made on a Jasco J-600 spectropolarimeter, using quartz cells with a 0.1 cm pathlength. β-Lactoglobulin samples purified from sheep milk or from *K. lactis* culture supernatant, both at a concentration of 0.8 mg/ml, were scanned from 260 to 190 nm at a temperature of 20 °C. The CD spectra were measured at least three times and the results averaged. Molar ellipticities were calculated assuming a value of 112 for the mean residue weight [20]. Fluorescence spectra were recorded on a Perkin-Elmer LS-3B fluorimeter at 20 °C with a 1 cm pathlength quartz cuvette. The excitation wavelength was 292 nm, and the emission was scanned over 300–400 nm. The protein concentration was 0.7 mg/ml.

N-terminal amino acid sequencing

Secreted recombinant β-lactoglobulin was purified for N-terminal sequence analysis by reverse-phase HPLC using an Applied Biosystems 130A separation system, and sequencing was done using an Applied Biosystems 477A pulsed-liquid microsequencer as described previously [21].

RESULTS

Expression and secretion of β-lactoglobulin

An intronless form of the full pre-β-lactoglobulin coding sequence was obtained as a *Sph*I–*Sma*I fragment inserted into pUC19. Several subcloning steps were used to prepare a *Bam*HI restriction fragment (Figure 1a) that was then inserted into the *Bgl*II site of the multicopy shuttle vector pMA91, with the concomitant loss of that site. The resulting plasmid (pMABLG, Figure 1b) has the entire β-lactoglobulin coding sequence, including the signal peptide. Expression is under the control of the phosphoglycerate kinase upstream and downstream flanking sequences. This expression plasmid is similar to that previously used for calf chymosin in *S. cerevisiae* [22], although there was no secretion in that system.

The strain dependence of levels of expression was investigated by comparison of three different laboratory strains of *S. cerevisiae*. In addition, the possible contribution of heat shock as a strategy to increase expression was tested because the upstream flanking region of the phosphoglycerate kinase expression cassette contains a heat-shock-responsive element [23]. The results are summarized in Table 1. It can be seen that the best levels of expression were with strain AH22, and that in all cases heat shock improved the expression. It was possible with this approach to obtain the expression of approx. 3–4 mg of β-lactoglobulin per litre of culture supernatant, eventually yielding about 2 mg of pure protein (results not shown).

However, this level of expression was insufficient for our protein-engineering studies and it was decided to investigate whether better results might be obtained with the yeast *K. lactis*. This yeast has been shown to be strikingly tolerant of secreting highly expressed heterologous proteins such as prochymosin, interleukin-1β and human serum albumin ([24–26]; reviewed in [27]). Figure 2 shows the results of SDS/PAGE analysis of samples of culture supernatant from *K. lactis* transformed with pDRBLG. These results demonstrated that it was possible to obtain the expression of approx. 40–50 mg of β-lactoglobulin per

Table 1 Effects of strain and heat shock on levels of expression of β-lactoglobulin in *S. cerevisiae*

Expression and secretion of β-lactoglobulin in different strains of *S. cerevisiae* was from plasmid pMABLG. Samples of culture supernatant were dialysed and subjected to SDS/PAGE. The amount of β-lactoglobulin was estimated by densitometry.

	Expression (mg/l)		
	No heat shock	With heat shock	Fold increase
JRY188	0.6	1.8	3.0
BJ5464	0.4	0.5	1.3
AH22	2.4	3.6	1.5

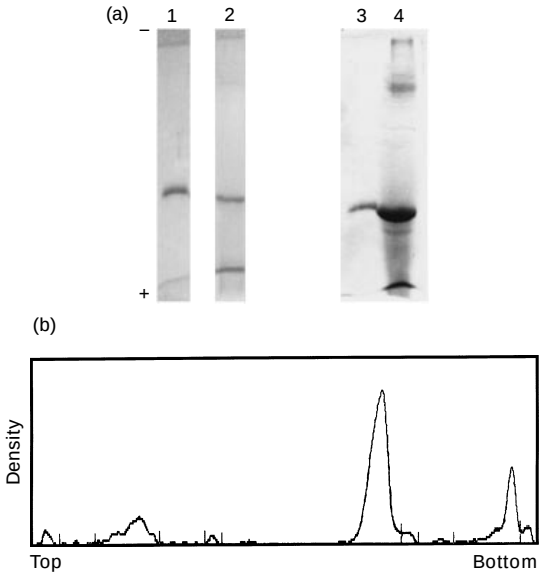

Figure 2 SDS/PAGE analysis of β-lactoglobulin expressed in *K. lactis*

(**a**) Lane 1, 1 μg of bovine β-lactoglobulin; lane 2, 20 μl of unconcentrated culture supernatant; lane 3, 1 μg of bovine β-lactoglobulin; lane 4, 1 μl of culture supernatant concentrated 50-fold by ammonium sulphate precipitation followed by dialysis. (**b**) Densitometric scan of lane 4. The peak areas were integrated and showed that the β-lactoglobulin accounted for 72% of the total protein, disregarding the material associated with the dye front.

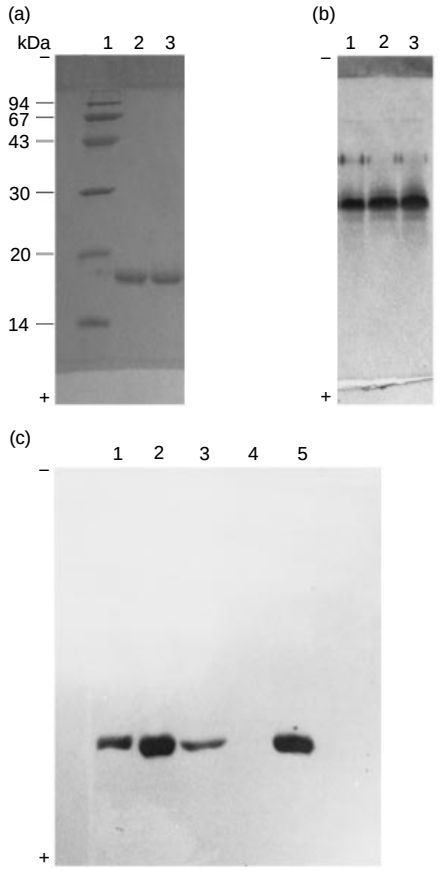

Figure 3 Electrophoretic characterization of β-lactoglobulins

(**a**) SDS/PAGE of β-lactoglobulin purified from sheep milk (lane 2) and from *K. lactis* (lane 3). Molecular mass markers (kDa) are shown in lane 1. (**b**) Electrophoresis under non-denaturing conditions of β-lactoglobulin from sheep milk (lane 1), β-lactoglobulin from *K. lactis* (lane 2) and a mixture of the two β-lactoglobulins (lane 3). (**c**) Western blotting of bovine β-lactoglobulin (Sigma) (lane 1), β-lactoglobulin from sheep milk (lane 2), culture supernatant and cell extract from the *K. lactis* expression system (lanes 3 and 4) and β-lactoglobulin purified from the *K. lactis* expression system (lane 5).

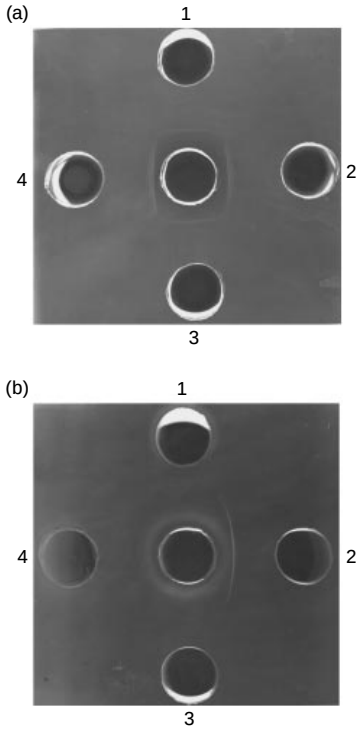

Figure 4 Ouchterlony double diffusion of recombinant and milk β-lactoglobulins

The wells contained the following samples: (**a**) wells 1 and 3, β-lactoglobulin purified from sheep milk; wells 2 and 4, recombinant β-lactoglobulin secreted by *K. lactis*; central well, antibodies prepared against purified β-lactoglobulin from sheep milk. (**b**) Well 1, cell extract from *K. lactis* with plasmid pDRBLG; well 2, culture supernatant from *K. lactis* with plasmid pDRBLG; well 3, culture supernatant from *K. lactis* without plasmid pDRBLG; well 4, cell extract from *K. lactis* without plasmid pDRBLG; central well, antibodies prepared against purified β-lactoglobulin from sheep milk.

litre of culture, and moreover that the protein accounted for about 72% of the total protein, and thus required little further purification. This straightforward change of yeast has enabled a massive improvement in the expression system, and indicates that *K. lactis* may frequently be the organism of choice for the expression and secretion of soluble heterologous proteins.

Purification and characterization of recombinant *K. lactis* β-lactoglobulin

A simple purification procedure was developed for β-lactoglobulin from the *K. lactis* expression system. The culture supernatant was concentrated by cross-flow filtration. The proteins present were then collected by ammonium sulphate precipitation, fractionated and then desalted on Sephadex G-75. Fractions shown to be pure by SDS/PAGE were pooled, and the β-lactoglobulin was precipitated by ammonium sulphate for long-term storage. It was noted that β-lactoglobulin prior to ammonium sulphate precipitation had a yellow colour. Preliminary fluorescence and UV spectroscopy measurements indicated that this was due to the binding of some molecule present in the culture medium, possibly a vitamin or other hydrophobic molecule which β-lactoglobulin is known to bind very tightly [4,7].

Electrophoretic characterization of the purified recombinant β-lactoglobulin and of β-lactoglobulin purified from sheep milk is shown in Figure 3. SDS/PAGE of both proteins gave single sharp bands with a molecular mass of 18.2 kDa. Electrophoresis under non-denaturing conditions showed that the recombinant and milk proteins are indistinguishable. Western blotting of proteins from crude cell extracts and culture supernatants (Figure 3c) clearly demonstrates that all the expressed β-lactoglobulin is secreted in a form with subunit molecular mass of 18.2 kDa.

Comparison of recombinant and milk β-lactoglobulins by Ouchterlony double diffusion showed that they have identical reactivity to polyclonal antibodies prepared against β-lactoglobulin from sheep milk (Figure 4a). There was no detectable spur formation. This technique showed in addition that the antibodies were specific for β-lactoglobulin and had no reactivity against *K. lactis* itself (Figure 4b). Moreover, all the β-lactoglobulin was in the secreted form, with none detectable within the cells.

Figure 5 shows the spectroscopic properties of both the milk and recombinant β-lactoglobulins. It can be seen that the CD spectra are virtually the same, as are those from the fluorescence measurements. These results provide strong evidence that the recombinant β-lactoglobulin has folded into a native structure that is essentially indistinguishable from the milk protein. By

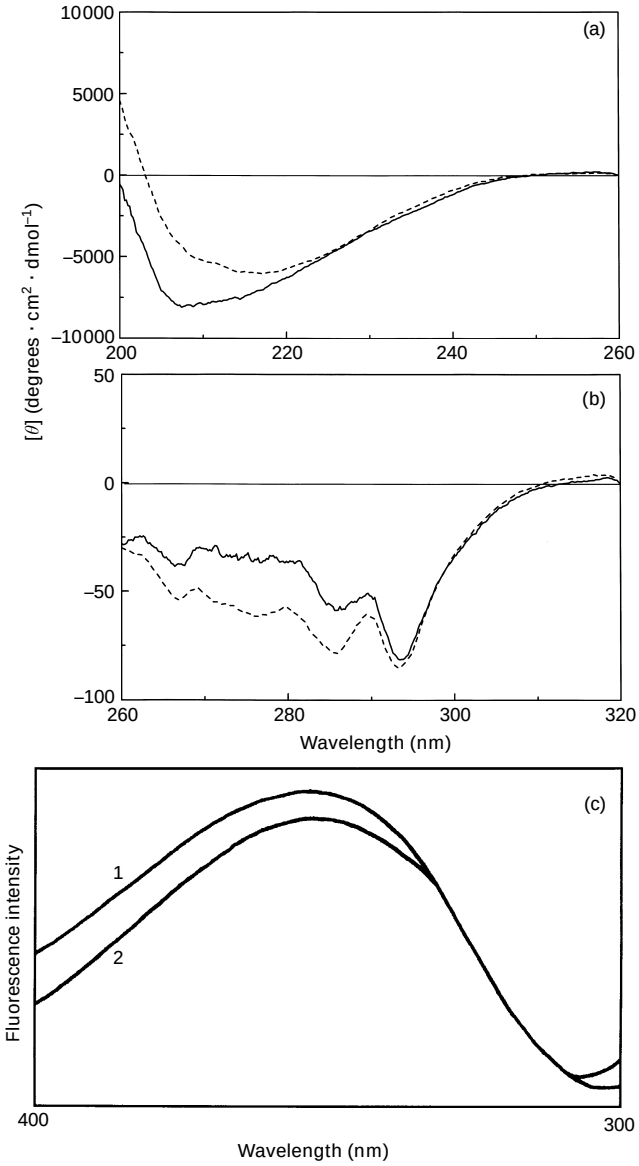

Figure 5 Spectroscopic characterization of β-lactoglobulins

(**a**) Far-UV and (**b**) near-UV CD spectra of milk (solid line) and recombinant (broken line) β-lactoglobulins. Molar ellipticities ([θ]) are given in units of degrees·cm^2·dmol^{-1}. (**c**) Fluorescence spectra of milk (1) and recombinant (rec) (2) β-lactoglobulins.

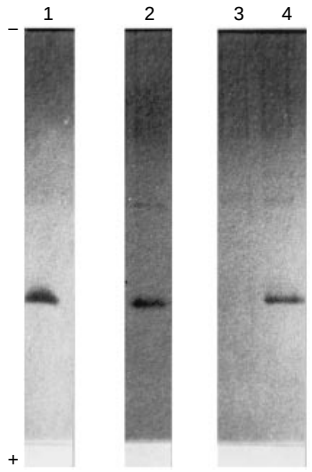

Figure 6 SDS/PAGE analysis of the secretion of wild-type and cysteine mutant forms of β-lactoglobulin

Samples from culture supernatants were concentrated 20-fold by Amicon ultrafiltration, and 10 μl aliquots loaded on to the gel. Lane 1, bovine β-lactoglobulin; lane 2, wild-type β-lactoglobulin; lane 3, mutant C119S; lane 4, mutant C121S.

Disulphide bond formation

Site-directed mutagenesis was used to show that correct disulphide bond formation is required for secretion of β-lactoglobulin from *S. cerevisiae*. β-Lactoglobulin possesses five half-cystine residues (66, 106, 119, 121 and 160) which are arranged into two disulphide bonds and one free thiol group. The disulphide bridge between residues 66 and 160 appears to be stable and uncontroversial, but there is conflicting evidence on whether residue 106 forms a disulphide bond with residue 119 or with residue 121 [3,28]. Mutant forms of β-lactoglobulin were prepared in which cysteine residues 119 and 121 were separately mutated to serine residues (designated C119S and C121S). Figure 6 shows SDS/PAGE analysis of the culture supernatants of *S. cerevisiae* transformed with plasmids carrying these two mutations or wild-type β-lactoglobulin. It is clear that mutant C119S was unique in not being secreted, and it is thus reasonable to suggest that correct disulphide bond formation between residues 106 and 119 is required for secretion. This result supports the crystal structure evidence ([3]; S. Brownlow, personal communication) that the native form of β-lactoglobulin has a disulphide bridge between residues 106 and 119.

DISCUSSION

The yeast *K. lactis* has provided us with a breakthrough in our quest for a convenient high-capacity expression and secretion system for recombinant ovine β-lactoglobulin. The use of this system provides 40–50 mg of secreted β-lactoglobulin for every litre of culture, and is thus a striking improvement over expression systems based on *S. cerevisiae*. Moreover, *K. lactis* secretes almost no proteins itself, and the secreted β-lactoglobulin thus only requires a simple procedure to achieve purification. The ovine β-lactoglobulin expressed and secreted by *K. lactis* is virtually indistinguishable from the protein purified from sheep milk by native PAGE and SDS/PAGE, reactivity to antibodies, CD, fluorescence spectroscopy and N-terminal sequencing.

Previous experiments to express recombinant β-lactoglobulin have used both yeast and *E. coli* systems. Totsuka et al. [29] contrast, recombinant β-lactoglobulin purified by Amicon ultrafiltration instead of ammonium sulphate precipitation not only retained a yellow colour, but also showed marked spectral differences from the milk protein (results not shown).

N-terminal sequence analysis of recombinant β-lactoglobulin purified by microbore HPLC gave the following unique sequence for ten cycles of Edman degradation: Ile-Ile-Val-Thr-Gln-Thr-Met-Lys-Gly-Leu. The initial yield was 500 pmol, and the repetitive yield was 89%. This sequence is identical to the N-terminal sequence of the mature form of β-lactoglobulin found in sheep milk [20], and thus shows that the *K. lactis* cells have correctly recognized and processed the sheep secretion signal peptide. Similar experiments with the recombinant β-lactoglobulin secreted by the *S. cerevisiae* system gave the same result.

reported an expression/secretion system in *S. cerevisiae* that yielded approx. 1 mg of bovine β-lactoglobulin per litre of culture. By coincidence, this was found to be increased 5-fold by engineering a tryptophan to tyrosine replacement at residue 19 [30]. The system can also be improved 3–4-fold by strain choice and the use of heat shock as shown above. Bovine β-lactoglobulin can be expressed in *E. coli* at levels of up to approx. 50 mg per litre of culture [11]. It is primarily present in the form of inclusion bodies, and requires 8 M guanidine hydrochloride for reconstitution. However, the requirement for denaturation and renaturation is a major disadvantage for protein-engineering studies in which mutant versions of the protein may have altered stability or folding characteristics. Furthermore, β-lactoglobulin is prone to disulphide interchange [28], and this must be a concern for a successful reconstitution process, especially as chaotropic agents like urea and guanidine hydrochloride are known to encourage the interchange of disulphides [31].

The results reported here demonstrate that the yeast *K. lactis* has compelling advantages over *S. cerevisiae* and *E. coli* for the expression and secretion of recombinant heterologous proteins such as ovine β-lactoglobulin.

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REFERENCES

1 North, A. C. T. (1991) Biochem. Soc. Symp. **57**, 35–48
2 Sawyer, L. (1992) in Protein Engineering: Proceedings 1st AFRC Conference on Protein Engineering in the Agricultural and Food Industries (Goodenough, P., ed.), pp. 116–125, CPL Scientific, Newbury, U.K.
3 Papiz, M. Z., Sawyer, L., Eliopoulos, E. E., North, A. C. T., Findlay, J. B. C., Sivaprasadaro, R., Jones, T. A., Newcomer, M. E. and Kraulis, P. J. (1986) Nature (London) **324**, 383–385
4 Hambling, S. G., McAlpine, A. W. and Sawyer, L. (1992) β-Lactoglobulin in Advanced Dairy Chemistry I (Fox, P. F., ed.), pp. 141–190, Elsevier, Amsterdam
5 Aschaffenburg, R. (1968) J. Dairy Res. **35**, 447–460
6 Perez, M. D. and Calvo, M. (1995) J. Dairy Sci., **78**, 978–988
7 Creamer, L. K. (1995) Biochemistry, **34**, 7170–7176
8 McKenzie, H. A. (1967) Adv. Protein Chem. **22**, 147–234
9 McKenzie, H. A. (1971) β-Lactoglobulins in Milk Proteins (McKenzie, H. A., ed.), vol. II, pp. 257–330, Academic Press, New York
10 Lyster, R. L. J. (1972) J. Dairy Res. **39**, 279–318
11 Batt, C. A., Rabson, L. D., Wong, D. W. S. and Kinsella, J. E. (1990) Agric. Biol. Chem. **54**, 949–955
12 Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 488–492
13 Kingsman, S. M., Cousens, D., Stanway, C. A., Chambers, A., Wilson, M. and Kingsman, A. J. (1990) Methods Enzymol. **185**, 329–341
14 Bianchi, M. M., Falcone, C., Chen, X. J., Weslowski-Louvel, M., Frontali, L. and Fukuhara, H. (1987) Curr. Genet. **12**, 185–192
15 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor
16 Gietz, D., St. Jean, A., Woods, R. A. and Schiestl, R. H. (1992) Nucleic Acids Res. **20**, 1425
17 Taylor, J. W., Ott, J. and Eckstein, F. (1985) Nucleic Acids Res. **13**, 8764–8785
18 Sedmak, J. J. and Grossburg, S. E. (1977) Anal. Biochem. **79**, 544–552
19 Laemmli, U. K. (1970) Nature (London) **277**, 680–685
20 Gaye, P., Hue-Delahaie, D., Mercier, J.-C., Soulier, S., Vilotte, J.-L. and Furet, J.-P. (1986) Biochimie **68**, 1097–1107
21 Hayes, J. D., Kerr, L. A. and Cronshaw, A. D. (1989) Biochem. J. **264**, 437–445
22 Mellor, J., Dobson, M. J., Roberts, N. A., Tuite, M. F., Emtage, J. S., White, S., Lowe, P. A., Patel, T., Kingsman, A. J. and Kingsman, S. M. (1983) Gene **24**, 1–14
23 Piper, P. W., Curan, B., Davies, M. W., Hirst, K., Lockheart, A., Ogden, J. E., Stanway, C. A., Kingsman, A. J. and Kingsman, S. M. (1988) Nucleic Acids Res. **16**, 1333–1348
24 van den Berg, J. A., van der Laken, K. J., van Ooyen, A. J. J., Renniers, T. C. H. M., Rietveld, K., Schaap, A., Brake, A. J., Bishop, R. J., Schultz, K., Moyer, D., Richman, M. and Shuster, J. R. (1990) Bio/Technology **8**, 135–139
25 Fleer, R., Chen, X. J., Amellal, N., Yeh, P., Fournier, A., Guinet, F., Gault, B., Faucher, D., Folliard, F., Fukuhara, H. and Mayaux, J. F. (1991) Gene **107**, 285–295
26 Fleer, R., Yeh, P., Amellal, N., Maury, I., Fournier, A., Bacchetta, F., Baduel, P., Jung, G., L'Hôte, H., Becquart, J., Fukuhara, H. and Mayaux, J. F. (1991) Bio/Technology **9**, 968–975
27 Swinkels, B. W., van Ooyen, A. J. J. and Bonekamp, F. J. (1993) Antonie van Leeuwenhoek **64**, 187–201
28 McKenzie, H. A., Ralston, G. B. and Shaw, D. C. (1972) Biochemistry **11**, 4539–4547
29 Totsuka, M., Katakura, Y., Shimizu, M., Kumagai, I., Miura, K. and Kaminogawa, S. (1990) Agric. Biol. Chem. **54**, 3111–3116
30 Katakura, Y., Totsuka, M., Ametani, A. and Kaminogawa, S. (1994) Biochim. Biophys. Acta **1207**, 58–67
31 Weissman, J. S. and Kim, P. S. (1991) Science **253**, 1386–1393

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