Expression and Secretion of Bovine β -Lactoglobulin in *Saccharomyces cerevisiae*

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A bovine β -lactoglobulin (β -LG) was expressed in *Saccharomyces cerevisiae* carrying bovine pre- β -LG cDNA and secreted into its growth medium. The expression plasmid was constructed by inserting the whole coding region of the cDNA encoding pre- β -LG between the promoter and terminator of the yeast glyceraldehyde 3-phosphate dehydrogenase gene of pYG100, a yeast expression vector. In the supernatant of the yeast growth medium, β -LG with a native conformation was detected by sandwich ELISA, and its amount was estimated to be 1.1 mg/l. A Western-immunoblotting analysis revealed that β -LG secreted in the growth medium had the same mobility as that of authentic bovine β -LG. The N-terminal sequence was also identical with that of authentic mature bovine β -LG.

β -Lactoglobulin (β -LG) is a major whey protein found in the milk of ruminants, and has also been identified in the milk of horses, dogs and pigs.¹⁾ β -LG from ruminants exists as stable dimers of two identical subunits of 162 amino acid residues, each with a molecular weight of *c.* 18000 and two disulfide bonds. The biological function of β -LG is still not clear but is thought to be for binding and transporting retinol to the neonate, because of its homology in tertiary structure to the retinol binding protein revealed by an X-ray crystallographic study.²⁾ Recent studies have also suggested that β -LG is a member of a protein superfamily, whose function is binding and/or transporting small hydrophobic molecules such as retinol, cholesterol esters and billins.³⁾

Bovine β -LG has been used as a model for physicochemical studies of protein. We have previously reported the local conformational change or denaturation process of a β -LG molecule from heat denaturation, which was

revealed by using five monoclonal antibodies (McAb) against bovine β -LG as probes.⁴⁾

β -LG is also a major allergen in bovine milk. The antibody binding sites and T-cell recognition sites in β -LG have been partly identified by using chemically modified protein or its enzymatic digests.⁵⁾

Protein engineering techniques such as site-directed mutagenesis would give more detailed information on the relationship between the structure of β -LG and its functions. However, no attempt has been so far made to produce mutant proteins of β -LG. To determine the role of individual residues in the various functions and physicochemical characteristics of β -LG, we cloned and sequenced the cDNA for bovine β -LG, and constructed its secretion system in *Saccharomyces cerevisiae*.

Materials and Methods

Construction of a bovine mammary gland cDNA library. Bovine mammary gland total RNAs were isolated from a

mammary gland by the guanidine thiocyanate method of Okayama *et al.*⁶⁾ Poly (A)⁺ RNAs were purified from total RNAs by chromatography on oligo (dT)-cellulose, using an mRNA purification kit (Pharmacia). Double-strand cDNAs (ds-cDNA) were synthesized from purified poly (A)⁺ RNAs with cDNA synthesis kit (Pharmacia) according to the manufacturer's manual, which is based on the method of Gubler and Hoffman.⁷⁾ An *EcoRI* adapter, AATTCGGCACGAG, was ligated to each end GCCGTGCTCp

of the blunt-ended ds-cDNAs. The cDNAs with *EcoRI* adapters were then inserted into the *EcoRI* site of pUC118, and the recombinant plasmids were used to transform *E. coli* DH5 α according to the procedure of Hanahan.⁸⁾

Cloning and sequencing of bovine pre- β -LG cDNA. cDNAs encoding pre- β -LG were selected by colony hybridization, using two ³²P-end-labeled oligodeoxyribonucleotide probes, 5'-CAGAAATGGGAGAACGG-3' A G G A T

and 5'-ACCCAAACCATGAAGG-3', corresponding to T G T A

⁵⁹Gln-⁶⁴Gly and ⁴Thr-⁹Gly, respectively. Among the 7 positive clones, the plasmid which had the longest cDNA insert was identified and designated as pBB29. The nucleotide sequence of the cDNA insert of pBB29 was determined by the dideoxynucleotide chain termination method,⁹⁾ using recombinant pUC118 plasmids as templates.¹⁰⁾ A series of pUC118 subclones containing deleted cDNA inserts was prepared by a deletion kit for kilo-sequencing (Takara Shuzo), and a pUC118 subclone containing the *SmaI* fragment of pBB29 was also prepared. Four oligodeoxyribonucleotides (17 mer) used as primers for sequencing were synthesized by MilliGen.

Construction of the expression plasmid for bovine β -LG in yeast. pBB29 was digested to completion with *SacI* and *SmaI*, and the ends of the cDNA fragment were blunted with T4 DNA polymerase. The blunt-ended cDNA fragments were then inserted into the *SaII* site situated between the yeast glyceraldehyde 3-phosphate dehydrogenase (GAPD) promoter and the terminator of pYG100¹¹⁾ (Fig. 2). The yeast expression vector pYG100 is the YE_p-type shuttle vector between *E. coli* and yeast.

The resulting pYBSS1 expression plasmid was introduced into *S. cerevisiae* AH22 (*a, leu2, his4, can1*) by the lithium acetate method.¹²⁾ Leu⁺ transformants were selected and grown at 30°C by shaking for three days in a yeast minimal medium (YMM) supplemented with histidine (20 μ g/ml).

Sandwich ELISA. After the yeast growth medium had been centrifuged, its supernatant was filtrated and serially diluted with phosphate-buffered saline containing 0.05% Tween 20. β -LG in the supernatant was detected by two systems of sandwich ELISA,¹³⁾ which were established by Enomoto *et al.* These detected the native and denatured β -LG in sera of mice fed on whey-protein isolate diet

(manuscript in preparation). In brief, native β -LG was detected by sandwich ELISA, using anti-bovine β -LG McAb 62A6⁴⁾ and alkaline phosphatase (Sigma)-labeled anti-bovine β -LG McAb 61C1⁴⁾ (62A6/61C1-Alp), with which reduced carboxymethylated (denatured) bovine β -LG (RCM- β -LG) could not be detected. The denatured β -LG was detected with the other system of sandwich ELISA, using anti-bovine β -LG McAb 21B3^{4,14)} and alkaline phosphatase-labeled anti-bovine β -LG McAb 31A4⁴⁾ (21B3/31A4-Alp), which could not detect native β -LG. The amounts of native and denatured β -LG secreted into the growth medium were estimated from a standard curve drawn by plotting the ELISA value against the log-concentration of native bovine β -LG or RCM- β -LG. The detection sensitivity of these systems was 10⁻⁵ to 10⁻⁶ g/l of native β -LG for the 62A6/61C1-Alp system, and 10⁻⁵ to 10⁻⁶ g/l of RCM- β -LG for the 21B3/31A4-Alp system.

Bovine β -LG, RCM- β -LG and McAbs were prepared as described previously.⁴⁾

Western-immunoblotting analysis. The supernatant of the yeast growth medium (YMM) was concentrated by ultrafiltration (PTGC OLC M2; mol. wt. cutoff, 10,000; Millipore) and then dialyzed. After lyophilizing, the lyophilizate was applied to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) performed on a 10–20% gradient gel by the method of Laemmli.¹⁵⁾ The gel was subjected to electroblotting to a PVDF membrane (Immobilon, Millipore), which was followed by an immunological analysis, using anti-bovine β -LG McAbs 62A6 and 31A4⁴⁾ as primary antibodies and a blotting detection kit for mouse IgG (Amersham).

N-Terminal amino acid sequence analysis. The lyophilized supernatant of the yeast growth medium was dissolved in 50 mM imidazole-HCl (pH 6.7) buffer and applied to a DEAE-Sephacel (Pharmacia) column. β -LG was eluted with a linear gradient of 0 to 0.5 M NaCl in 50 mM imidazole-HCl (pH 6.7) buffer. The partially purified recombinant β -LG was desalted by gel filtration on a prepacked Sephadex G-25 column (PD-10, Pharmacia) and then lyophilized. The lyophilizate was applied to SDS-PAGE, which was performed on an 8% gel by the method of Laemmli¹⁵⁾ and electroblotted to a PVDF membrane. About 30 μ g of authentic or recombinant β -LG on the membrane was applied to an automated Edman degradation gas-phase protein sequencer (Model PSQ-1, Shimadzu).

Results

Molecular cloning and nucleotide sequence of bovine pre- β -LG cDNA

cDNAs were synthesized from poly(A)⁺

RNAs, which were purified from bovine mammary gland total RNAs, by the method of Gubler and Hoffman. cDNAs with *EcoRI*

Fig. 1. Restriction Enzyme Cleavage Map of the Bovine Pre- β -lactoglobulin cDNA Insert of pBB29.

The solid, hatched and open boxes represent the coding region for the signal peptide, the coding region for mature protein of bovine β -lactoglobulin, and the 5',3'-noncoding region, respectively. The bold and thin lines represent the *EcoRI* adapter and pUC118, respectively.

adapters were inserted into the *EcoRI* site of pUC118 and introduced into *E. coli* DH5 α . The cDNA library was screened by clony hybridization with two ^{32}P -end-labeled oligomers. Among the 7 positive clones, the plasmid which had the longest bovine pre- β -LG cDNA insert was identified and designated as pBB29. The nucleotide sequence of the cDNA insert of pBB29 was determined by the dideoxy sequencing method. The restriction enzyme map of the bovine pre- β -LG cDNA insert of pBB29 is shown in Fig. 1. The cDNA was 773 nucleotides long, and had the 534bp coding region flanked by 27bp of 5' and 212bp of 3'-noncoding region.

By comparing the nucleotide sequence of

Fig. 2. Construction of the Bovine β -Lactoglobulin Expression Plasmid in Yeast, pYBSS1.

The construction of the expression plasmid was performed as described in the Materials and Methods section. The solid and open arrows represent the promoter and terminator of the yeast glyceraldehyde 3-phosphate dehydrogenase (GAPD) gene. The hatched region and the solid box in the hatched region represent the bovine pre- β -lactoglobulin cDNA and its coding region, respectively. The direction of the protein coding region of cDNA is indicated by the thin arrow. Amp^r, ampicillin resistance gene; *LEU2*, *LEU2* gene of *S. cerevisiae*; 2 μ ori, replication origin of 2 μ m DNA.

the cDNA obtained in this study with the sequences reported previously by three groups,^{16–18)} our sequence was identical with the sequence reported by Alexander *et al.*,¹⁸⁾ except a difference at position 406 on the sequence reported by Alexander *et al.*, where we found T instead of G and the lack of 18 bp at the 5'-end in our cDNA. The amino acid sequence deduced from our cDNA sequence agrees precisely with the sequence of bovine β -LG genetic variant A determined by a protein sequence analysis,¹⁹⁾ although the substitution at position 406 caused an amino acid substitution (¹⁰⁵Phe in our sequence instead of ¹⁰⁵Val in that of Alexander *et al.*¹⁸⁾).

Expression of bovine β -LG in yeast

The expression plasmid for bovine β -LG in yeast, pYBSS1, was constructed as shown in Fig. 2. pYBSS1 carries the whole coding region of the cDNA (*Sac*I–*Sma*I fragment of pBB29) between the promoter and terminator of the yeast GAPD gene. pYBSS1 was introduced into *S. cerevisiae* AH22 by the lithium acetate method, and Leu⁺ transformants were grown at 30°C by shaking for three days in YMM supplemented with histidine.

The recombinant β -LG secreted into the growth medium was detected by two systems of sandwich ELISA, with which native and denatured β -LG could be identified (described in the materials and methods section). Immunoreactive molecules were detected in the supernatant of the growth medium of yeast harboring pYBSS1 by 62A6/61C1-Alp sandwich ELISA. The amount was estimated to be 1.1 mg/l of native β -LG, using native bovine β -LG as a standard (Table I). Denatured β -LG, even in a supernatant concentrated 10-fold by ultrafiltration, was below the detection sensitivity (10^{-5} to 10^{-6} g/l) for 21B3/31B4-Alp sandwich ELISA (Table I). No immunoreactive substance was detected with either system in the supernatant of the growth medium of yeast harboring pYG100 (Table I). These results show that the denatured β -LG in the supernatant was, if any, less than 0.1% of

Table I. LEVELS OF NATIVE AND DENATURED β -LACTOGLOBULIN (β -LG) IN THE SUPERNATANT OF THE YEAST CULTURE

	β -LG (mg per liter culture)		
	Plasmids		
McAbs ^a	pYBSS1	pYG100	
62A6/61C1-Alp	1.1 ^b	N.D.	
21B3/31A4-Alp	N.D. ^c	N.D.	

Sandwich ELISA was performed as follows: 1, coat the wells of a polystyrene microtiter plate with McAbs 62A6 or 21B3; 2, add the diluted (or concentrated) supernatant containing β -lactoglobulin, which binds to McAbs on the plate; 3, add the solution containing the alkaline phosphatase-labeled McAbs 61C1-Alp or 31A4-Alp to be bound to β -lactoglobulin combined with McAb on the plate; 4, add a 0.1% solution of *p*-nitrophenylphosphate disodium salt, the substrate of alkaline phosphatase; 5, measure the absorbance of each well at 405 nm.

N.D., not detected.

^a Monoclonal antibodies used for sandwich ELISA.

^b The amount of β -lactoglobulin was estimated from the standard curve of native bovine β -lactoglobulin.

^c The supernatant concentrated 10-fold by ultrafiltration was assayed.

Fig. 3. Western-immunoblotting Analysis of the Yeast Culture Supernatants.

Lane A, 200 ng of the lyophilized supernatant of *S. cerevisiae* AH22 harboring pYBSS1, the bovine β -lactoglobulin expression plasmid; lane B, 20 ng of authentic bovine β -lactoglobulin; lane C, 5 ng of authentic bovine β -lactoglobulin; lane D, 200 ng of the lyophilized supernatant of *S. cerevisiae* AH22 harboring pYG100.

Table II. N-TERMINAL AMINO ACID SEQUENCE OF AUTHENTIC AND RECOMBINANT β -LACTOGLOBULIN

Authentic bovine β -LG	¹ Leu-Ile-Val-Thr- ⁵ Gln-Thr-Met-Lys-Gly- ¹⁰ Leu-Asp-Ile-Gln-Lys- ¹⁵ Val-Ala-Gly-
Recombinant β -LG	¹ Leu-Ile-Val-(Thr)- ⁵ Gln-Thr-Met-Lys-Gly- ¹⁰ Leu-Asp-Ile-Gln-Lys- ¹⁵ Val-Ala-Gly-

The concentrated supernatant of a culture medium of yeast harboring pYBSS1 was applied to a DEAE-Sephacel column and eluted with a linear gradient of 0 to 0.5 M NaCl in 50 mM imidazole-HCl (pH 6.7) buffer. The fractions containing β -lactoglobulin were desalted and lyophilized. The lyophilizate and authentic bovine β -lactoglobulin were applied to SDS-PAGE and electroblotted to a PVDF membrane. Authentic and recombinant β -lactoglobulin on the membrane were applied to a gas-phase protein sequencer.

The fourth residue of the recombinant β -lactoglobulin in parentheses is identified as "Thr"; however, "Tyr" was also detected with almost equal intensity of the signal.

β -LG with a native conformation. Therefore, most of the recombinant β -LG molecules obtained in this study are supposed to have a conformation similar to native β -LG, although the detailed conformation remains to be elucidated.

To determine the molecular size of the recombinant β -LG, SDS-PAGE and a Western-immunoblotting analysis were performed (Fig. 3). Authentic bovine β -LG and lyophilized supernatants of the growth media of yeast harboring pYBSS1 or pYG100 were subjected to SDS-PAGE and electroblotted to a membrane. After immuno-staining, a polypeptide co-migrating with authentic bovine β -LG was detected as a single band in a dialyzed and lyophilized supernatant of the growth medium of yeast harboring pYBSS1 (lane A, Fig. 3). No band was detected in the supernatant of yeast harboring pYG100 (lane D, Fig. 3). This result shows that the recombinant β -LG had the same molecular weight as authentic β -LG, and suggests that this molecule was not glycosylated.

To confirm that the recombinant pre- β -LG containing 16 amino acid residues of signal peptide had been processed correctly in the yeast, an N-terminal amino acid sequence analysis was performed. The N-terminal amino acid sequences of authentic and recombinant β -LG determined by a gas-phase protein sequencer are shown in Table II. Although the fourth residue was only tentatively identified as Thr because of putative background noise, the N-terminal sequence of the recombinant

β -LG agreed with that of authentic mature bovine β -LG. This indicates the correct processing of bovine pre- β -LG to mature β -LG during the secretion of the protein in yeast.

Discussion

The full length or partial sequences of cDNA for bovine β -LG have been reported by three groups.¹⁶⁻¹⁸⁾ The sequences, however, differed significantly from one another, especially in the 3'-noncoding region, and it is not clear what caused the difference. Alexander *et al.*¹⁸⁾ pointed out that the major differences were all deletions, which might have resulted from cloning/sequencing artifacts. They also claimed that their sequence was correct as it was identical with the corresponding segment of the gene, and was similar to ovine β -LG cDNA. This claim is supported by our cDNA sequence, which was almost identical to the sequence reported by Alexander *et al.*¹⁸⁾

Simons *et al.* reported the expression and secretion of ovine β -LG into the milk of transgenic mice.²⁰⁾ The concentration of ovine β -LG in the milk of a transgenic mouse was estimated to be up to 23 mg/ml. A large amount of β -LG may be obtained in the milk of transgenic animals, but transgenic animals are less efficient for producing many different mutant proteins than microorganisms, because of the large effort needed for obtaining transgenic animals. Many heterologous proteins produced in *E. coli* often form inclusion bodies, and in order to obtain correctly folded

molecules, it is necessary to solubilize the inclusion body with denaturants and then to dialyze for refolding. It was revealed by the experiments using McAbs as probes that completely denatured β -LG recovered its retinol binding activity and rough tertiary structure through refolding, but that the local conformation did not return to that of the native protein (Hattori *et al.*, manuscript in preparation). So a β -LG molecule produced by *E. coli* would not have a conformation exactly identical to the native β -LG required for studying the roles of individual residues in protein folding or the antigenic properties of β -LG.

We chose the yeast secretion system for expressing β -LG cDNA. Many eukaryotic secretory proteins with their own signal peptides expressed in yeast such as human interferon α^{21} and hen egg white lysozyme²² have been processed correctly and secreted into a growth medium and being folded correctly. The recombinant pre- β -LG with 16 amino acid residues of its own signal peptide was also processed correctly. The mature protein had the same molecular weight as authentic bovine β -LG, and is supposed to have a similar conformation to that of native β -LG. The mutant proteins derived from the recombinant β -LG would be useful for an advanced study of protein folding or of the relationships between structure and function such as retinol binding activity and allergenicity.

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References

- 1) R. Jenness, "Developments in Dairy Chemistry," Vol. 1, ed. by P. F. Fox, Applied Science Publishers, Barking, Essex, 1983, pp. 87-114.
- 2) M. Z. Papiz, L. Sawyer, E. E. Eliopoulos, A. C. T. North, J. B. C. Findlay, R. Sivaprasadarao, T. A. Jones, M. E. Newcomer and P. J. Kraulis, *Nature*, **324**, 383 (1986).
- 3) J. Godovac-Zimmermann, *Trends in Biochem. Sci.*, **13**, 64 (1988).
- 4) S. Kaminogawa, M. Shimizu, A. Ametani, M. Hattori, O. Ando, S. Hachimura, Y. Nakamura, M. Totsuka and K. Yamauchi, *Biochim. Biophys. Acta*, **998**, 50 (1989).
- 5) T. Takahashi, S. Kaminogawa, T. Kuwata, O. Ando and K. Yamauchi, *Agric. Biol. Chem.*, **52**, 2485 (1988).
- 6) H. Okayama, M. Kawaichi, M. Brownstein, F. Lee, T. Yokota and K. Arai, *Methods in Enzymol.*, **154**, 3 (1987).
- 7) U. Gubler and B. J. Hoffman, *Gene*, **25**, 263 (1983).
- 8) D. Hanahan, "DNA Cloning," Vol. I, IRL Press, Oxford, 1987, pp. 109-135.
- 9) F. Sanger, S. Nicklen and A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 5463 (1977).
- 10) M. Hattori and Y. Sakaki, *Annal. Biochem.*, **152**, 232 (1983).
- 11) I. Kumagai and K. Miura, *J. Biochem.*, **105**, 946 (1989).
- 12) H. Ito, Y. Fukuda, K. Murata and A. Kimura, *J. Bacteriol.*, **153**, 163 (1983).
- 13) C. Blake and B. J. Gould, *Analyst*, **109**, 533 (1984).
- 14) S. Kaminogawa, M. Hattori, O. Ando, J. Kurisaki and K. Yamauchi, *Agric. Biol. Chem.*, **51**, 797 (1987).
- 15) U. K. Laemmli, *Nature*, **227**, 680 (1970).
- 16) A. C. Jamieson, M. A. Vandeyar, Y. C. Kang, J. E. Kinsella and C. A. Batt, *Gene*, **61**, 85 (1987).
- 17) V. N. Ivanov, E. S. Judinkova and S. I. Gorodetsky, *Biol. Chem. Hoppe-Seyler*, **369**, 425 (1988).
- 18) L. J. Alexander, G. Hayes, M. J. Pearse, C. W. Beattie, A. F. Stewart, I. M. Willis and A. G. Mackinlay, *Nucl. Acids Res.*, **17**, 6739 (1989).
- 19) G. Braunitzer, R. Chen, B. Schrank and A. Stangl, *Hoppe-Seyler's Z. Physiol. Chem.*, **354**, 867 (1973).
- 20) J. P. Simons, M. McClenaghan and A. J. Clark, *Nature*, **328**, 530 (1987).
- 21) R. A. Hitzeman, D. W. Leung, L. J. Perry, W. J. Kohr, H. L. Levine and D. V. Goeddel, *Science*, **219**, 620 (1983).
- 22) I. Kumagai, S. Kojima, E. Tamaki and K. Miura, *J. Biochem.*, **102**, 733 (1987).