

Efficient expression of bovine α -lactalbumin in *Saccharomyces cerevisiae*

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A synthetic gene encoding the mature bovine α -lactalbumin fused to the preproregion of the yeast α -mating factor has been expressed and secreted at high level in *Saccharomyces cerevisiae* under the control of the α -mating promotor. Growth conditions were found to be critical for the expression: recombinant α -lactalbumin could only be detected in the medium provided the culture was grown at neutral pH. The secreted bovine α -lactalbumin is enzymatically active and identical to the whey protein, as confirmed by SDS/PAGE, IEF, ultraviolet and CD spectral analysis, and amino-terminal sequence determination.

The whey protein α -lactalbumin retains the interest of many researchers in different fields of protein chemistry. First, although it has been known for many years that it is a cofactor of galactosyltransferase in the synthesis of lactose, the binding locus of this enzyme on the α -lactalbumin molecule is still not defined [1]. Moreover, the mechanism explaining the influence of α -lactalbumin on the catalytic properties of galactosyltransferase has not yet been elucidated. Second, the 0.17-nm resolution crystallographic analysis of baboon α -lactalbumin [2, 3], defining the exact structure of the calcium binding loop ('the elbow'), does not clarify the exact nature of the binding sites for Zn^{2+} and Cu^{2+} ions. Third, the previous observations and the similarity between the primary structures of α -lactalbumin and hen egg-white lysozyme are at the origin of a number of investigations concerning the molecular evolution of both proteins (for review see [4]). Fourth, immunological studies have located the main antigenic epitopes on hen egg-white lysozyme [5] but not on α -lactalbumin, although different antibodies against bovine α -lactalbumin have been prepared (e.g. Joniau, M. et al., unpublished data). Fifth, α -lactalbumin and lysozyme are used in many investigations as a model for the study of the mechanism by which the amino acid sequence of a protein directs the folding in the native conformation. In the same context, the stability against thermal and chemical denaturation for both proteins has also been compared [6, 7]. Finally, the binding of different cations to these proteins is also under investigation in thermodynamic studies in order to separate the conformational and binding parts of the thermodynamic state functions (Desmet, J. et al., unpublished results) [8–10].

The goal of all these studies is to determine the role of individual amino acids in each of these phenomena. The techniques of genetic engineering are useful tools in such studies, provided the DNA coding for the protein is cloned and ex-

pressed. This has been done a number of years ago for different lysozymes [11, 12] and was started very recently for α -lactalbumin. Wang et al. [13] expressed bovine α -lactalbumin at high level as a fusion protein in *Escherichia coli*. Cleavage of the fusion protein at its junction by trypsin produced an α -lactalbumin which was indistinguishable from the native protein, provided that the protein was converted to its native form by treatment with mixtures of reduced and oxidized glutathione in the presence of Ca^{2+} . Indeed, it was proven earlier by Rao and Brew [14] that calcium regulates the folding of α -lactalbumin and the formation of its disulfide bonds. Similarly, goat α -lactalbumin was expressed as a fusion protein in *E. coli* and refolded into a biologically active conformation [15].

In the conclusion of their paper, Wang et al. [13] stated that there may be a potential disadvantage in their method to obtain recombinant α -lactalbumin because it relies on the conformation-limited proteolytic cleavage of the fusion protein and the ability of the split product to refold correctly. Indeed, mutations that reduce the stability of the protein, as for example a substitution of Asp87 or Asp88 that impairs the Ca^{2+} binding potential, may result in a less specific cleavage of the folded protein. In order to overcome these difficulties, we have engineered *Saccharomyces cerevisiae* in order to obtain secretion of active bovine α -lactalbumin. Our results not only demonstrate efficient secretion of the protein but also that the recombinant α -lactalbumin produced is identical to the milk protein.

EXPERIMENTAL PROCEDURES

Enzymes, chemicals and plasmids

Restriction enzymes were purchased from Boehringer Mannheim, BRL and New England Biolabs. Klenow fragment of *E. coli* DNA polymerase I, T_4 polynucleotide kinase and β -glucuronidase/arylsulfatase were from Boehringer Mannheim. Zymolyase 100T was obtained from Kirin Brewery (Tokyo, Japan). Deoxyadenosine 5'- α -[^{35}S]thiotriphosphate (>1000 Ci/mmol) was obtained from Amersham. Other chemicals used were of analytical or biochemical grade.

The plasmids pSCMF4 and pUDT2HIL202 were gifts from R. Contreras (University of Gent, Belgium). The oligonucleotides were purchased from the Laboratory for

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Enzymes. Lysozyme (EC 3.2.1.17); β -galactosyltransferase (EC 2.4.1.151); DNA polymerase I (EC 2.7.7.7); T_4 DNA ligase (EC 6.5.1.1); T_4 polynucleotide kinase (EC 2.7.1.78); β -glucuronidase/arylsulfatase (EC 3.2.1.31/3.1.6.1); endo-1,3- β -glucanase, zymolyase (EC 3.2.1.39).

Note. The novel nucleotide sequence data published here have been deposited with the EMBL sequence data bank.

Genetics (University of Gent, Belgium). Plasmid pGV479 is a derivative of pGV451 [16]: the difference between these two plasmids is basically situated in the multiple cloning site as shown in Fig. 1. The special feature of pGV479 is the presence of a unique *DpnI* restriction site (GATC) flanked by the *MnII* sites (CCTC and GAGG) at the left and right side, respectively. The distance between *DpnI* and *MnII* is such that any blunt end restriction fragment, cloned into pGV479, can be excised with *MnII* at exactly the same positions where it was inserted [17]. Thus, this site allows amplification of a DNA fragment by cloning.

1	ACGCCAGCAACGCGGCCGGAAGCTTATCGGTGACCCCTGACTAAGTCGAGCCCAATTCCCG	pGV451
	ACGCCAGCAACGCGGCCGGAAGCTTATCGGTGACCCCTGACTAAGTCGAGCCCAATTCCCG	pGV479
61	GGGATCCGTCGACCTCGACGCAAGCT GGGCTCGACTCAGTCAGGGCGATGATAAGCTGT	pGV451

	CTCGAGAAGATCTTCTCGAGGGCGCTCGGGCTCGACTCAGTCAGGGCGATGATAAGCTGT	pGV479
	↑	
120	CAAACATGAGAATTGAAGATCTTCGAAGATCTTCAATCTTGAAGACGAAAGGCGCTCGT	pGV451

	CAAACATGAGAA TCTTGAAGACGAAAGGCGCTCGT	pGV479
180	GATACGCCTATTTTATAGGTTAATGTCATGATAAATAGTTTCTTAGACGTCAGGTGC	pGV451
	GATACGCCTATTTTATAGGTTAATGTCATGATAAATAGTTTCTTAGACGTCAGGTGC	pGV479
240	CACCTTTTCGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTCTAAATACATTCAA	pGV451

	CACCTTTTCGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTCTAAATACATTCAA	pGV479
300	TATGTATCCGCTCATGAGACAATAACCCCTGATCGAGATTTTCAGGAGCTAAGGAAGCTAA	pGV451
	TATGTATCCGCTCATGAGACAATAACCCCT GAGATTTTCAGGAGCTAAGGAAGCTAA	pGV479

Fig. 1. Comparison between pGV451 and pGV479. Differences are marked by asterisks. Numbering follows the pGV451 sequence (only the relevant part is shown). In pGV479, the *MnII* sites are underlined. Cleavage by *DpnI* is shown by an upward arrow

Strains and media

Escherichia coli cells used as hosts for transformation of recombinant DNA were DH5 α [*supE44 lacU169* (Φ 80*lacZ* Δ M15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*]; MC1061 (*hsdR mcrB araD139* Δ (*araABC-leu*)7679 *lacX74 galU galK rpsL thi*); GM119 (*dam-3 dcm-6 lacY1 metB1 supE44 galK2 galT22 mtl-1? thi-1? fhuA32? tsx-78*). Cells were grown in Luria broth [1% Bacto tryptone (Difco), 0.5% Bacto yeast extract (Difco), 1% NaCl] or agar (Luria broth + 1.5% Bacto agar). Ampicillin (50 μ g/ml) or chloramphenicol (30 μ g/ml) were added to the media when necessary.

Saccharomyces cerevisiae strain GRF181 (*Mat α leu2-212 his3-15 ura3 Δ CAN cir⁺*) [18] was used as a host for the expression of bovine α -lactalbumin. Uracil-selective medium [0.67% yeast nitrogen base without amino acids (Difco), 0.5% casamino acids (Difco), 3% sucrose] was used to select for plasmid-containing yeast cells and to prepare inocula for expression. Growth and expression of α -lactalbumin was carried out in uracil-selective or in YPD medium [2% Bacto pepton (Difco), 1% Bacto yeast extract, 3% sucrose], supplemented with 50 mM Mes and adjusted to pH 7 with 2 M NaOH.

Transformation procedures for *E. coli* and *S. cerevisiae* were performed as described by Sambrook et al. [19] and Hinnen et al. [20] respectively.

Preparation of the oligonucleotides (Fig. 2)

A synthetic gene was designed as described under Results and Discussion. Four gene fragments were constructed by synthesis of pairs of partially overlapping oligomers of about 55 nucleotides. Each pair of oligomers was rendered double-stranded by an enzymatic filling-in reaction: 0.5 μ g of each of the two oligomers were mixed in water, denatured at 95°C

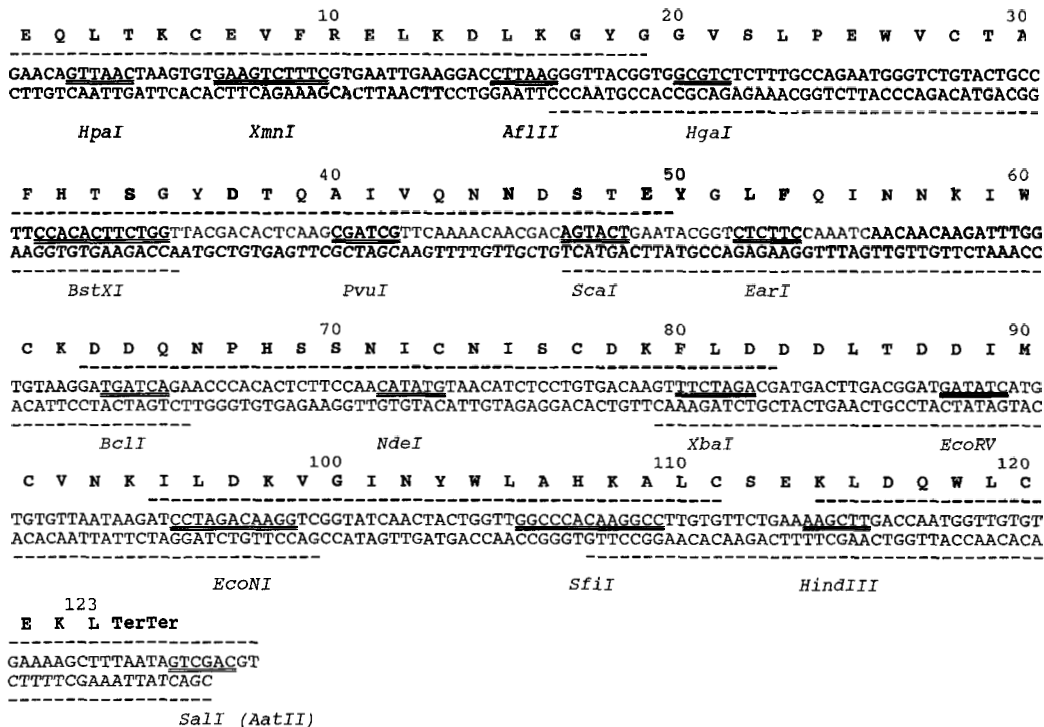


Fig. 2. Amino-acid and DNA sequence of the synthetic bovine α -lactalbumin gene. The synthetic approach enabled us (a) to introduce unique restriction sites (underlined) at regular distances within the gene and (b) to adapt usage to an optimal bias for *S. cerevisiae*, the organism used as expression host. The dashed lines (---) above and under the nucleotide strands correspond to the sequences of the oligomers synthesized to assemble the gene

for 5 min and placed on ice. Annealing buffer was added to a final concentration of 7 mM Tris/HCl pH 7.5, 33 mM NaCl, 7 mM MgCl₂, 3.5 mM dithiothreitol. Annealing was carried out in a volume of 30 µl by an initial incubation at 37°C for 10 min followed by a slow cooling to room temperature (20 min). Subsequently, the single-stranded ends were filled in by mixing 4 µl annealing mixture, 1 unit Klenow polymerase (1 µl), 8 µl of a dNTP solution (each 2.5 mM in water) and 17 µl buffer to a final concentration of 50 mM Tris/HCl pH 7.5, 100 mM MgSO₄, 1 mM dithiothreitol, and 50 µg/ml bovine serum albumin. The reaction was carried out at 16°C for 10 min and subsequently the enzyme was inactivated at 65°C. The resulting double-stranded, blunt-end fragments were phosphorylated using T₄ polynucleotide kinase and ATP [19]. The reaction mixture was desalted on a Sephadex G-50 spin column [21].

Cloning of the synthetic fragments and subsequent reconstruction of the gene (Fig. 3)

Each of the subfragments 1, 2 and 3 were mixed at a 3:1 ratio with *DpnI*-cleaved and dephosphorylated pGV479 and ligated with T₄ DNA ligase. The orientation of the insert was checked by restriction analysis. The resulting plasmids containing the first, the second, etc., subfragment were named pVA1, pVA2, etc., respectively for the orientation corresponding clockwise to the coding direction of bovine α -lactalbumin.

All subsequent ligations and DNA manipulation techniques were performed using standard protocols [19]. Fragments 1 and 2 were combined in pVA6 by cleavage of pVA1 and pVA2 with *Bst*XI and *Pvu*II and recloning of the ligation product. Similarly pVA7 was derived from pVA3 and pVA6: it contains the fragments 1 + 2 + 3 in the correct order. As described under Results and Discussion, the fourth subfragment was digested with *Hind*III prior to ligation into *Sma*I + *Hind*III-digested pUC19. The plasmid containing the *Eco*NI site was named pUC19-4CD. The fifth plasmid pVA5 was generated by annealing two fully complementary oligomers with sticky ends for *Hind*III and *Aat*II and cloned into *Hind*III + *Aat*II-digested pGV479. pSCMF4-5 was constructed by inserting the 36-bp *Hind*III – *Sal*I fragment from pVA5 into *Hind*III + *Sal*I-cleaved pSCMF4. Finally, pSCMFsBLA was obtained by the ligation of the following fragments: pSCMF4-5 cleaved with *Stu*I and *Hind*III, the 291-bp *Mn*II – *Eco*NI fragment from pVA7 and the 50-bp *Eco*NI – *Hind*III fragment from pUC19-4CD. The 1566-bp *Bgl*II – *Bam*HI fragment of pSCMFsBLA was inserted into the unique *Bam*HI site of pUDT2, a derivative of pUDT2HIL202 [22]. The resulting plasmid, containing the synthetic gene with the coding direction in an orientation clockwise with respect to the cytochrome *c* terminator, was named pUDTsBLA (Fig. 3).

Antibodies to bovine α -lactalbumin

Polyclonal antibodies (195B) were raised in a rabbit by injecting native bovine α -lactalbumin, purified from the commercial product (Sigma L5385) by gel filtration on Sephadex G-100. They were affinity-purified from serum pools by chromatography on bovine α -lactalbumin–Sephacrose. Monoclonal antibodies were obtained from a single fusion, using spleen cells from mice injected with the same, native bovine α -lactalbumin and the mouse myeloma Sp2/0-Ag-14 (kindly obtained from Flow through courtesy of Drs C. Milstein and H. Köhler). Three clones were selected (23/4B1, 23/4E2 and 23/5D1) for induction of ascites. From these,

purified antibodies were isolated by affinity chromatography as described above.

Purification of bovine α -lactalbumin from yeast culture media by affinity chromatography

Supernatants from yeast cultures in YPD medium, buffered with 50 mM Mes pH 7.0, were collected by centrifugation. After further addition of Tris/HCl (to ensure a pH $\geq$ 7.0) and NaN₃, 1–1.5-l aliquots were run at about 30 ml/h on single 10-ml antibody–Sephacrose columns, equipped with a 10-ml PD-10 column (Pharmacia) as a prefilter. Two types of affinity matrices were made, by coupling 40 mg of either polyclonal rabbit anti-(bovine α -lactalbumin) (195B) or monoclonal anti-(bovine α -lactalbumin) (clone 23/4B1) to 3 g CNBr-activated Sepharose (Pharmacia). After adsorption at 4°C, the columns were washed with Tris/saline pH 7.5 till negligible absorbance of the eluate. Further impurities were washed off with 1 M NaCl in Tris/saline. Elution of adsorbed α -lactalbumin was done using 20-ml pulses of 0.2 M citrate pH 3.5, followed by 0.2 M glycine pH 2.7 and, occasionally, 0.2 M glycine pH 2.0. Fractions were monitored for α -lactalbumin content by radioimmunoassay. Positive fractions were pooled, dialysed against distilled water and lyophilized. They were redissolved in 5 mM Tris/HCl pH 7.5 and their total protein concentration was determined according to Lowry et al. [23] and by absorbance at 280 nm. As some of the samples were slightly turbid, the latter data were corrected for light dispersion by extrapolation from absorbance at 320 nm.

Radioimmunoassay of bovine α -lactalbumin

The concentration of bovine α -lactalbumin in yeast culture supernatants and in chromatographic fractions was measured by an immunoradiometric technique. Flexible, round-bottom PVC microtiter plates (Falcon, Becton Dickinson) were coated using 100 µl of 10 µg/ml affinity-purified, polyclonal rabbit anti-(bovine α -lactalbumin) antibodies in 0.01 M bicarbonate pH 9.8 for overnight at 4°C. Postcoating was done with bovine serum albumin at 5 mg/ml in Tris/saline. Properly diluted 25-µl samples were incubated in the wells for 1 h at room temperature. The cups were washed with TBS, containing 0.05% Tween-20. ¹²⁵I-labeled affinity-purified anti-(bovine α -lactalbumin) antibodies were applied (25 µl of a 1-µg/ml solution, representing about 0.1 µCi). Either polyclonal rabbit (195B) or one of the three monoclonal mouse antibodies were used as detectors. After thoroughly washing, the cups were cut from the plate and ¹²⁵I measured individually in a crystal scintillation counter. Standard curves were obtained with purified bovine α -lactalbumin (see above) and calculated by linear regression analysis from logit/log curves.

Spectroscopy

Circular dichroic measurements were carried out with a Jasco J-600 spectropolarimeter using cuvettes of 1-mm or 10-mm path length in the far and near ultraviolet respectively [10]. Absorption spectra were recorded on a Beckman DU-70 spectrophotometer.

Amino-terminal sequence determination

The presence of the correct amino-terminal sequence for the recombinant bovine α -lactalbumin was confirmed, using classical Edman chemistry, in an Applied Biosystems (type 477A) gas-phase sequencer. This manipulation was kindly

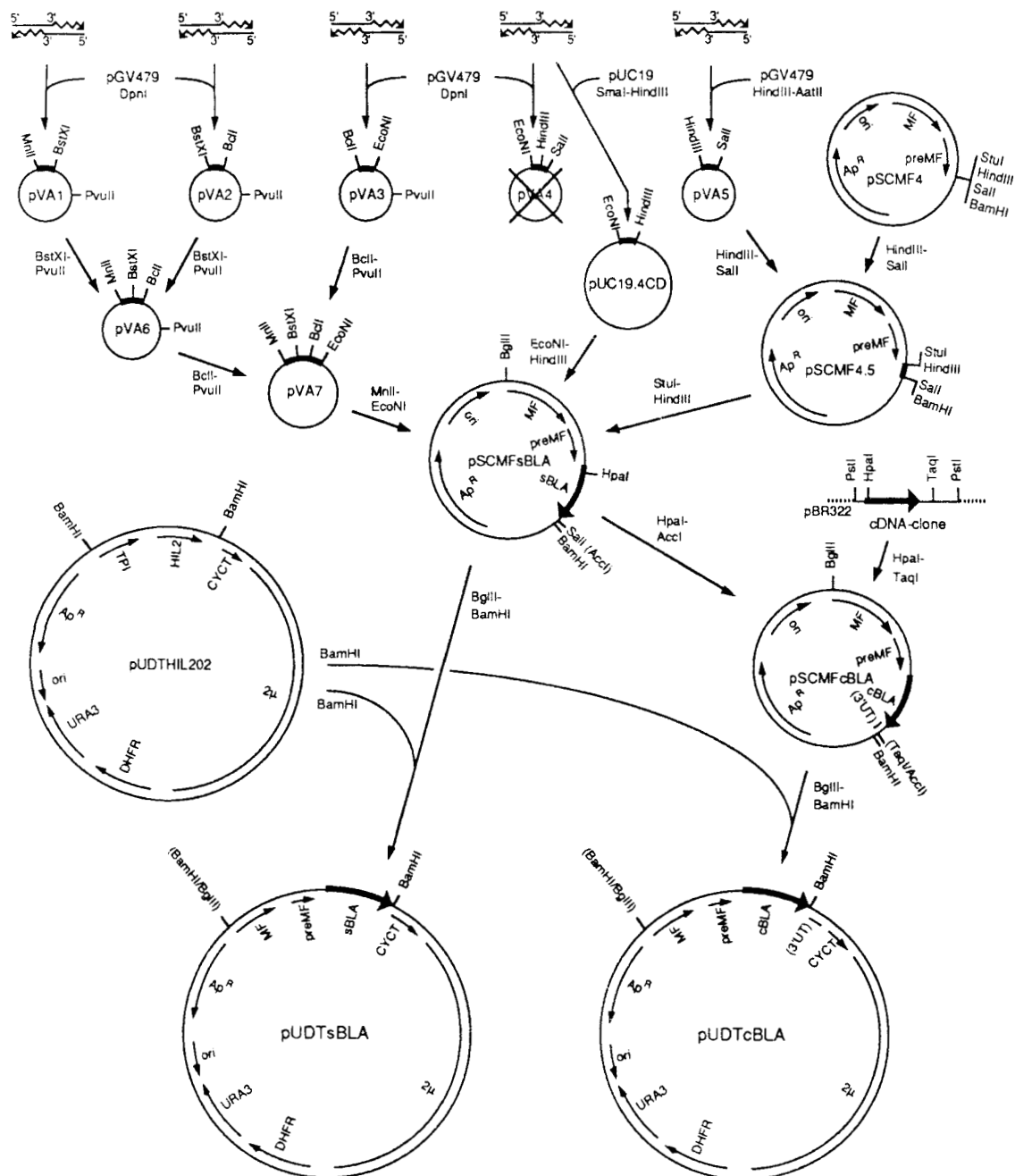


Fig. 3. Cloning strategy for the synthetic bovine α -lactalbumin gene. Each of the five subfragments was generated individually by annealing and filling-in of two partially complementary oligonucleotides, followed by ligation into *Dpn*I-cleaved pGV479. As fragment 4 failed to be inserted into pGV479, this fragment was cleaved with *Hind*III and the first part was ligated into *Sma*I + *Hind*III-digested pUC19, resulting in pUC19-4CD. A fifth fragment coding for the last part of the gene, including two stop codons, was obtained by annealing two fully complementary oligonucleotides with *Hind*III and *Aat*II sticky ends and cloning into *Hind*III + *Aat*II-cleaved pGV479. The fragments were subsequently connected in the correct order as indicated in the figure. The total reconstruction of the synthetic gene took place in pSCMF4, where the multi-linker sequence provided all the needed sites. The *S. cerevisiae* expression vector, pUDTsBLA, was constructed by substituting the *Bam*HI fragment of pUDT2HIL202 for the *Bgl*II – *Bam*HI fragment of pSCMFsBLA. The construction of the second expression vector pUDTcBLA is illustrated in the same figure, and contains the bovine α -lactalbumin coding sequence derived from the cDNA clone. Abbreviations: MF, α -mating factor promoter; preMF, α -mating factor preproregion; CYCT, cytochrome *c* terminator; 3'UT, 3' untranslated region.

carried out by Dr Jo Van Damme at the Rega Institute (K.U. Leuven, Belgium).

RESULTS AND DISCUSSION

Design and cloning of the gene

The small size of bovine α -lactalbumin (123 amino acid residues) offers the possibility to synthesize the gene chemi-

cally. This allows unique restriction recognition sites to be introduced at regular distances within the gene for further use in cassette mutagenesis. Moreover, codon usage can be adapted to an optimal bias for *S. cerevisiae*, the organism envisaged as expression host.

The reverse-translation computer program SYNVAR (G. Volckaert, unpublished) allows one to determine all possible restriction recognition sequences matching a given amino acid

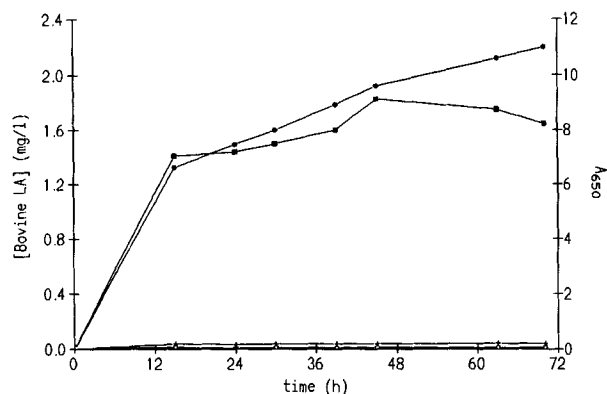


Fig. 4. Localisation of bovine α -lactalbumin (LA) in the yeast cell. *S. cerevisiae* GRF 181 carrying pUDTsBLA was first grown in uracil-selective medium to stationary phase. The culture was diluted 1:50 in fresh YPD medium (300 ml) containing 50 mM Mes pH 7 and the cells were grown at 28°C with shaking. Samples (10 ml) of cells were taken in the course of time as indicated and the supernatants were obtained by centrifugation ('medium' fractions). The pellets were washed twice with 1.2 M sorbitol and 10 mM KH_2PO_4 pH 6.8, resuspended in 2 ml 1.2 M sorbitol, 10 mM KH_2PO_4 pH 6.8, 0.6% zymolyase 100T and 0.4 mM phenylmethylsulfonyl fluoride and incubated at 30°C for 30 min. After centrifugation, the supernatants were obtained ('periplasmic space' fractions). The pellets were dissolved in 2 ml water (containing 0.4 mM phenylmethylsulfonyl fluoride) to give the spheroplasts an osmotic shock, centrifuged and the supernatants were collected ('intracellular' fraction). Bovine α -lactalbumin contents (in mg/l original culture) were measured by radioimmunoassay. (●) Cell growth ($A_{650 \text{ nm}}$); (■) bovine α -lactalbumin in the medium; (+) bovine α -lactalbumin in the periplasmic space; (○) bovine α -lactalbumin intracellular

sequence, including all six codon possibilities for Leu, Ser and Arg. A set of sites for the synthetic gene was chosen so that unique sites are available when the gene is present in pGV479. Some amino acid codons are fixed by this procedure. The remaining codons were filled in from the highest scores according to a comparison of highly expressed genes in yeast [24] (Fig. 2).

Assembly of the gene was planned with four overlapping fragments. Each pair of adjacent subfragments shared a unique restriction site in the overlapping region. Cleavage at this site and a second cleavage at a unique restriction recognition site in the vector part allowed recombination of the subfragments to reconstitute the whole gene. Each subfragment was generated individually and cloned into the unique *DpnI* site of pGV479. By cloning in this site, and recleavage with *MnII*, the same blunt-ended fragment can be recovered as was inserted in the *DpnI* site in the first place (Fig. 1). In this way, the first amino acid of the synthetic bovine α -lactalbumin gene was made available for subsequent cloning.

Unexpectedly, the fourth subfragment (starting two nucleotides in front of the *EcoNI* site and spanning the end of the coding region) failed to be cloned into pGV479. Therefore, we digested this subfragment with *HindIII* (an internal site of this fragment) and cloned the subsubfragments into *SamI* + *HindIII*-digested pUC19. The larger one (containing the *EcoNI* site: pUC19-4CD) was used for subsequent manipulation. This means that the cloning strategy was slightly modified and a fifth subfragment had to be synthesized to complete the gene. The final reconstruction of the synthetic gene was done with vector pSCMF4 by recloning of relevant fragments in the correct order (Fig. 3).

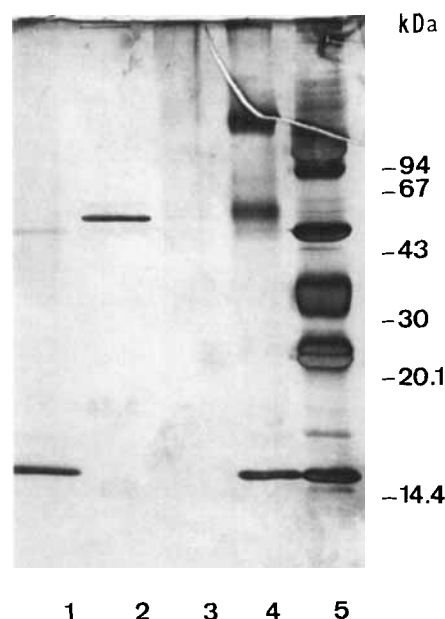


Fig. 5. SDS/PAGE of recombinant bovine α -lactalbumin. The protein was purified from yeast growth medium by affinity chromatography on a Sepharose column to which either the mouse monoclonal (lanes 1 and 2) or the rabbit polyclonal antibodies (lanes 3 and 4) were bound. The fractions were collected and run on a 5–15% acrylamide gradient gel. Lanes 1 and 3, fractions eluted at pH 3.5; lanes 2 and 4, fractions eluted at pH 2.7; lane 5, molecular mass markers (from bottom: α -lactalbumin, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase b). The gel was silver-stained

Expression of bovine α -lactalbumin

A multicopy plasmid pUDTsBLA was constructed to direct the synthesis and secretion of bovine α -lactalbumin in yeast. This plasmid contains the α -mating factor preproregion fused to the synthetic gene coding for α -lactalbumin. Expression of the α -lactalbumin gene takes place under the control of the α -mating factor promoter; the transcription termination signal was provided by the *CYC1* terminator.

The yeast strain GRF181 transformed with this plasmid was grown in uracil-selective medium and the amount of bovine α -lactalbumin secreted in the culture medium was measured by radioimmunoassay, using polyclonal antibodies raised against commercially available bovine α -lactalbumin. Initially no α -lactalbumin could be detected in the medium. A possible explanation for this observation could be a conformational change of the protein due to the low pH (≤ 3), since yeast is known to acidify the culture medium. At this low pH, α -lactalbumin probably appears in its unfolded conformation, showing increased hydrophobicity [25] and sticking to the yeast cell wall. Indeed, we found that α -lactalbumin, added to a culture of non-producing cells, sticks to the cells presumably in its calcium-free hydrophobic form. Nevertheless, 50–60% of the added protein could be recovered by titrating the culture to neutral pH. If the yeast cells were grown under conditions of controlled neutral pH (50 mM Mes pH 7), the α -lactalbumin added to the culture remained soluble. Subsequently, all growth media were supplemented with this buffer. Under these conditions, production levels of 0.5 mg/l of secreted α -lactalbumin were obtained. An increase in production was noticed by growing the cells in YPD medium; expression levels of up to 2 mg/l were noted.

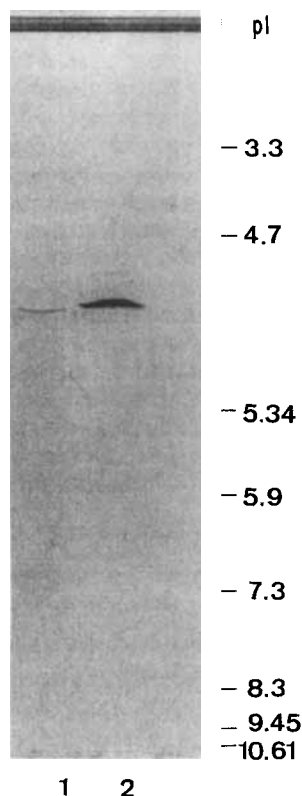


Fig. 6. IEF of recombinant bovine α -lactalbumin. IEF was performed on Servalyte pre-coats, carrying ampholines with pI in the range 3–10, and stained with Coomassie brilliant blue. Lane 1, recombinant bovine α -lactalbumin; lane 2, commercial bovine α -lactalbumin. Proteins with reference pI values used were, from bottom: cytochrome c, ribonuclease, horse myoglobin, whale myoglobin, conalbumin, β -lactoglobulin, serum albumin and amyloglucosidase

As shown in Fig. 4, the expression of the α -lactalbumin gene directed by the α -factor promoter occurs in the log phase during cell growth and does not increase during stationary phase. Also secretion seems to be very efficient, since almost no α -lactalbumin was found in the cytoplasm and periplasmic space at different moments during the growth of the culture.

Purification and characterization of recombinant α -lactalbumin

Secreted α -lactalbumin was purified by affinity chromatography using either polyclonal rabbit or monoclonal mouse anti-(bovine α -lactalbumin) antibodies (as described in Experimental Procedures) and subjected to SDS/PAGE. As shown in Fig. 5 (lanes 1 and 4), some of the acid-eluted fractions contain α -lactalbumin as the major component. The antigen is more easily eluted (at higher pH) from the monoclonal than from the polyclonal antibody. The additional bands, observed in the more acidic fractions, probably result from leakage of the antibodies from the solid matrix. All further studies were done with recombinant α -lactalbumin eluted at pH 3.5 (lane 1) from the affinity column loaded with monoclonal antibodies. This fraction shows a single band on IEF co-migrating with the commercial product (Sigma) (Fig. 6). Both electrophoretic results demonstrate the identity of the recombinant protein with the natural product.

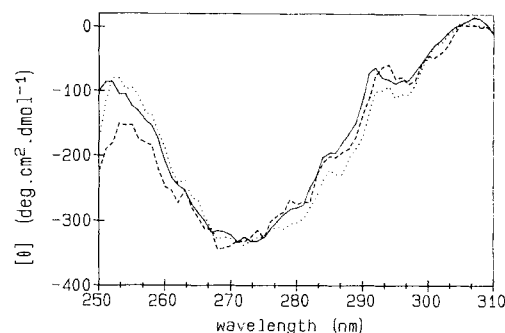


Fig. 7. Circular-dichroic spectra of bovine α -lactalbumin preparations purified from yeast culture supernatant. Spectra were expressed as residual ellipticity $[\theta]$ as a function of wavelength. Only the 'aromatic' region is shown. Spectra obtained for α -lactalbumin prepared using the cDNA (—) or the synthetic gene (···) were normalized at 271 nm to the reference spectrum of bovine α -lactalbumin (Sigma; ---). All three spectra are indicative of the Ca^{2+} form

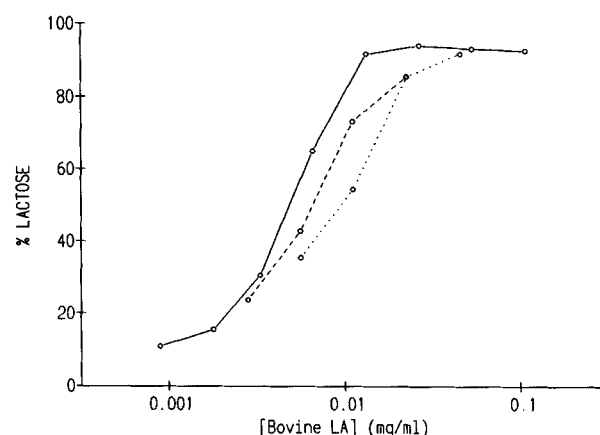


Fig. 8. Lactose synthesis by β -galactosyltransferase induced by bovine α -lactalbumin (LA) fractions purified from yeast culture supernatant. Lactose formed is expressed as a percentage of total $[^3\text{H}]\text{UDP-galactose}$ added. The protein fractions are the same as those in Fig. 5: α -lactalbumin prepared from the synthetic gene and purified on monoclonal antibody 23/4B1 (---) corresponds to lane 1 on the gel and that purified on polyclonal antibody 195B (···) corresponds to lane 4. Bovine α -lactalbumin (Sigma) was used as a reference (—). Protein concentrations were calculated from RIA measurements

When subjected to Edman degradation, recombinant α -lactalbumin shows an N-terminal amino acid sequence identical to the one expected from the DNA sequence, confirming the correct cleavage by yeast of the α -mating prepropeptide.

Circular dichroic analysis

In the presence of Ca^{2+} , bovine α -lactalbumin shows a very specific CD spectrum in the 270-nm range, due to a peculiar tertiary structure induced by Ca^{2+} binding [26]. Therefore, the affinity-purified α -lactalbumin (Fig. 5, lane 1) was subjected to CD spectral scanning in the wavelength range 250–310 nm. For this experiment, the CD spectrum of similarly purified recombinant α -lactalbumin, but obtained by expressing the cDNA gene (Fig. 3; pUDTcBLA), has been included. When compared with commercial α -lactalbumin, both recombinant proteins display the characteristic features of the Ca^{2+} -binding conformation with troughs of negative ellipticity at 270 nm and 295 nm (Fig. 7).

Enzymatic modulating activity

We also checked whether the affinity-purified bovine α -lactalbumin possessed the modulatory properties displayed by its natural counterpart in the lactose synthase complex. Serial dilutions of each of the main α -lactalbumin fractions were incubated with commercially available bovine β -galactosyltransferase, and [^{3} H]lactose synthesis was measured. Plots of lactose-synthesis-inducing capacity were constructed (Fig. 8) as a function of α -lactalbumin concentration: the determination of the recombinant α -lactalbumin concentration was done by RIA measurements. These results show that, similarly to the biological function of the natural protein, recombinant α -lactalbumin is capable of modulating the activity of β -galactosyltransferase, resulting in the synthesis of lactose.

CONCLUSION

Using *Saccharomyces cerevisiae* as a host, bovine α -lactalbumin was expressed and secreted into the culture medium under the control, respectively, of the α -mating type promotor and its prepropeptide sequence. Using a synthetic gene, up to 2 mg/l α -lactalbumin could be detected in the culture supernatant. More than 95% of the total production was found in the medium, illustrating a very efficient secretion directed by the α -mating factor preproregion [27]. Bovine α -lactalbumin in the medium could only be detected if special precautions were taken concerning the pH of the culture medium. This had to be buffered at pH 7, as below pH 5.5 no soluble α -lactalbumin could be detected.

Vilotte et al. [28] isolated the cDNA coding for bovine α -lactalbumin. In the course of this work they provided us with this material and we constructed a second secretion vector (pUDTcBLA), containing the cDNA gene under identical control as the synthetic gene in pUDTsBLA (see Fig. 3). The α -lactalbumin levels in the medium obtained from both coding sequences, were the same. It seems that the codon usage in the synthetic gene, that was based on an optimal bias for *S. cerevisiae*, had no decisive effect on the production [29].

Bovine α -lactalbumin produced by yeast can easily be purified from the growth medium through affinity chromatography, yielding a product which is enzymatically active and in all respects identical to the whey protein, as confirmed by SDS/PAGE, IEF, ultraviolet and CD spectral analysis. Moreover, no differences were found between the proteins obtained from both coding sequences. This production method is in contrast with the production in *E. coli* [13, 15] where the bovine α -lactalbumin has to be recovered from inclusion bodies by denaturation and renaturation.

At the same time as this paper was submitted, Takeda et al. [30] reported the production of goat α -lactalbumin by yeast. However, the production levels obtained by these authors are very low (< 0.1 mg/l) and only a few transformants express the protein. In our hands, no such expression instability was observed: all transformants secrete bovine α -lactalbumin. The discrepancy between their results and ours could be explained by the difference in culture conditions used to express α -lactalbumin which, as stated above, are critical for the conformation and stability of the protein. However, an undetected difference between goat and bovine α -lactalbumin cannot be ruled out.

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