

Production and Epitopic Characterization of Monoclonal Antibodies Against Bovine β -Lactoglobulin

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ABSTRACT

Sixty-nine murine monoclonal antibodies were produced against the bovine β -lactoglobulin (β -LG) variant B. Thirty-eight specificity groups of monoclonal antibodies were defined according to the reactivity in indirect or capture ELISA with bovine β -LG B, for bovine β -LG B coated at pH ranging from 2.3 to 9.4, and for β -LG with naturally occurring residue substitutions such as bovine β -LG variant A and ovine, caprine, porcine, and equine β -LG. Ten monoclonal antibodies appeared to be monospecific for bovine β -LG, 58 monoclonal antibodies recognized only ruminants β -LG, and 1 recognized all species. The specificity of the monoclonal antibodies was also studied with sequenced tryptic peptides of β -LG variant B. Critical residues involved in the epitopes of nine classes of monoclonal antibodies were characterized. Moreover, 5 monoclonal antibodies were able to discriminate between a fresh solution of β -LG and a solution that had been stored for ≥ 2 d at 4°C, and 3 monoclonal antibodies distinguished bovine β -LG that has been purified by gel filtration from that purified by salt fractionation. This array of monoclonal antibodies could be efficient probes for characterizing conformational structures involved in biological properties of β -LG (e.g., interaction with ligands and hypersensitivity reactions) and for studying conformational changes occurring upon physical treatments such as heat denaturation.

(**Key words:** β -lactoglobulin, monoclonal antibodies, epitopes, tryptic fragments)

Abbreviation key: MAb = monoclonal antibody,
PBS-Tween = PBS containing 0.02% Tween 20.

INTRODUCTION

When proteins possess excellent nutritional quality as well as various functional properties (emulsifying,

foaming, and gelling) and, therefore, are widely used as functional ingredients in many formulated bakery, dairy, and sausage products (19). These qualities are essentially related to β -LG, which is the most abundant protein of bovine whey. Except for rodents and humans, the presence of β -LG has been demonstrated in several mammals. In addition to its commercial utility, β -LG is considered to be a major allergen in infantile hypersensitivity to bovine milk (41, 43).

Numerous procedures have been designed to modify specifically the functional or allergenic properties of β -LG by various enzymatic, chemical, or physical treatments (19). To optimize processing procedures, a better fundamental understanding of the structural changes involved is required. Previously, physical techniques have been mostly used, including surface polarity measurements (36), surface hydrophobicity measurements (13, 18), fluorescence spectra (14, 36), differential scanning calorimetry (5, 37), circular dichroism (38), optical rotatory dispersion (40), or infrared spectroscopy (10). Such an approach depicts only the overall structural changes of the protein and requires the use of purified protein solutions but does not consider the extensive interactions that may occur with other milk components. In contrast, protein purification is not a prerequisite to immunochemical methods. Thus, those methods provide the opportunity to study the conformational changes of proteins in complex media such as milk products. Some authors (21, 30, 33) have used polyclonal antibodies. However, that approach is relatively unsatisfactory because of the lack of convincing interpretation of antigen-antibody interaction involving polyclonal antisera. Hybridoma technology now yields homogeneous, monospecific antibodies, eliminating the problem of interpretation that is related to the heterogeneity of polyclonal antibodies. Consequently, monoclonal antibodies (MAb) may be powerful tools for protein conformation analysis. The MAb allow determination of the antigenic structure at a level that is not previously possible (11). However, the structural analysis of β -LG has been limited by the small number of MAb produced.

Received May 31, 1996.

Accepted February 24, 1997.

Kaminogawa et al. (16, 17) raised 5 MAb against β -LG and identified two epitopes on tryptic and chymotryptic peptides. Also, Kuzmanoff and Beattie (22) reported the production and characterization of 6 MAb.

This paper reports the production of 69 MAb against bovine β -LG and their initial characterization. The critical residues involved in the epitopes recognized by some of them were determined using two ELISA procedures, tryptic peptides of defined sequence, and β -LG from species that are closely related to beef cattle (sheep and goat) and β -LG from less related species (e.g., horse and pig) in which varying degrees of substitutions occur.

MATERIALS AND METHODS

Milk and Whey Samples

Equine colostrum, bulk bovine milk (genotype BB or AA for β -LG), and bulk ovine milk were obtained from the herds of the Research Centre of Clermont-Ferrand-Theix. Caprine milk was obtained from a local farmer. Porcine colostrum was kindly provided by S. Bernard (INRA, Tours-Nouzilly, France). The bovine, caprine, and ovine milks and the equine colostrum were coagulated with rennet at 37°C for 1 h. After centrifugation ($16,000 \times g$ for 20 min), the supernatant was collected, treated with 30% (vol/vol) Frigen (Behring, Friburg, Germany), and centrifuged at $2300 \times g$ for 20 min. Each whey retentate was filtered ($0.8 \mu\text{m}$) and kept frozen at -20°C until used. Porcine colostrum was defatted by centrifugation at 4°C ($1400 \times g$ for 20 min) and filtered ($0.8 \mu\text{m}$). After ultracentrifugation at $40,000 \times g$ for 30 min, the supernatant was collected and treated with Frigen as described.

Antigen Purification

Samples of β -LG were purified from their respective types of whey by gel permeation chromatography (95- $\times$ 5-cm column, Sephadex G-100; Pharmacia Biotechnology, Uppsala, Sweden) at a flow rate of 50 ml/h. The column had been equilibrated in 0.02 M Tris-HCl, pH 7.0. The β -LG fractions were concentrated by ultrafiltration (Sartorius, Goettingen, Germany; molecular mass cutoff, 10,000 Da) under helium pressure and loaded onto a Mono-Q anion-exchange column (HR 10/10; Pharmacia Biotech) equilibrated with the same buffer. Elution was performed at 1 ml/mm flow rate with a 0.0 to 0.5 M linear gradient using HPLC equipment (Gradient Former GF 425, Pump 420, Detector 430; Kontron Instrument, Montigny les Bretonneaux, France). As assessed from

PAGE analysis, the β -LG were at least 98% pure. Samples of bovine β -LG, purified by salt fractionation according to the method of Maillart and Ribadeau Dumas (25), were also used.

Polyclonal Antibodies

Rabbits were immunized at monthly intervals with purified bovine β -LG variant B. The protein, emulsified in Freund's complete (first injection) or incomplete (booster injections) adjuvant, was administered at doses of 1 mg per rabbit by multiple intradermal injections. Rabbits were bled 8 d after each booster injection. The specificity of the antisera was assessed by double-immunodiffusion and agar gel immunoelectrophoresis. The immunoglobulin fraction was obtained by anion-exchange chromatography (Q-Sepharose Fast-Flow; Pharmacia Biotech) using a concave gradient of NaCl (0.0 to 0.5 M) in 0.02 M Tris-HCl buffer at pH 8.2.

MAb

Female BALB/c mice (8 to 10 wk old) were immunized intraperitoneally with 0.2 ml of a 1:1 (vol/vol) mixture of Freund's complete adjuvant and purified bovine β -LG B ($100 \mu\text{g}$ in $100 \mu\text{l}$ of 0.01 M PBS, pH 7.2). Five weeks later, the mice received $100 \mu\text{g}$ of protein in $200 \mu\text{l}$ of PBS with Freund's incomplete adjuvant. After a resting period of 2 mo, mice that demonstrated a high serum antibody response were injected intravenously with $100 \mu\text{g}$ of protein in PBS without adjuvant. Spleens were removed for fusion 3 d later and were gently squeezed in a sterile mortar in the presence of Dulbecco's Modified Eagle's Medium (Gibco BRL, 95160 Eragny, France). After washing, spleen cells were mixed with exponentially growing murine myeloma cells X63-Ag.8-6.5.3 in a 1:5 (vol/vol) ratio. This mixture was centrifuged for 5 min at $300 \times g$, and the medium was removed. Fusion was then conducted as described previously (23). After 12 to 14 d, the supernatants of the hybridoma population were screened for specific antibody production by indirect and capture ELISA with bovine β -LG B that had been diluted in PBS and using a peroxidase-conjugated rabbit anti-mouse IgG-Fc (Nordic, Tilburg, The Netherlands). Positive hybrids were cloned by limiting dilution.

ELISA Techniques

For indirect ELISA, flat-bottomed polystyrene microtiter plates (Maxisorp; Nunc, Kamstrup, Denmark) were coated overnight at 4°C with $100 \mu\text{l}$ of the

different purified β -LG at a concentration of 10 μ g/ml in PBS. In the assay with varying pH, the following buffers were used: 0.05 M borate, pH 9.4; 0.01 M phosphate, pH 7.3; 0.02 M citrate-phosphate, pH 4.5 and 0.05 M glycine-HCl, pH 2.3. The assay with peptides was performed with Immulon 2 plates (Dynatech, St. Cloud, France) and PBS as coating buffer. All subsequent steps were performed at room temperature (20°C). Four washes with PBS containing 0.02% Tween 20 (**PBS-Tween**) were followed by addition of the hybridoma supernatants (100 μ l/well) diluted in PBS-Tween. After 1 h of incubation, unbound antibody was removed by washing as described, before addition of peroxidase-conjugated rabbit anti-mouse IgG Fc (Nordic) that had been adequately diluted in PBS-Tween containing 1% normal rabbit serum (100 μ l per well). A further 1-h incubation followed, after which the plates were washed. Freshly prepared substrate *o*-phenylenediamine (0.4 mg/ml in 0.05 M citrate and 0.1 M phosphate buffer, pH 5.0, plus 0.015% H₂O₂) was added. After 30 min, the reaction was stopped by addition of 2.5 M H₂SO₄ (50 μ l per well). Absorbance

was recorded at 492 nm using an IEMS microplate reader (Labsystem, Helsinki, Finland).

For capture ELISA, plates were coated overnight at 4°C with 100 μ l of purified anti-bovine β -LG rabbit IgG that had been diluted in PBS. The optimal concentration of capture antibody was determined by titration. Further steps were performed at room temperature (20°C). After four washes with PBS-Tween, 100 μ l of purified bovine β -LG B at a concentration of 10 μ g/ml in PBS containing 1% normal rabbit serum were added and incubated for 1 h. The procedure was then carried out as described for the indirect assay.

Peptides

The production of tryptic peptides was accomplished essentially as described by Dalgalarondo et al. (9). Purified β -LG (5 mg/ml) was digested at 37°C for 24 h with trypsin at a final enzyme to substrate ratio of 1%. The peptides were separated by reversed-phase HPLC, lyophilized, and stored as a powder at 4°C. The peptides were identified by analy-

TABLE 1. Classification of the 69 monoclonal antibodies (MAb) raised against bovine β -LG.

ELISA ¹	Class ²	A	B	C	D	E	F	G	H	I	J	K
	β -LG bovine A	**	*	**	**	**	*	**	**	**	**	**
	β -LG bovine B	*	**	**	**	**	*	*	*	*	*	**
	β -LG ovine	—	—	—	**	*	**	**	*	***	**	**
	β -LG caprine	—	—	—	**	*	**	**	*	***	***	**
	β -LG porcine	—	—	—	—	—	—	—	—	—	—	**
	β -LG equine	—	—	—	—	—	—	—	—	—	—	**
Indirect +, pH I ³					29			13				
Capture +, pH II					51 116	73	137	111		17 27		
						70	93			49 140		
	pH III				28 38		18 154					
					14		36 153					
	pH IV			17		100		7				102
	pH V					96	71	60 123		128		
								50 64 91		8 120		
	pH VI				65 37							
	pH VII	45			87			79 81 86 66		46		
					129			121 143		61		
Indirect +, pH I						20						
Capture —, pH II				47				147				
	pH V				63 109	19					134	
	pH VI		126 124		31							
	pH VII			3	26 48		25 34			56		
	pH VIII	156										
Indirect —, Capture +			88 105						75			
			138						155			

¹The MAb were classified into three groups according to their reactivity in indirect and capture ELISA (indirect positive and capture positive, indirect positive and capture negative, and indirect negative, and capture positive). Numbers are MAb references.

²The MAb were classified in 11 classes (A through K) according to their relative reactivity (— = negative, * = low, ** = medium, *** = high; see Figure 5) in indirect ELISA using evolutionarily related β -LG: bovine β -LG A and B, and ovine, caprine, porcine, and equine β -LG).

³For each ELISA group, eight classes (I through VIII) were defined according to the binding patterns of the MAb in indirect ELISA using bovine β -LG B coated at different pH values.

sis of amino acid composition and by Edman sequencing of the first 3 N-terminal residues.

RESULTS AND DISCUSSION

Production and Characterization of MAb

The fusion of the immune splenocytes with the murine myeloma cells resulted in 156 hybrids that were positive in the ELISA tests. Titration curves allowed the selection, for cloning and further characterization, of 69 hybrids secreting MAb of the IgG isotype with high binding activity (OD >0.2 at a 10^{-2} dilution).

The MAb reactivity for bovine β -LG was compared using an indirect ELISA and a capture ELISA. In the indirect ELISA, the protein may adopt a specific orientation when interacting with the plastic well, which would cause antigenic determinants to be hidden. Moreover, this adsorption may induce complete or partial denaturation of the protein, which would expose some areas that usually are hidden in the native form of the protein (11). Antibodies reacting with such antigenic determinants can be produced if emulsification or subsequent *in vivo* events leading up to the immune response appreciably perturb the conformation of the protein surface (39). These problems are avoided when a capture procedure is applied because antigen is held to the plate via polyclonal antiserum, which is expected to be directed against the immunodominant antigenic determinants distributed all over the antigen.

Three groups of MAb were defined according to their reactivity in the two ELISA tests: MAb that were positive in the indirect test only ($n = 17$), MAb that were positive in the capture test only ($n = 5$), and MAb that were positive in both tests ($n = 47$) (Table 1). The high proportion (25%) of false-positive of ELISA-specific (26) MAb that react only with the coated β -LG is quite striking and indicates a propensity of the β -LG molecule to undergo conformational changes upon adsorption onto the plastic surfaces. In comparison, only 9% of such ELISA-specific MAb were observed for myoglobin (39), a protein of the same size. This difference could be related to the high content of β -sheets in β -LG compared with the high content of α -helices in myoglobin.

The indirect ELISA, applied to the 69 MAb, revealed striking differences in binding for MAb 47, 124, and 156, depending on whether the β -LG used for coating was purified by salting out at low pH according to the method of Maillart and Ribadeau Dumas (25) or by gel permeation chromatography (Table 2). This result indicates that purification procedures led to slightly different conformational states of the molecule and that the method used to purify β -LG

must be considered when MAb reactivities are compared. Furthermore, MAb 17, 45, 116, 138, and 153 exhibited lower binding when the β -LG solution (2 mg/ml in PBS) that was used for coating was stored >2 d at 4°C (Table 3), indicating that conformational changes appeared promptly upon aging.

Binding of MAb to β -LG Coated at Varying pH

Conformational changes of β -LG can be induced by variations in pH (3, 40). To determine whether these changes in conformation affect interactions between antigen and antibodies, the reactivities of all 64 MAb that were positive in indirect ELISA were analyzed with β -LG B that had been coated at varying pH. On the basis of binding patterns, MAb were grouped into eight classes (Table 1 and Figure 1). Class I (3 MAb) did not show significant variations of β -LG binding as a function of pH in the range 9.4 to 4.5 and show a decrease at pH 2.3. In class II (13 MAb), the binding was maximal at pH 7.3 and then gradually declined at more acidic pH. The 7 MAb of class III showed an increase in binding from pH 9.4 to 7.3; the signal was constant between 7.3 and 4.5, but decreased at pH 2.3. In class IV (4 MAb), the binding was constant from pH 9.4 to 7.3, decreased slowly between pH 7.3 and 4.5, and decreased sharply from 4.5 to 2.3. The 14 class V MAb exhibited constant binding from pH 9.4 to 7.3 and a strong decrease from 7.3 to 2.3. In classes VI (5 MAb) and VII (17 MAb), the binding decreased either slightly (VI) or strongly (VII) as pH decreased. The class VIII correspond to 1 MAb that had a slight increase in binding between pH 9.4 and 7.3, decreased drastically from pH 7.3 to

TABLE 2. Reactivity of three monoclonal antibodies (MAb) in indirect ELISA (absorbance at 492 nm) using bovine β -LG B purified by salt fractionation or by gel filtration.¹

MAb	β -LG B	
	Gel filtration	Salt fractionation
47	3.72	0.07
124	1.03	0.29
156	1.26	0.06

¹The β -LG were coated at 10 μ g/ml in 0.05 M borate buffer, pH 9.4. The MAb were diluted 1:3 (vol/vol) in PBS containing 0.02% Tween 20. The binding of the MAb was revealed by a peroxidase-conjugated rabbit anti-mouse IgG Fc. Absorbance was expressed as the mean of duplicate samples.

4.5, and then remained constant from pH 4.5 to 2.3. These pH effects may be explained by different phenomena. It is known that the oligomerization of β -LG is highly dependent on pH and that the binding of the different oligomers to plates can vary. Moreover, the orientation of the oligomers can be different, and some epitopes can be either hindered or exposed. The biggest differences are obtained around pK values of ϵ -amino groups (pH 9.4 to 7.3), pK of imidazole or histidine (pH 7.3 to 4.5), and below pK of side-chain carboxylates (pH 2.3).

Similar binding dependence on pH has been reported by Kuzmanoff and Beattie (22) using 6 MAb that were specific for β -LG. However, those authors (22) used a limited range of pH (5.5 to 7.5) for β -LG coating. Moreover, in contrast with our experiments, their MAb were incubated at the chosen pH, which can modify their binding independently to the effect on the β -LG structure.

Binding of MAb to Peptides

Several studies of the antigenic structure of proteins have relied heavily on proteolytically or chemically generated peptides. In order to determine the epitopes that were recognized by the MAb obtained, bovine β -LG B was cleaved with trypsin. The tryptic peptides were separated by reversed-phase HPLC (Figure 2), identified (sequences given in Table 4), and tested for MAb binding by indirect ELISA. Seven MAb bound significantly to one or a few of the purified peptides (Table 4). Two (MAb 126 and 128) bound slightly to several unrelated peptides and did not allow epitope delineation; MAb 3 bound to peptide f 41–60 and to native β -LG. Moderate binding was observed for MAb 37 to peptide f 61–69 or 70 linked to f 149–162 and to peptide f 149–162, indicating that the epitope is located on the C-terminal fragment of the protein. Sequence f 61–70 may be involved in the

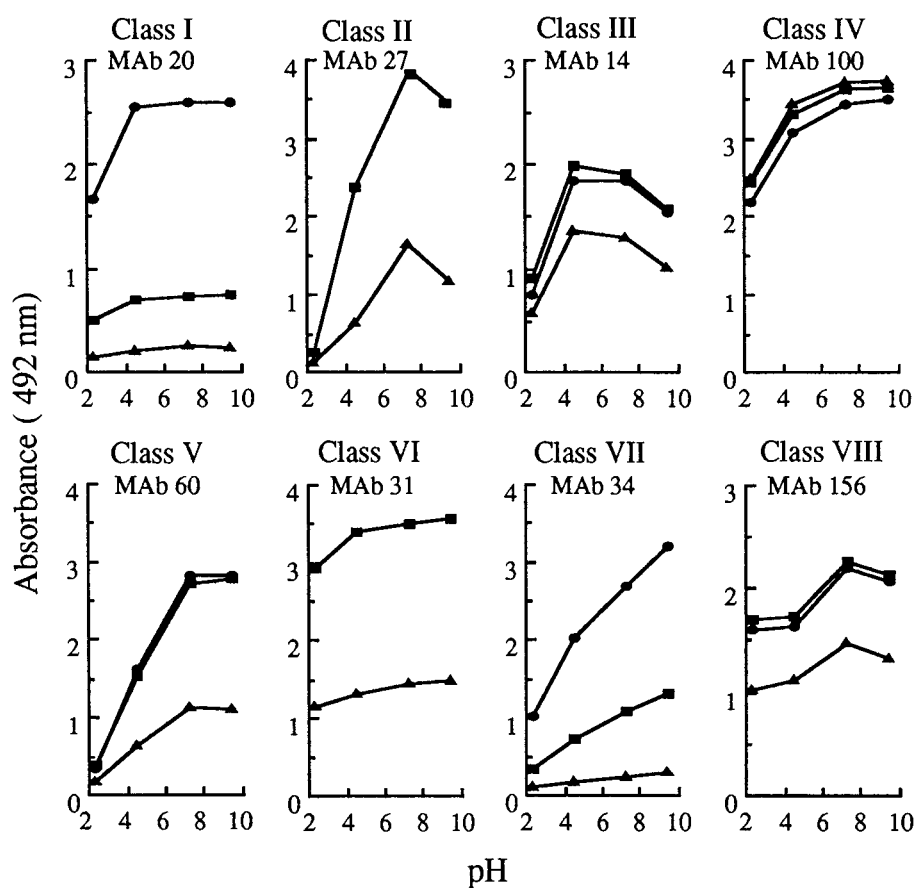


Figure 1. Typical ELISA indirect profiles illustrating the binding curves of monoclonal antibodies (MAb) to bovine β -LG B that were coated at various pH values. The β -LG was coated at 10 μ g/ml in the following buffers: 0.05 M borate, pH 9.4; 0.01 M phosphate, pH 7.3; 0.02 M citrate-phosphate, pH 4.5; or 0.05 M glycine-HCl, pH 2.3. The MAb were tested diluted (vol/vol) 1:3 (■), 1:10 (●), and 1:100 (▲) in PBS containing 0.02% Tween 20. The binding of the MAb was revealed by a peroxidase-conjugated rabbit anti-mouse IgG Fc. Absorbance was expressed as the mean of duplicate samples.

TABLE 3. Reactivity of 5 monoclonal antibodies (MAb) in indirect ELISA (absorbance at 492 nm) using bovine β -LG B that had been stored for various amounts of time.¹

MAb	β -LG B		
	Fresh	48 h	10 d
17	0.67	0.17	ND ²
45	1.45	0.99	ND
116	2.41	ND	0.36
138	1.26	0.6	ND
153	2.44	ND	0.39

¹The β -LG were coated at 10 μ g/ml in 0.05 M borate buffer, pH 9.4. The MAb were diluted 1:3 (vol/vol) in PBS containing 0.02% Tween 20. The binding of the MAb was revealed by a peroxidase-conjugated rabbit anti-mouse IgG Fc. Absorbance was expressed as the mean of duplicate samples.

²Not determined.

epitope formation because MAb 64 reacted with peptide f 61–70 linked to peptide f 149–162 by a disulfide bond, but not with peptide f 149–162. However, the absorbance reached was relatively low compared with the very strong reactivity of this MAb for native β -LG. Consequently, sequence f 61–70 could be considered either as only a small fragment of the epitope for MAb 64 or as a partly modified conformation of this portion of the epitope. Both MAb 102 and MAb 129 displayed strong binding profiles for peptide f 21–40 and moderate binding to peptide f 41–60 and f 61–69 linked to peptide f 149–162. These two MAb, however, were distinct in their binding dependence on pH (see Table 1 and Figure 1; MAb 102, class D, stronger influence of carboxylate protonation or monomeric form; MAb 129, class G, uniform influence of protein protonation upon binding). This result indicates the presence of a conformational aspect of the epitope that is lost when the protein is digested with trypsin.

Not surprisingly, only 10% of our MAb reacted with tryptic peptides of β -LG, which illustrates well the limits of using peptides in the assay (31, 32).

Crossreactivity of Evolutionarily Related β -LG

Closely related variants were purified that differed in a limited number of residues, such as bovine β -LG A and B, ovine and caprine β -LG, and more differentiated porcine and equine β -LG. Reactivities of these variants toward the different MAb were analyzed in indirect and capture ELISA. The results were analyzed for mutated and conserved residues that could change the affinity of the studied proteins (2, 42, 44). As expected, different binding patterns were observed, and 11 classes of MAb specificity could be defined (Table 1).

Classes A, B, and C involved 10 MAb that were strictly specific to bovine β -LG and that differed in their affinity for the variants A and B. The epitopes for these MAb must be defined by residues that are characteristic of the bovine β -LG sequence. In the β -LG from ruminants (Bovidae, Ovidae, and Capridae), the amino acid substitutions are situated at positions 1, 53, 130, 150, 158, and 162 (Figure 3). Residue 1 can be ignored because it is too far from the other residues in the three-dimensional structure (27, 34) (Figure 4). Mutation of residue 53 (Asp for Asn) is a relatively isolated substitution in a highly conserved sequence. According to the analysis of the available three-dimensional structure (Figure 4), this region is relatively hindered, and residue 53 may be thus totally inaccessible. Residue 130 is exposed on the edge of α -helix and on a loop that has been reported to be immunogenic (6, 28). Residues 150, 158, and 162 are well exposed on the surface, and the folding of the chain brings them near residue 130 with which they could shape a conformational epitope (Figure 4). The 2 MAb in class A bound the bovine variant B more strongly (illustrated on Figure 5 for MAb 45), and the 5 MAb in class B reacted better with bovine β -LG A. Bovine variants A and B differ only at residues 64 (substitution of Asp for Gly) and 118 (substitution of Val for Ala, of limited impact) (Figure 3). According to three-dimensional data, residue 64 is exposed on the protein surface and is spatially contiguous to residues 150, 158, and 162 (Figure 4), which have been established as plausible residues for the epitopes of class A, B, and C MAb. Consequently, the epitope

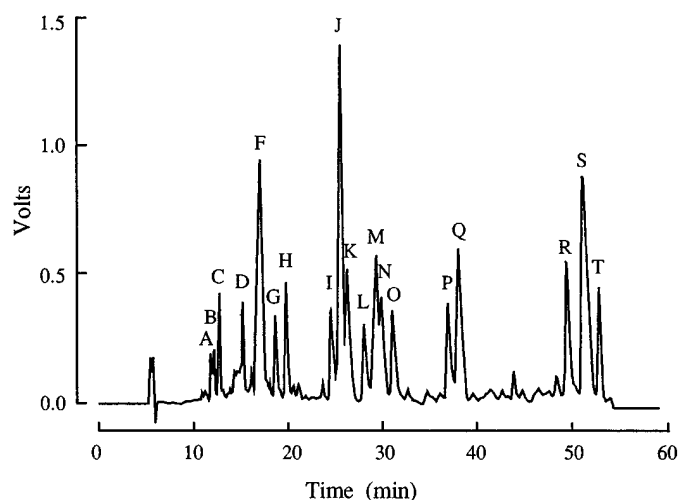


Figure 2. Separation of tryptic hydrolysate of bovine β -LG by HPLC on a 25- $\times$ 0.45-cm Nucleosil C₁₈ column eluted with a linear gradient: A (0.1% trifluoroacetic acid in H₂O, pH 2.5) to B (60% acetonitrile, 40% H₂O, and 0.1% trifluoroacetic acid) at a flow rate of 1 ml/min.

defining the specificity of MAb grouped in classes A and B could comprise residues 64, 150, 158, and 162, which are ≤ 15 Å apart from each other. The 3 MAb in class C cannot discriminate between the two variants of bovine β -LG. Thus, residue 64 is not included in the epitope or the epitopes of these MAb. Residues 53, 150, and 158 may be involved in its formation. Residue 53 may define the specificity of MAb 3 and 47 because they react only in the indirect ELISA. As discussed previously, this procedure is known to unfold areas that are hidden in the native protein and that are undetectable by a method that has less of an effect on structure. This observation is consistent with the finding that MAb 3 binds the peptide f 41–60 (Table 4). With both the indirect and capture techniques, MAb 17 is active, and its epitope is likely to involve residues 150 and 158, which are spatially close and are exposed on the protein surface.

Classes D through J regroup 58 of the 69 characterized MAb (84%). Those MAb crossreacted with different intensities with all of the ruminant β -LG but failed to bind porcine and equine β -LG. Consequently, these MAb bound to regions conserved between bovine, ovine, and caprine β -LG, which excluded 36 residues altered in porcine and equine β -LG that are distributed over entire sequence (Figure 3).

The MAb in classes D, E, and F do not discriminate between the bovine β -LG variants A and B, which

implies that the epitopes involved exclude residues 64 and 118. The MAb in class D ($n = 15$) do not differentiate among the four ruminant β -LG (Figure 5 for MAb 37), which suggests that residues 1, 53, 130, 150, 158, and 162 are not involved in their epitopes. These observations, combined with the results of the peptide analysis (Table 4), allow the determination of putative epitopes for two MAb of this group: MAb 37 and 129. Because the peptide f 149–162 is recognized by MAb 37, the region f 151–157 likely constitutes its epitope; MAb 129 bound strongly the peptide f 21–40. Fragment 34–37 is shared by the four ruminant β -LG and is altered in porcine and equine β -LG (Figure 3). Hence, this fragment might form the epitope that is recognized by this MAb.

Class E (6 MAb) recognized bovine β -LG better than it recognized ovine and caprine β -LG (Figure 5, MAb 100). The opposite was observed for Class F (9 MAb), suggesting that the mutations of residues 53, 130, 150, 158, or 162 are somehow responsible for the formation of the epitopes that are recognized by these two classes of MAb.

Classes G, H, I, and J show higher specificity for bovine variant B than variant A, pointing to residues 64 or 118 as the constituents of their epitopes. Class G (14 MAb) shows a similar binding pattern for bovine β -LG variant B, ovine β -LG, and caprine β -LG

TABLE 4. Reactivity of monoclonal antibodies (MAb) in indirect ELISA using peptides from a tryptic digest of bovine β -LG.

Peptide ¹		MAb						
Sequence	Peak ²	3	37	64	102	126	128	129
f 1–8	I	— ³	—	—	—	—	—	—
f 9–14	G	—	—	—	—	—	—	—
f 15–20	J	—	—	—	—	—	—	—
f 21–40	R	—	—	—	1.47	—	—	1.15
f 41–60	S	0.37	—	—	0.51	—	—	0.18
f 61–69 <-> f 149–162	Q	—	0.67	—	0.66	0.14	—	0.23
f 61–70 <-> f 149–162	P	—	0.64	0.14	—	—	—	—
f 71–75	D	—	—	—	—	—	—	—
f 78–83	N	—	—	—	—	0.12	0.13	—
f 84–91	F	—	—	—	—	—	—	—
f 92–100	O	—	—	—	—	—	—	—
f 92–101	L	—	—	—	—	0.15	0.16	—
f 125–135	H	—	—	—	—	—	—	—
f 125–135 + f 136–138	M	—	—	—	—	0.16	0.15	—
f 136–138	C	—	—	—	—	—	—	—
f 139–141	A	—	—	—	—	—	—	—
f 139–141 + f 142–148	B	—	—	—	—	—	—	—
f 142–148	K	—	—	—	—	—	—	—
f 149–162	T	—	0.44	—	0.25	—	—	—
β -LG		0.25	1.45	3.32	1.24	0.12	0.70	1.05

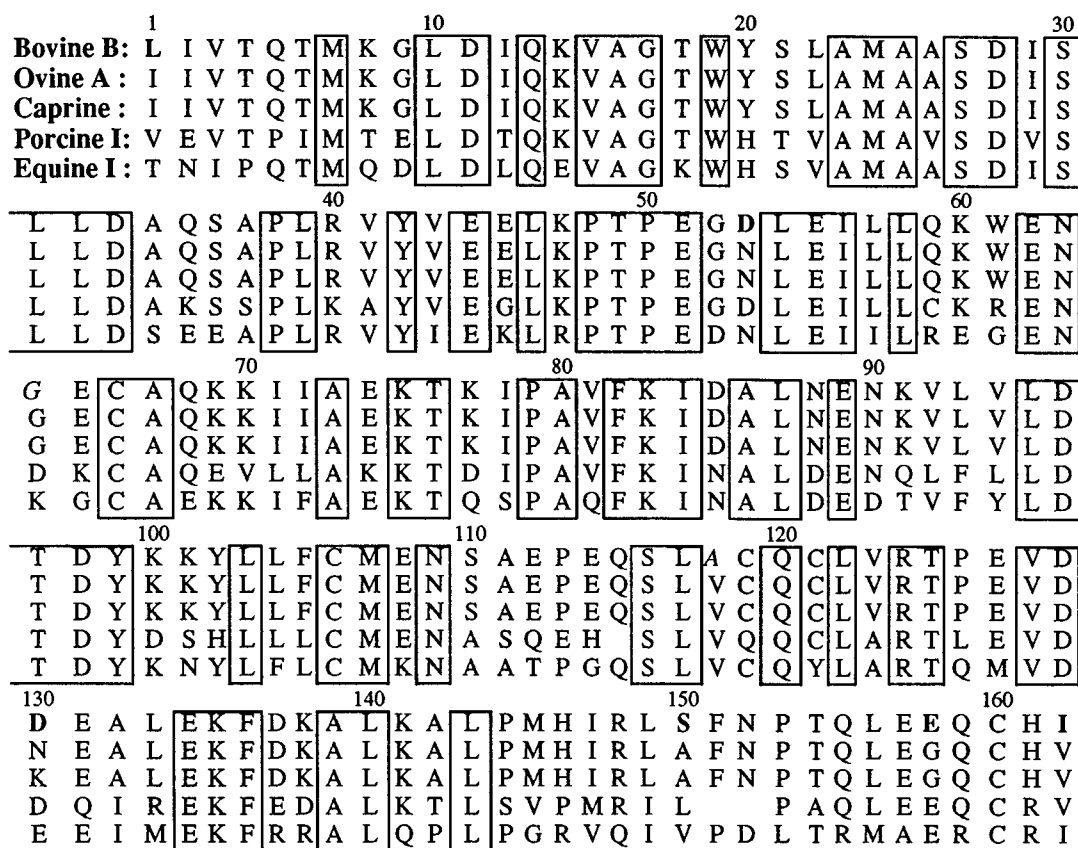
¹The peptides were coated at 10 μ g/ml in 0.05 M borate buffer, pH 9.4. The MAb were diluted 1:3 (vol/vol) in PBS containing 0.02% Tween 20. The binding of the MAb was revealed by a peroxidase-conjugated rabbit anti-mouse IgG Fc. Absorbance was expressed as the mean of duplicate samples.

²Peaks designated in Figure 2.

³Negative reactivity.

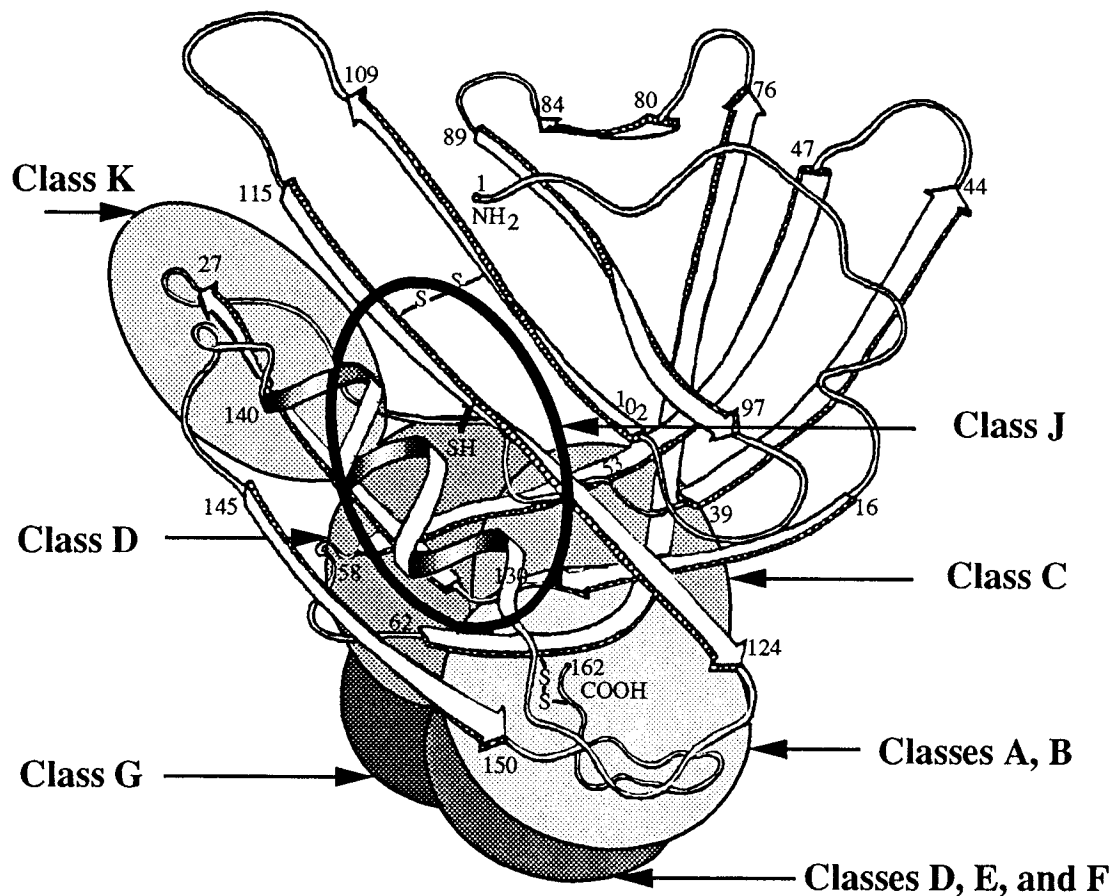
ruled out because an epitope including residues 130 and 64 would include also residue 150, which is spatially intercalated between them (Figure 4) but cannot be part of the epitope because that residue is common to caprine and ovine β -LG (Figure 3). Consequently, the epitope for MAb 134 is more likely to include residues 118 and 130.

This analysis of the different classes of MAb shows 38 patterns of reactivity and may imply the existence of at least 38 different epitopes. Previous studies



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data were not presented. Residues 15 and 29 are placed at the opposite portions of the globulin, and the sequence f 18–24 is buried in the structure and is inaccessible. Therefore, the epitope that is defined by this MAb could be closely related to that epitope recognized by our MAb 102 (residues 26–31). Reddy et al. (35) reported on 1 MAb raised against bovine β -LG that reacted with human serum retinol-binding protein and recognized the sequence f 96–99. Native β -LG did not inhibit this MAb binding in their ELISA, indicating that this sequence is not exposed on the native structure. Kuzmanoff and Beattie (22) reported on the production of 6 MAb that recognized three different epitopes of bovine β -LG variant A. Only 1 of these MAb was monospecific for the bovine β -LG, a proportion similar to ours (10 of 69 MAb was monospecific for bovine β -LG). The 5 remaining MAb crossreacted with whey fractions of caprine and porcine milk, but none recognized ovine whey proteins. These results are surprising considering the sequence homology between these species (1 substitution in



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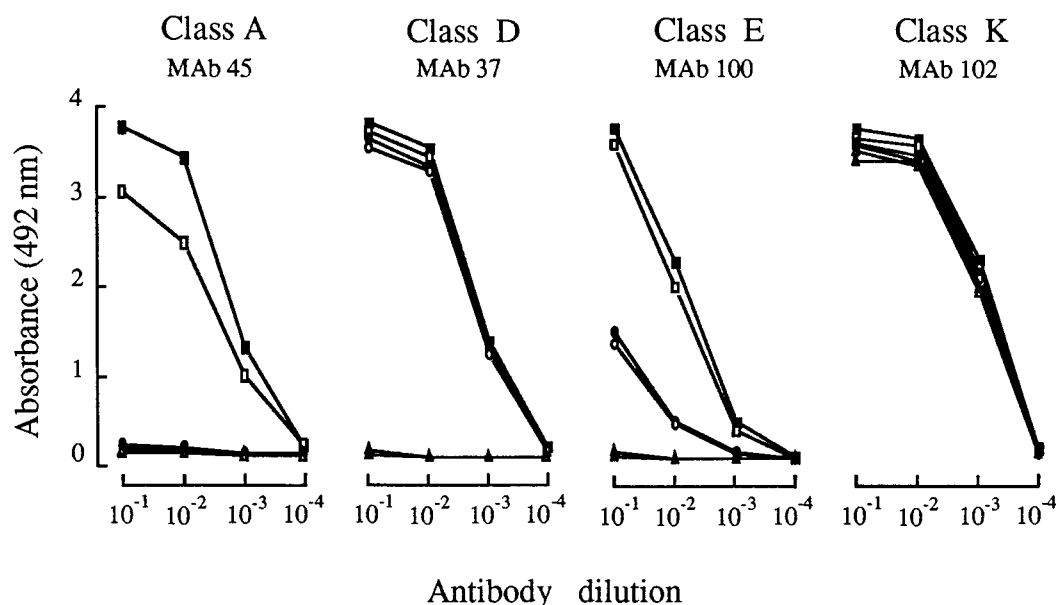


Figure 5. The ELISA indirect binding curves of monoclonal antibodies (MAB) to evolutionarily related β -LG: bovine variant B (■), bovine variant A (□), ovine (●), caprine (○), porcine (△), and equine (▲). The β -LG were coated at 10 μ g/ml in 0.05 M borate buffer, pH 9.4. The MAB were diluted (vol/vol) 1:3, 1:10, and 1:100 in PBS containing 0.02% Tween 20. The binding of the MAB was revealed by a peroxidase-conjugated rabbit anti-mouse IgG Fc. Absorbance was expressed as the mean of duplicate samples.

position 130 between caprine and ovine β -LG versus 62 substitutions between caprine and porcine β -LG). The results obtained here are different because the 59 MAB reacting with caprine β -LG all crossreacted with ovine β -LG, but only 1 MAB also recognized the porcine and equine β -LG. Moreover, for the 69 MAB that have been described in this work, the results are consistent with the known and reported β -LG sequence homologies: 95% for ovine and caprine β -LG calculated in relation to bovine β -LG (20), 62% between porcine and bovine β -LG (8) and 56% between equine and bovine β -LG (7). Results agree also with previous analyses of the immunological relationships between species that have been realized with polyclonal rabbit antisera (15, 24). The discrepancy between our results and the results reported by Kuzmanoff and Beattie (22) may result from the particular methodology used by those authors (22) for immunization of mice (prior immunosuppression with cyclophosphamide, α -LA, and caseins).

The epitopes recognized by the MAB that have been described here are not entirely defined. Further investigations are necessary to achieve full delineation of all residues constituting the determinants. However, some of the MAB have been characterized sufficiently to be already used as efficient probes in studying the heat-induced conformational changes of β -LG in milk products (17) or to study the allergenicity of β -LG

(29). Two of the MAB have already been used as sensitive reagents in the development of an ELISA for the detection of bovine milk addition to caprine or ovine milk (23).

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