Detection of Conformational Modifications of Heated β-Lactoglobulin by Immunochemical Methods

Anne Laligant, Jacques Marti, Jean-Claude Cheftel, Eliane Dumay, and Jean-Louis Cuq*

Laboratoire de Génie Biologique et Sciences des Aliments, Université Montpellier II, Place E. Bataillon, 34095 Montpellier Cedex 2, France

β-Lactoglobulin (βLG) was submitted to various thermal treatments (75 and 90 °C), and its conformational modifications were assessed by competitive radioimmunoassay (RIA) with rabbit antisera against native βLG (βLG_{NAT}) or chemically modified βLG obtained by performic oxidation (βLG_{OX}). Heat-processed βLG reacted with both antisera. Molecule alteration was accompanied by an increased binding of polyclonal antibodies. Whatever the serum used, the RIA test revealed a thermal dependence that was influenced by pH (6.5 and 7.5). However, anti-βLG_{OX} antiserum was shown to be the most useful tool for monitoring slight denaturation structural changes after heating. The test accurately detected local modifications of βLG epitopes that occurred after thermal processing of an industrial βLG isolate.

Keywords: *β-Lactoglobulin; thermal treatment; conformational modification; immunochemical detection*

INTRODUCTION

Bovine β-lactoglobulin (βLG) is an important whey protein largely used as an additive in foodstuffs to stabilize emulsions, retain water, or form gel structures. This protein is known as one of the principal allergenic components in cows' milk (Baldo, 1984; Taylor, 1986; Bahna, 1991). To date, the antigenic structure of βLG has not been revealed in detail. The native protein contains at least four antigenic reactive regions composed of discontinuous (conformational) sites maintained by intramolecular disulfide bonds (Kurisaki et al., 1985; Otani, 1988). Moreover, it has been suggested that the βLG molecule must have at least two allergenic determinants, which are not identical to antigenic ones and comprise a relatively restricted part of the molecule, in contrast to the antigenic determinants (Huang et al., 1985; Malik et al., 1988; Takahashi et al., 1990).

Technological means were introduced to reduce or abolish milk/whey protein allergenicity and produce "nonsensitizing" or "hypoallergenic" baby milk formula (Pahud et al., 1985; Jost et al., 1987; Poulsen et al., 1987; Asselin et al., 1988; Guesry et al., 1989). The enzymatic hydrolysis in vitro of the polypeptide chain splits the sequential epitopes and collapses the allergenic structure (Huang et al., 1985), while heat treatments affect the higher order of protein structure or the conformational epitopes. The partial loss of βLG allergenicity depends on the process intensity (Jost et al., 1987). The main risk associated with technological processing is the emergence of new allergenic sites (Bleumink and Berrens, 1966; Poulsen et al., 1987; Host and Samuelsson, 1988). This forces infant formula manufacturers to optimize their operational conditions and to check immunological properties of their new modified proteins. Hence, they must be provided currently available tools for predicting clinical performance of hypoallergenic substitutes (Bahna, 1991; Cordle, 1994).

To preserve nutritional quality and achieve optimal immunological benefits, a combination of enzymatic hydrolysis and heating methods was developed with an additional ultrafiltration step to remove large peptides (Nakamura et al., 1992). Another new technique consists of using high-pressure proteolytic digestion to selectively eliminate β-lactoglobulin in whey concentrates from bovine milk, whereas α-lactalbumin appears to be resistant to high pressure (Okamoto et al., 1991; Nakamura et al., 1993).

Heat treatments such as pasteurization, sterilization, concentration, or spray-drying are important steps in milk and milk products processing. βLG undergoes conformational changes and protein–protein associations when submitted to heating (Dupont, 1965; Sawyer, 1968; MacKenzie, 1971; Mills, 1976; Green et al., 1979; Dumay, 1988; Laligant et al., 1991). Such partial denaturation affects the physicochemical and functional properties of βLG (Mangino, 1984; Schmidt et al., 1984; Dumay and Cheftel, 1986; Dumay, 1988; De Wit, 1989) and also the antigenic/allergenic ones (Ratner et al., 1958; MacLaughlan et al., 1981; Kilshaw et al., 1982; Heppell et al., 1984). Physicochemical changes in heated βLG were studied by analytical methods such as electrophoresis, chromatography, and spectrophotometry. Immunological methods could be alternative procedures for studying protein heat denaturation and obtaining information on protein structure. Publications in this field, using monoclonal antibodies (Mab) against native βLG, concerned the species and variants (A and B) immunospecificity, the pH dependence of antibody–antigen binding (Kuzmanoff et al., 1991), the preservation of the α-helix portion in the βLG retained at an emulsified oil surface (Shimizu, 1995), the location of the core sequence present in βLG and retinol binding protein constituting the common epitope (Reddy et al., 1992), and the indispensable role of Trp_{19} in the structural stability of βLG (Katakura et al., 1994). Most recent studies on βLG immunodetection were concerned primarily with structures expressed in native protein. Most of the antibodies induced by immunization from a native protein appeared to be mainly directed against assembled conformational sites rather than sequential sites. Poor cross-reactions were observed between denatured proteins and these antibodies. Thus, the competitive inhibition ELISA test with antisera raised from purified βLG, able to discriminate whey samples heated at 100 °C for 10 min, showed a decreased

0021-8561/95/1443-2896$09.00/0

response with increasing severity of heat treatment (Heppell, 1985). When denatured protein was used as the immunogen, one could expect antibodies more reactive with the unfolded polypeptide chain. Nevertheless, few studies elicited antibodies against heated βLG: anti-βLG from UHT processed milk (Otani et al., 1984), anti-lactose-βLG (Otani, 1980), and anti-reduced-carboxymethylated-βLG (Otani and Hosono, 1987; Takahashi et al., 1990).

To follow irreversible changes occurring in βLG structure above the critical denaturation temperature of 70 °C (Sawyer, 1968; Mills, 1976; De Wit and Klarenbeek, 1981; Laligant et al., 1991), we attempted to develop an immunassay by using polyclonal antibodies. The two intramolecular disulfide bonds of bovine βLG, between residues 106 and 119 and residues 66 and 160 (MacKenzie et al., 1972; Papiz et al., 1986; Monaco et al., 1987), are extremely important in conformational epitopes. Moreover, the free SH and the two disulfide bonds play a major role in heat denaturation. Since chemical modifications affect the protein conformation and stability, we chose oxidated βLG (βLG_{OX}) as immunogen, with alteration of SH and disulfide groups to elicit the potential markers of denatured forms. Thus, the usefulness and limitations of the immunological method for the investigation of thermal effects on proteins are examined by radioimmunological assay (RIA) using rabbit polyclonal antisera to βLG_{NAT} and to βLG_{OX}. The present paper describes the influence of a combination of heating and pH which are of practical interest on the immunological recorded response.

MATERIALS AND METHODS

Materials. β-Lactoglobulin (βLG, L-2506, batch 52F-8035, noncrystallized) was purchased from Sigma Chemical Co. (St. Louis, MO). Whey protein isolate (WPI) came from Union Laitière Normande (Condé sur Vire, France) and contained (on a dry basis) 89% total proteins, with 74% βLG plus 6.5% α-lactalbumin (αLA) per 100 g of protein, as evaluated by liquid chromatography. The concentration of βLG from Sigma was determined at 280 nm using an $\epsilon^{1\%}_{1cm}$ of 9.5 (MacKenzie, 1971). All other chemicals used in this study were of analytical grade.

Chemical and Thermal Modifications of βLG. Aqueous dispersions (1% protein w/v) of βLG in distilled water were adjusted to pH 6.5 or 7.5 with 0.1 N NaOH or HCl. Ten milliliters of dispersion was placed in a glass tube (18 mm i.d., 180 mm in height), heated for 3, 6, 10, or 20 min in a water bath at 75 or 90 °C, then rapidly cooled to room temperature with tap water, and kept at 4 °C before analysis. The irreversible unfolding followed by aggregation of βLG was analyzed by liquid chromatography, electrophoresis, or RIA by using polyclonal antibodies.

The oxidation of βLG was carried out in performic acid following the procedure of Hirs (1956). One hundred fifty milligrams of βLG was dissolved in 6 mL of formic acid (98–100%) and 12 mL of performic acid. After 3 h at 0 °C, 40 mL of deionized water was added to the solution. The reaction mixture was evaporated to remove the excess reagents. The final derivative (βLG_{OX}) was dissolved in 6 mL of deionized water, then lyophilized, and stored at 4 °C. The effects of the modification on the amino acid residues and protein conformation were analyzed by liquid chromatography. Moreover, βLG_{OX} was used to immunize rabbits.

Fluorescence Spectra. Fluorescence spectra of native or chemically modified βLG were measured on a JY3 spectrofluorometer (Jobin-Yvon, Paris) at room temperature. Both protein solutions [63.2×10^{-6} M] were prepared in 20 mM phosphate buffer, pH 7.5. The βLG_{OX} suspension (4.6 mg mL^{-1}) was first sonicated for 15 min and centrifuged at 18800*g* for 15 min, and the supernatant was diluted (1:4). Solutions of βLG_{NAT} and βLG_{OX} were excited from 200 to 600 nm, and the fluorescence spectra were scanned from 300 to 700 nm.

Liquid Chromatography. The quantitation of soluble proteins was carried out by gel filtration chromatography (GFC) using a TSK 3000 column (7.5 × 300 mm, Toyo Soda, Tokyo) or by ion exchange chromatography on a Mono Q column (HR 5/5, Pharmacia) as previously described by Dumay and Cheftel (1989). Protein samples were diluted as needed, centrifuged at 18800*g* for 15 min, and filtered on Durapore membranes (0.22 μm, Millipore, Bedford, MA) to remove insoluble proteins. The resulting clear filtrates were analyzed by liquid chromatography. Gel filtration chromatography was carried out with a 0.06 M phosphate buffer, pH 6.0, containing 0.15 M Na_2SO_4, and at a flow rate of 0.5 mL min^{-1}. Ion exchange chromatography was done at a flow rate of 1 mL min^{-1} with a 0.02 M piperazine buffer, pH 6.0, containing 0.02–1 M NaCl with a linear gradient (0.02–0.14 M in 11 min, 0.14–0.35 M in 8 min, 0.35–1 M in 5 min). The concentration of protein in the eluate was monitored at 280 nm.

The amino acid quantitation of βLG_{NAT} and βLG_{OX} was carried out by ion exchange chromatography on a Chromobeads C_2 column (0.6 × 75 cm) kept at 60 °C, according to the procedure of Spackman et al. (1958). Amino acid analyses were performed after hydrolysis of samples with 6 N HCl at 110 °C for 24 h in evacuated, sealed tubes. The amino acid composition was determined with a NC1 Technicon autoanalyzer. The eluate was monitored by measuring the absorbance at 440 and 570 nm after color development with ninhydrin reagent.

Rabbit Antisera. Antisera to βLG_{NAT} and βLG_{OX} were prepared by immunizing two groups of rabbits over a period of 5 months. Antisera were raised by subcutaneous injections of the antigen (βLG_{NAT} or βLG_{OX}, 1 mg mL^{-1}) in 0.15 M NaCl emulsified with an equal volume of Freund's complete adjuvant. The same procedure was repeated 14, 22, 35, 123, 128, and 157 days after the first injection. Each rabbit received 1 mg antigen per injection, except for the last injection, when animals received 250 μg. Bleedings were performed 7 days after the last immunization, and the antisera were separated by centrifugation (5000*g*, 20 min, 4 °C). Pooled antisera to βLG_{OX} or to βLG_{NAT} were stored at 4 °C and contained, in the final solution, 1 mM phenylmethanesulfonyl fluoride (PMSF) dissolved in dimethyl sulfoxide (DMSO) and 0.1% (w/v) sodium azide.

Radioiodination by Chloramine T Method. βLG was iodinated (^{125}I) using the chloramine T method (MacConahey and Dixon, 1980). Briefly, 4 μg of βLG in 0.1 M phosphate buffer, pH 7.4, was labeled with 200 μCi of ^{125}I. The reaction was stopped after 3 min by adding 3 μg of sodium metabisulfite. Free ^{125}I was removed by Sephadex G-25 chromatography. The equilibrated buffer and the eluant were 0.1 M phosphate buffer, pH 7.4, containing 0.1% BSA. The βLG-bound radioiodine was isolated in the void volume, and the radioactivity was screened with a Kontron type MR-480 γ-counter. The solution was adjusted to 10^6 cpm mL^{-1} in PBS, pH 7.4, containing 0.1% BSA (w/v) and 0.01% sodium azide (w/v). Aliquots were stored at 4 °C.

Immunodiffusion Analysis. Antibody production was monitored by double immunodiffusion tests (Ouchterlony, 1953), performed in 1% (w/v) agarose gel in veronal buffer, pH 8.2, with 0.02% (w/v) sodium azide.

Titration of Antiserum. The dilution of antiserum to be used in RIA was adjusted to an amount capable of binding 50–80% of the added radiolabeled βLG at the selected incubation conditions. Assays were performed in 1.5 mL polypropylene test tubes initially incubated at room temperature for 1 h with 1% dichlorodimethylsilane in dichloromethane and then washed with distilled water. One hundred microliters of each antiserum, 3-fold diluted in 0.15 M PBS at pH 7.4, was incubated with 100 μL of [^{125}I]βLG (approximately 2×10^5 cpm) at room temperature for 2 h and then at 4 °C for another 46 h. Antibody-bound antigen was then precipitated together with normal rabbit serum (150 μL) by the addition of 450 μL of 1.85 M ammonium sulfate followed by an incubation at room temperature for 4 h. Then, the immune complexes were centrifuged at 10000*g* for 5 min and the supernatants discarded. After three washings of the precipitates with 1.2 M ammonium sulfate (200 μL), the radioactivity was measured by a γ-counter. A correction for nonspecific precipitation was

made by analyzing controls containing only antibody or antigen. Titers were expressed as the serum dilution giving 50% binding of the labeled antigen. Results are the average of two or three determinations.

Competitive Radioimmunoassay. Inhibition curves were constructed to investigate the cross-reactivity of antibodies against heat-processed βLG. All reagents were diluted in 0.1 M PBS. Serial dilutions of antigen (native βLG standard or heated βLG) were prepared to cover the range from 9 mg to 10 ng of protein per milliliter. To anti-βLG_{NAT} serum or anti-βLG_{OX} serum (appropriately diluted 1:450 and 1:90, respectively), the same volume (100 μL) of competitive inhibitor at various concentrations (native βLG standard or heated βLG) was added in duplicate, followed by an equal volume of labeled antigen (2×10^5 cpm). Samples were incubated at room temperature for 2 h and then at 4 °C for another 46 h. A further 150 μL of normal rabbit serum was added to assist the precipitation of the complex, followed by the addition of 100 μL of PBS. Combined antigen–antibody complexes were separated from unbound antigen by the addition of 250 μL of ammonium sulfate to a 1.2 M final concentration for 700 μL of total volume. After another incubation at room temperature for 4 h, the immune precipitate formed was removed according to the same procedure mentioned above. Results are the average of three determinations.

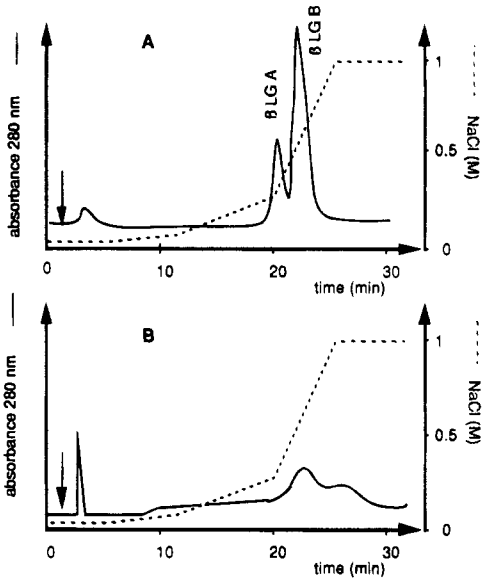

Figure 1. Ion exchange chromatography (Mono Q column) of native βLG control (A) or of performic acid oxidized βLG (B). Chromatographic conditions are given under Materials and Methods.

RESULTS AND DISCUSSION

Characterization of Chemically Modified βLG (βLG_{OX}). To select the most appropriate antibodies as a conformational probe of the denatured structure of βLG, the adopted strategy passed through chemical modification of βLG. Thus, it appeared to be important to understand well the contribution of the chemical treatment to the epitope on the immunogen. For this purpose, an early characterization of βLG_{OX} structure was investigated.

Performic acid oxidation of βLG induced the formation of a partially insoluble product (βLG_{OX}) in aqueous medium (as assessed by gel permeation chromatography at pH 6.0, data not shown). At this pH, βLG is known to exist essentially as dimers. As a consequence of the changes resulting from oxidation, the major peak, representative of βLG, disappeared, while significant modifications were observed. The fraction eluted at the void volume corresponds to large aggregates of protein with molecular masses >600 000 Da. The major/second peak represented basically the remaining dimers, which are eluted with new molecular species and therefore exhibit considerable heterogeneity in molecular masses (small aggregates: tri, tetra-, and polymers). This major peak was followed by some lighter material, indicated by the observed trailing edge, and this could correspond to denatured monomers. This monomer did not reassociate into dimers when the pH was readjusted to pH 6.0 for chromatographic analysis. The reduced height of peak βLG_{OX} might result from the diminution of the molar extinction coefficient as a result of modifications of some aromatic amino acid residues.

The net charge distribution profiles determined by ion exchange chromatography at pH 6.0 are shown in Figure 1. At this pH, the βLG dimer had a negative net charge. The separation of both variants is attributed to an Asp residue in βLG A substituted by a Gly residue in βLG B. Chromatography of the βLG_{OX} soluble fraction resulted in the separation of two distinct peaks and confirmed the loss in soluble protein (variant A plus B) after the chemical treatment with the appearance of material eluted at void volume.

A considerable change in the spectral properties of soluble βLG was observed after the oxidative treatment. The absorbance at 280 nm decreased by 72% (figure not shown), indicating a destruction of aromatic amino acid residues and a significant formation of insoluble protein material. The shape and intensity of the βLG_{OX} absorbance were considerably modified compared to that of βLG. The UV spectra, resulting mainly from the absorbance of tyrosine and tryptophan residues in the 250–300 nm range, indicated a decrease in the molar extinction coefficient due to alterations of these amino acid residues. On the other hand, a sloping baseline results in the 310–400 nm region. This result usually originates from light scattering, suggesting that large particles such as aggregates were present in the solution.

The significant damage to Trp was supported by the fluorescence data for the soluble protein fraction, usually dominated by the contribution of tryptophan residues. Excitation and emission spectra (Figure 2A,B) were significantly shifted from shorter wavelengths to higher wavelengths, revealing the formation of new species.

The amino acid composition of βLG_{OX} on total fraction (soluble and insoluble portions) compared to that of βLG_{NAT} was determined by ion exchange chromatography after acid hydrolysis (Table 1). Oxidation of sulfhydryl groups was previously derived from the reduction of two disulfide bonds and yielded five residues of cysteic acid. In addition, methionine was essentially oxidized to methionine sulfone and the four tyrosine residues were totally destroyed.

So, chemical modification of βLG side groups decreased the conformational stability with unexpectedly partial insolubilization of new derivative. The breakup of –SS– bridges and sulfonate group formation were the main important contributors to an exposure of hydrophobic groups to the aqueous solvent, which provided a driving force for subsequent aggregation. Nevertheless, this denatured structure seemed to be a good tool to select antibodies as most appropriate conformational probe of the denatured structure.

Characteristic Immunological Properties of Oxidized βLG. To develop a convenient and simple test, the chloramine T method was applied to provide radiolabeled protein as a tracer in RIA. The labeling yield of βLG was more than 25% and led a labeled βLG

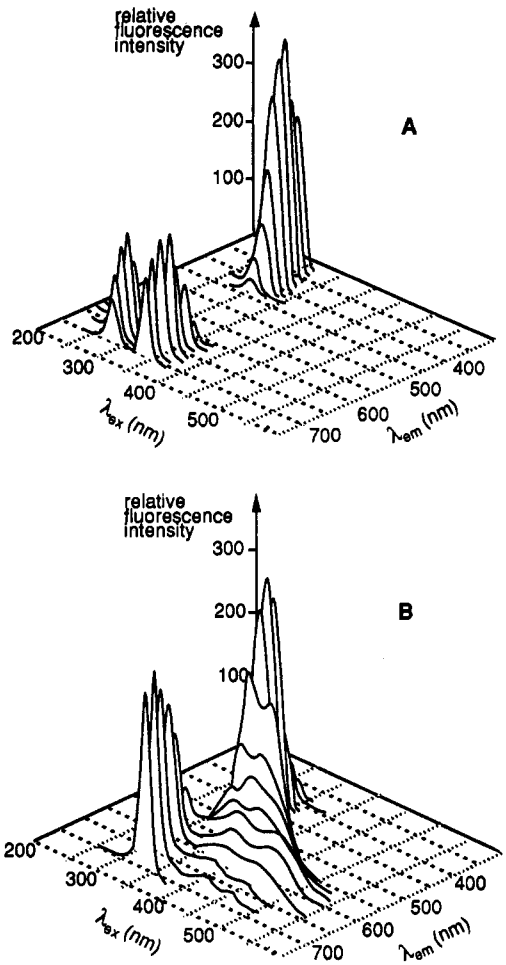

Figure 2. Fluorescence spectra of native βLG (βLG_{NAT}, A) and of performic acid oxidized βLG (βLG_{OX}, B).

Table 1. Amino Acid Compositions of Native βLG (βLG_{NAT}) and Performic Acid Oxidized βLG (βLG_{OX})

	mol of residues/mol of protein		
amino acid	βLG_{NAT}	βLG_{OX}	βLG_{NAT} (Eigel et al., 1994)
cysteic acid	0	4.7	0
methionine sulfoxide	0.6	0	0
methionine sulfone	0	2.8	0
aspartic acid	17.3	18	15
threonine	7.5	7.9	8
serine	6	6.5	7
glutamic acid	27.1	28.7	25
proline	8.7	8.1	8
glycine	3.4	3.4	4
alanine	13.7	14	15
valine	10.5	9.2	9
half-cystine	4.6	0	5
methionine	3.1	0	4
isoleucine	9.3	9.4	10
leucine	22.2	22	22
tyrosine	3.7	0	4
phenylalanine	4	4.2	4
lysine	14	14.8	15
histidine	2.1	2.1	2
arginine	3.1	3.1	3
tryptophan			2

activity of about 30 000 cpm ng^{-1}. A precipitation test of the labeled protein in the presence of 12% (w/v) trichloroacetic acid followed by centrifugation (100000*g*, 10 min) showed that free ^{125}I represented only 5% of the initial radioactivity, ensuring the quality of the tracer. Finally, an SDS–PAGE analysis of stock solution confirmed that βLG was specifically labeled.

To determine whether labeling could modify the physical and biological properties of the βLG molecule, fractional precipitation curves of [^{125}I]βLG were carried out with increasing concentration of ammonium sulfate. The effect of salt on the efficiency of fractional precipitation for [^{125}I]βLG free antigen and bound antigen to polyclonal antibodies was followed by RIA. One hundred microliters of anti-βLG serum (diluted 1:9 in the PBS buffer) was incubated for 48 h with 100 μL of [^{125}I]βLG. Then, 150 μL of preimmune rabbit serum and 350 μL of ammonium sulfate (final concentration ranging between 0 and 2 M) were added. After incubation for 4 h at 20 °C, the tubes were centrifuged (5 min, 10000*g*) and the precipitate was washed with ammonium sulfate solution at the same final concentration as that used in the test. Total and bound radioactivities were determined by a γ-counter. Because the binding activity of polyclonal antibodies was preserved, the iodination via tyrosyl groups did not destroy the biological properties of the immunserum. The range of 1.2–1.3 M ammonium sulfate permitted precipitation of the antigen–antibody complex without any significant background due to indesirable precipitation of the free antigen.

The titers of each polyclonal antibody, defined as the dilution that induces 50% binding of a definite quantity of labeled antigen, were evaluated by antisera dilution curves. Both antisera were able to bind the tracer. These data confirmed the immunogenicity of βLG_{NAT} and the antigenicity of the modified protein. Nevertheless, a considerably different antibody binding was obtained with both proteins, with titer values about 10-fold higher for βLG_{NAT} (1:944) than for βLG_{OX} (1:106).

The antigenic reactivity of βLG_{NAT} and βLG_{OX} with antisera was also followed by the immunodiffusion method. βLG formed a single precipitin arc with antiserum to βLG_{NAT} as well as antiserum to βLG_{OX}, whereas βLG_{OX} formed no discernible arc with either anti-βLG_{NAT} serum or anti-βLG_{OX} serum. The immunoprecipitation results, which indicated that a considerable amount of antibody directed against βLG could be produced by immunizing rabbits with βLG, are compatible with titer observations.

Further experiments were carried out to check the antigenicity of modified βLG. To assess whether injections of βLG_{NAT} elicited the production of anti-βLG_{OX} antibodies, the antiserum was tested by a peroxidase probe method. The precipitin curves showed three zones during which the amount of formed precipitate increased to a maximum and then decreased (curves not shown). As the dilution of antisera was increased, the equivalence zone progressively shifted to a lower activity and a lower amount of βLG_{OX}. Hence, the equivalence points decreased to 2.5, 1, and 0.5 μg of antigen per tube, respectively, for antisera dilutions of 1:1, 1:2, and 1:4. The activity, which decreased rapidly with the dilution of antiserum, tended to have a negligible value at a dilution of 1:10. The essential point of this result was that some population of anti-βLG_{NAT} polyclonal antibodies reacted with modified βLG (βLG_{OX}).

So, the oxidation of βLG did not prohibit native antibodies eliciting, which was supported by titer and immunodiffusion results. This cross-reactivity between anti-βLG_{OX} and βLG_{NAT} agrees with earlier findings on anti-RCM-βLG (reduced carboxymethylated). Takahashi et al. (1990) explained that the unfolding of the βLG molecule after reduction and carboxymethylation of intramolecular –SS– bonds retained stable antigenic sites which did not totally abolish the antibody binding on βLG. Nevertheless, the lower titer that we have registered with anti-βLG_{OX} was probably caused by several factors. First, it could be an expression of a

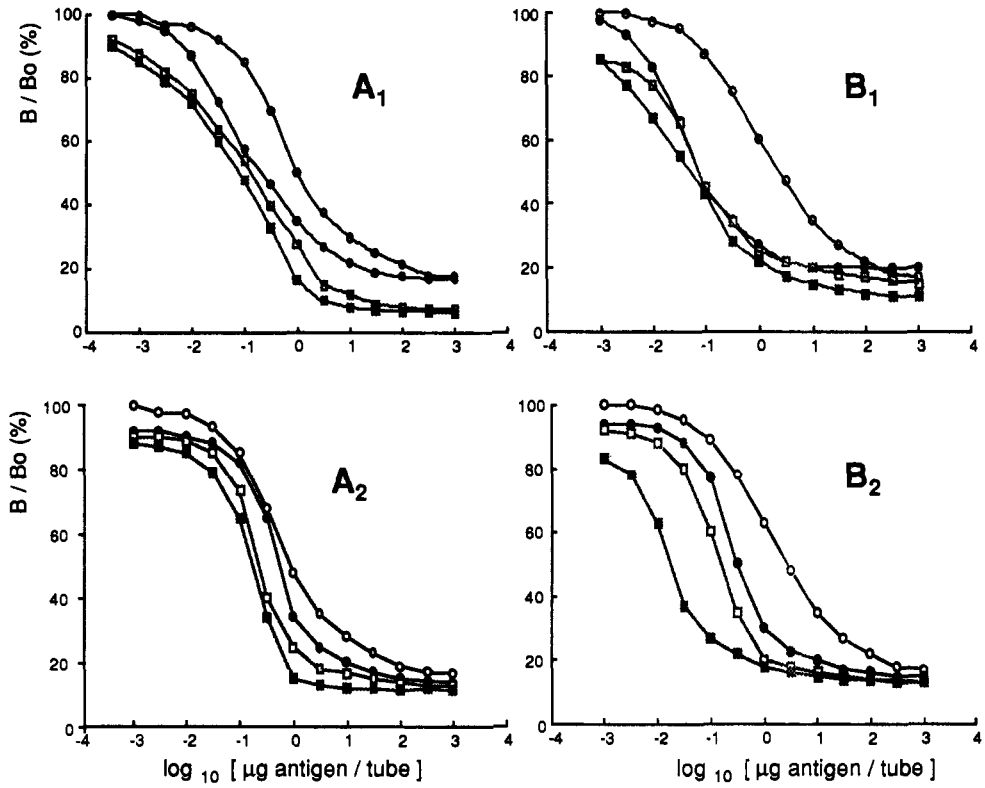

Figure 3. Binding curves of polyclonal antibodies to [^{125}I]βLG in the presence of heat-treated βLG at 90 °C. RIA conditions: A1, A2, anti-βLG$_{NAT}$ polyclonal antibodies (1/450); B1, B2, anti-βLG$_{OX}$ polyclonal antibodies (1/90). Heating conditions: control with no heating (○); 3 (●), 10 (□), and 20 min (■) of heating. A1, B1, pH 7.5; A2, B2, pH 6.5. B/B_0, bound [^{125}I]βLG to polyclonal antibodies in the presence of unlabeled antigens (competitors)/bound [^{125}I]βLG to polyclonal in the absence of competitors.

Table 2. Effects of Heat Treatments on the Relative Denaturation Index (B_{50})[a] of βLG in Pure Solution or Whey Protein Isolate (WPI) Tested by RIA in the Presence of Anti-βLG$_{NAT}$ Serum or Anti-βLG$_{OX}$ Serum

			heat treatment					
			90 °C			75 °C		antibody
sample	pH	reference	3 min	10 min	20 min	3 min	10 min	used in RIA
βLG	6.5	890	400	180	160			anti-βLG$_{NAT}$
	7.5	890	200	125	80			
βLG	6.5	2510	250	135	15	840	560	anti-βLG$_{OX}$
	7.5	2510	55	75	65	500	180	
WPI	6.5	1780	240	160				anti-βLG$_{OX}$
	7.5	1780	400	315				

[a] B_{50} is expressed as nanograms of antigen per tube.

slight recognition of βLG$_{NAT}$ by anti-βLG$_{OX}$ antibodies, probably due to a complete disruption of the conformation and immunochemical properties of βLG$_{OX}$. This result was in support of the view expressed by Otani and Hosono (1987) which noted that antisera against RCM-βLG did not cross-react with βLG. Second, the lower immune response to βLG$_{OX}$ could be due to the state of protein aggregation, a factor involved in immunogenic potential, which induced a tolerant state in animals.

Reactivity of Heated βLG. To understand the relationships between the structure and the biological functionality of βLG, we attempted to analyze some irreversible effects of heat treatment on βLG or WPI aqueous solutions by a competitive RIA. Because the thiol reactivity of βLG increases by more than 2 orders of magnitude between pH 6.5 and 7.5 and the temperature parameter influences the reaction, the susceptibility of the molecule to heat denaturation was recorded at both pH values in two temperatures, 75 and 90 °C. The antigenic reactivity of heat-processed βLG was expressed in terms of their ability to neutralize antibodies specific to βLG$_{NAT}$ or βLG$_{OX}$. Inhibition of the reaction between βLG$_{NAT}$ and anti-βLG$_{NAT}$ antibodies diluted 1:450 (or anti-βLG$_{OX}$ antibodies, diluted 1:90) by the heated βLG (75 or 90 °C) is shown in Figure 3. All binding curves were reversed sigmoidal curves using this classical plotting method (Jensenius et al., 1983).

Regardless of the pH (6.5 or 7.5), heated βLG could completely substitute for native βLG. These data clearly indicated that the antibodies associated with either the anti-βLG$_{NAT}$ sera or the anti-βLG$_{OX}$ sera could recognize both native and heated βLG. Preliminary data of the immunodiffusion test and titer had already emphasized that some specific antibodies in the sera against βLG$_{OX}$ could be efficiently bound to the native protein. On the other hand, the inhibition binding curves shifted to the left compared to the curve for native βLG, and these behaviors increased as a function of heating times and temperature. At $B/B_0 = 1/2$, the amount of antigen approximately related to the affinity of antigen with antibody. Whatever the sera may be, this index decreased (Table 2). Therefore, the affinity of either anti-βLG$_{NAT}$ sera or anti-βLG$_{OX}$ sera to βLG increased considerably upon heating at 75 or 90 °C.

Recent studies have already reported that antibodies raised against native protein bind preferentially to the denatured antigen. Such results were obtained with

polyclonal antibodies tested in the presence of heated ovalbumin (Breton et al., 1988) and also with monoclonal antibodies anti-βLG (Kaminogawa et al., 1989) or anti-tryptophan synthetase (Friguet et al., 1984) tested in the presence of chemically modified antigen.

The index B/B_0 (Table 2) decreased with the intensity and length of the thermal treatment. Moreover, whereas slight structural changes of βLG were registered by hydrophobic chromatography and electrophoresis (Laligant et al., 1991), the RIA test was able of detect subtle modifications as early as 3 min at 75 °C, indicating that 3 min is enough to induce some conformational change in the molecule. In this context, the test will instead serve to complement instrumental analysis.

The increase in affinity for the immunsera was greater when solutions were heated at alkaline pH (7.5), except after 20 min at 90 °C in the case of anti-βLG_{OX} sera (Table 2). Changes registered at 90 °C are higher compared to those at 75 °C. These immunological responses reveal once more a decrease of thermal stability of βLG toward alkaline pH values from 6.5 to 7.5. Similar unstability was observed by investigators with ELISA and monoclonal antibodies (Kaminogawa et al., 1989) as well as with precipitin ring test and polyclonal antibodies (Otani and Tokita, 1980).

These data agree with DSC studies which revealed a transition temperature that was pH dependent. As a matter of fact, βLG exhibited a lower denaturation temperature at pH 8.0 than at pH 6.5 (60 °C instead of 80 °C) (De Wit and Klarenbeek, 1981; Park and Lund, 1984; Harwalkar and Ma, 1989). It is well-known that above pH 6.8 βLG undergoes reversible modifications involving the dissociation of dimer to monomer.

RIA results proved that the polyclonal antibodies indeed recognized the heated antigen better than native βLG. The phenomenon may be based on different types of antigenic determinants accompanying the denaturation mechanism. Thus, some epitopes which correspond to a sequence of amino acids located in the interior of the native molecule become exposed partly due to the disorganization of the compact native structure as a flexible area of the polypeptide chain in the non-native protein. Kaminogawa et al. (1989) argued that a monoclonal probe of the random coil region at Lys_8–Trp_{19} detected a new loose structure located on the heat-labile segment Lys_8–Trp_{19}, which is affected as early as 67 °C at pH 7.2. Hattori et al. (1993) found that βLG contains a hydrophobic region (Val_{15}–Ile_{29}) existing inside the molecule which is easily exposed and hard to refold. They suggested that this structural feature of βLG would induce the production of antibodies preferentially against the unfolded molecule, despite the antibodies being elicited by immunization with native material. Second, it is possible to obtain conformational determinants present in new stable species as a consequence of irreversible aggregation reactions. Some work has actually identified small immunoreactive aggregates (Otani, 1988). Furthermore, the nature and the diversity of heat-induced aggregates (as a function of time and temperature conditions) complicated the understanding of the mechanism. Besides the fact that the level of protein denaturation evaluated by hydrophobic chromatography analysis was lower at pH 6.5 than at pH 7.5, partly due to lower reactivity of the thiol group, the appearance of high molecular mass aggregates at pH 6.5, promoted by hydrophobic interactions (as shown by gel permeation chromatography; Laligant et al., 1991), could induce a loss of some recognized antigenic determinants which become partially hidden. Kaminogawa et al. (1989) have already observed that some

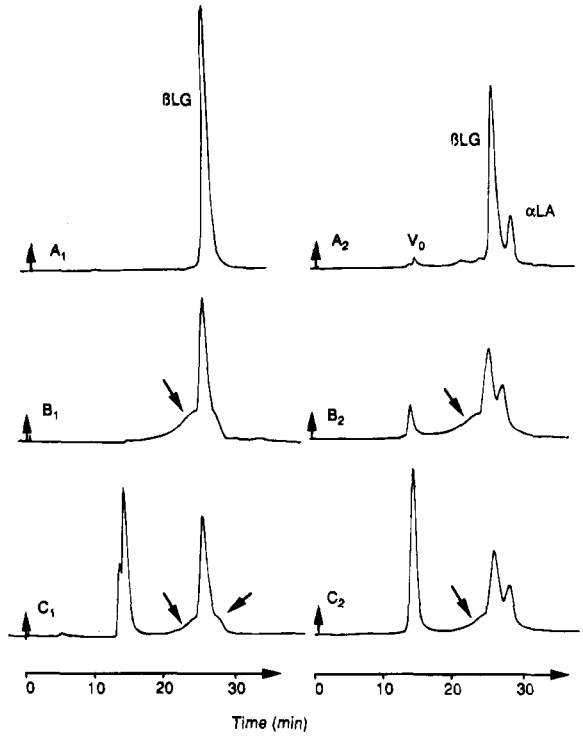

Figure 4. Gel permeation chromatography of βLG (A1–C1) or WPI (A2–C2) heated at pH 7.5 or 6.5 for 6 min at 90 °C. Heating conditions: A1, A2, control with no heating; B1, B2, heated at pH 7.5; C1, C2, heated at pH 6.5. Changes in elution patterns are indicated by short arrows.

epitopes for the monoclonal antibodies were destroyed by heating.

Since the anti-βLG_{OX} serum is able to detect modifications of heat-sensitive βLG as early as 3 min at 75 °C and the immunological decreased response is more marked compared to that with anti-βLG_{NAT} serum, we therefore selected anti-βLG_{OX} to assay the antigenic reactivity of WPI by RIA. The standard index (Table 2) for WPI was lower (B_{50} = 1780) than that of βLG (B_{50} = 2510). This difference could be due to the lower level of βLG protein in the WPI compared to purified βLG. The gel permeation chromatography of WPI (Figure 4) suggested a partial denaturation of βLG and a concomitant presence of new species eluted in the void volume. Thus, some denaturation of βLG during the purification process of WPI may occur, and the standard index for WPI tended to indicate some modifications.

In all cases, besides the fact that the antigenicity of βLG was affected by heating (Table 2), the increase in reactivity associated with WPI proteins seemed to be smaller than that observed for purified βLG. Moreover, compared to the above results with aqueous solution, heating at pH 6.5 affected the response much more than did heating at pH 7.5. Analogous results were reported by Otani and Tokita (1979), who used a precipitin ring test to study the effect of heat treatments at 96 °C on the antigenicity of βLG. They observed that βLG lost its antigenicity in MacIlvaine's buffer (pH 6.6) compared to the antigenicity of βLG heated in whey.

In an attempt to study the important physicochemical parameters that control the functional properties of whey proteins, the emphasis in many investigations has been on the potential effects of a variety of environmental conditions such as $FeCl_3$ (Watanabe et al., 1985) and lactose (Bleumink and Berrens, 1966; Otani et al., 1984; Otani, 1988). The heat denaturation (90 °C, 15 min) of βLG A in $FeCl_3$ media (pH 1.5) produced a partially disordered structure which retained some antigenicities

by immunoprecipitation using a rabbit anti-βLG A serum; Watanabe et al. (1985) concluded that $FeCl_3$ promoted an irreversible denaturation of proteins, which was a combined effect with heating. To provide information about the antigenic properties of βLG in milk products, Otani et al. (1984) studied the antigenic reactivity of RCM-βLG (reduced carboxymethylated) with antiserum against βLG from ultrahigh temperature (UHT) processing milk. They demonstrated that some of the antigenic sites of βLG in UHT milk were different from those of native βLG. However, to date, no αLA effect has been followed.

As previosly shown (Laligant et al., 1991), heating pure βLG solutions at pH 7.5 and 90 °C led to oligomers and polymers with molecular masses ranging from 40 000–200 000 Da (Figure 4B1), whereas WPI induced soluble high molecular mass aggregates (600 000–1 000 000 Da) (Figure 4B2). Heat treatment of βLG solution or WPI at 90 °C and pH 6.5 induces the formation of soluble high molecular mass aggregates (Figure 4C1,C2). SDS–PAGE of βLG solutions heated at pH 6.5 or 7.5 indicated also an increasing formation of βLG polymers (Laligant et al., 1991). These polymers not dissociated by SDS correspond probably to dimers, trimers, and higher polymers formed through SH/SS interchange reaction.

CONCLUSION

The competitive RIA procedure established in this study involved polyvalent polyclonal antibodies against denatured/oxidized structure, consisting of a large panel of antibody clones, each of which reacts with a specific antigenic site. Therefore, they are expected to target the most widely variable intermediate states, which correspond to partially or totally denatured protein, and to yield useful infomation on the structure in relation to heat processing. To our knowledge, in spite of partial insolubilization of the immunogenic molecule and polyclonal characteristics of our antibodies, this is the first use of an immunserum against denatured structure offering highly specific detection and quantitation of βLG dependent on its degree of denaturation.

The test is capable of discriminating between samples of βLG receiving only small increments in the level of heat treatment, and the procedure detects the pH effect of the treatment. These findings agree with the conformational changes reported in the literature. Moreover, its application is not limited to the qualitative screening of pure solutions but appears to be suitable for heated βLG in complex media such as whey.

Finally, sample preparation protocols are much simpler and quicker than those for conventional analytical procedures: no centrifugation step is required prior to the analytical procedure to release aggregate species and prevent undesirable phenomena, such as clogging in chromatography or diffusion in spectrofluorescence (Laligant et al., 1991). Thus, the main advantage of the procedure, compared to conventional detection methods, is the possibility of studying the true states present in protein samples: soluble and insoluble fractions.

The test could be applied to control each step in food processing used to scale up the fractionation of whey proteins in industry or to prevent postproduction problems such as the chemical/biochemical degradation of proteins. In this way, these screening tools for qualitatively detecting the processing parameters could contribute tremendously to quality control. In the following years, future developments will consist of establishing structure–function relationships to predict the functionality of proteins, which is affected during manufacturing by various factors such as the denaturation state of βLG.

LITERATURE CITED

Asselin, J.; Amiot, J.; Gauthier, S. F.; Mourad, W.; Hebert, J. Immunogenicity and allergenicity of whey protein hydrolysates. *J. Food Sci.* **1988**, *53*, 1208–1211.

Bahna, S. L. Breast milk and special formulas in prevention of milk allergy. In *Immunology of Milk and the Neonate*; Mestecky, J., Blair, C., Ogra, P. L., Eds.; Plenum Press: New York, 1991; pp 445–451.

Baldo, B. A. Milk allergies. *Aust. J. Dairy Technol.* **1984**, *39*, 120–128.

Bleumink, E.; Berrens, L. Synthetic approaches to the biological activity of β-lactoglobulin in human allergy to cows' milk. *Nature* **1966**, *212*, 541–543.

Breton, C.; Phan Than, L.; Paraf, A. Immunochemical properties of native and heat denatured ovalbumin. *J. Food Sci.* **1988**, *53*, 222–225.

Cordle, C. T. Control of food allergies using protein hydrolysates. *Food Technol.* **1994**, Oct, 72–76.

De Wit, J. N. Functional milk protein. In *Developments in Dairy Chemistry: Properties of Whey Proteins*; Fox, P. F., Ed.; Elsevier Applied Science: London, 1989; pp 285–322.

De Wit, J. N.; Klarenbeek, G. A. Differential scanning calorimetric study of the thermal behaviour of bovine β-lactoglobulin at temperature up to 160 °C. *J. Dairy Res.* **1981**, *48*, 293–302.

Dumay, E. Heat denaturation of β-lactoglobulin and gelling properties of whey protein concentrates. *Cah. ENSBANA* **1988**, *6*, 67–87.

Dumay, E.; Cheftel, J-C. Emulsifying properties of whey protein concentrates. Correlations between different methods of evaluation. *Sci. Aliments* **1986**, *6*, 147–176.

Dumay, E.; Cheftel, J-C. Heat treatment of βLG concentrate at slightly alkaline pHs. Effects on the solubility and chromatographic behaviour of βLG and αLA. *Sci. Aliments* **1989**, *9*, 561–582.

Dupont, M. Comparison of the thermodenaturation of β-lactoglobulins A and B at pH 6.85. *Biochim. Biophys. Acta* **1965**, *94*, 573–575.

Friguet, B.; Djavadi-Ohaniance, L.; Goldberg, M. E. Some monoclonal antibodies raised with a native protein bind preferentially to the denatured antigen. *Mol. Immunol.* **1984**, *21*, 673–677.

Green, D. W.; Aschaffenburg, R.; Camerman, A.; Coppola, J. C.; Dunnill, P.; Simmons, R. M.; Komorowoski, E. S.; Sawyer, L.; Turner, E. M. C.; Woods, K. F. Structure of bovine β-lactoglobulin at 6 Å resolution. *J. Mol. Biol.* **1979**, *131*, 375–397.

Guesry, P. R.; Secretin, M. C.; Jost, R.; Pahud, J. J.; Monti, J. C. Hypoallergenic formulas. In *Food Intolerance in Infancy: Allergology, Immunology and Gastroenterology*; Robert, N., Ed.; Raven Press: New York, 1989; Vol. 1, pp 253–265.

Harwalkar, V. R.; Ma, C. Y. Effects of medium composition, preheating and chemical modification upon thermal behavior of oat globulin and βLG. In *Food Protein*; Kinsella, J. E., Soucie, W. G., Eds.; American Oil Chemists' Society: Champaign, IL, 1989; p 210.

Hattori, M.; Ametani, A.; Katakura, Y.; Shimizu, M.; Kaminogawa, S. Unfolding/refolding studies on bovine β-lactoglobulin with monoclonal antibodies probes. *J. Biol. Chem.* **1993**, *268*, 22214–22419.

Heppell, L. M. J. Determination of milk protein denaturation by an enzyme-linked immunosorbent assay. In *Immunoassays in Food Analysis*; Morris, B. A., Clifford, M. N.; Eds.; Elsevier Science Publishing: New York, 1985; pp 115–123.

Heppell, L. M. J.; Cant, A. J.; Kilshaw, P. J. Reduction in the antigenicity of whey proteins by heat treatment: a possible strategy for producing a hypoallergenic infant milk formula. *Br. J. Nutr.* **1984**, *51*, 29–36.

Hirs, C. H. W. The oxidation of ribonuclease with performic acid. *J. Biol. Chem.* **1956**, *219*, 611–621.

Host, A.; Samuelsson, E. G. Allergic reactions to raw, pasteurized, and homogenized/pasteurized cow milk: a comparison. *Allergy* **1988**, *43*, 113–118.

Huang, Q.; Coleman, J. W.; Stanworth, D. R. Investigation of the allergenicity of β-lactoglobulin and its cleavage fragments. *Int. Arch. Allergy Appl. Immunol.* **1985**, *78*, 337–344.

Jensenius, J. C.; Siersted, H. C.; Johnstone, A. P. Quantification of human immunoglobulins by semiautomatic polyethylene glycol precipitation radioimmunoassays. Estimation of circulating immune complexes and immunoglobin synthesized in vitro. *J. Immunol. Methods* **1983**, *56*, 19–32.

Jost, R.; Monti, J. C.; Pahud, J. J. Whey Protein allergenicity and its reduction by technological means. *Food Technol.* **1987**, *41*,118–121.

Kaminogawa, S.; Shimizu, M.; Ametani, A.; Hattori, M.; Ando, O.; Hachimura, S.; Nakamura, Y.; Totsuka, M.; Yamauchi, K. Monoclonal antibodies as probes for monitoring the denaturation process of bovine β-lactoglobulin. *Biochim. Biophys. Acta* **1989**, *998*, 50–56.

Katakura, Y.; Totsuka, M.; Ametani, A.; Kaminogawa, S. Tryptophan-19 of β-lactoglobulin, the only residue completely conserved in the lipocalin superfamily, is not essential for binding retinol, but relevant to stabilizing bound retinol and maintaining its structure. *Biochim. Biophys. Acta* **1994**, *1207*, 58–67.

Kilshaw, P. J.; Heppell, L. M. J.; Ford, J. E. Effects of heat treatment of cows' milk and whey on the nutritional quality and antigenic properties. *Arch. Dis. Childhood* **1982**, *57*, 842–847.

Kurisaki, J.; Nakamura, S.; Kaminogawa, S.; Yamauchi, K.; Watanabe, S.; Hotta, K.; Hattori, M. Antigenicity of modified β-lactoglobulin examined by three different assays. *Agric. Biol. Chem.* **1985**, *49*, 1733–1737.

Kuzmanoff, K. M.; Beattie, C. W. Isolation of monoclonal antibodies monospecific for bovine β-lactoglobulin. *J. Dairy Sci.* **1991**, *74*, 3731–3740.

Laligant, A.; Dumay, E.; Valencia, C. C.; Cuq, J. L.; Cheftel, J. C. Surface Hydrophobicity and aggregation of β-lactoglobulin heated near neutral pH. *J. Agric. Food Chem.* **1991**, *39*, 2147–2155.

MacConahey, P. J.; Dixon, F. J. Radioiodination of proteins by the use of the Chloramine T method. In *Methods in Enzymology: Immunochemical Techniques*; Van Vunakis, H., Langone, J. J., Eds.; Academic Press: New York, 1980; Vol. 70, pp 210–213.

MacKenzie, H. A. β-Lactoglobulins. In *Milk Proteins: Chemistry and Molecular Biology*; MacKenzie, H. A., Murphy, W., Eds; Academic Press: New York, 1971; Vol. II, pp 257–330.

MacKenzie, H. A.; Ralston, G. B.; Shaw, D. C. Location of sulfhydryl and disulfide groups in bovine β-lactoglobulin and effects of urea. *Biochemistry* **1972**, *11*, 4539–4547.

MacLaughlan, P.; Anderson, K. J.; Widdowson, E. M.; Coombs, R. R. A. Effect of heat on the anaphylactic-sensitising capacity of cows' milk, goats' milk and various infant formulae fed to guinea-pigs. *Arch. Dis. Childhood* **1981**, *56*, 165–171.

Malik, Z.; BottomLey, R.; Austen, B. Allergenicity of the major genetic variants of bovine β-lactoglobulin. *Biochem. Soc. Trans.* **1988**, *16*, 338–340.

Mangino, M. E. Physicochemical aspects of whey protein functionality. *J. Dairy Sci.* **1984**, *67*, 2711–2722.

Mills, O. E. Effect of temperature on tryptophan fluorescence of β-lactoglobulin B. *Biochim. Biophys. Acta* **1976**, *434*, 324–332.

Monaco, H. L.; Zanotti, G.; Spadon, P.; Bolognesi, M.; Sawyer, L.; Eliopoulos, E. E. Crystal structure of the trigonal form of bovine β-lactoglobulin and of its complex with retinol at 2.5 Å resolution. *J. Mol. Biol.* **1987**, *197*, 695–706.

Nakamura, T.; Sado, H.; Syukunobe, Y. Antigenicity of whey protein hydrolysates fractionated with ultrafiltration membrane. *Nippon Shokuhin Kogyo Gakkaishi* **1992**, *39*, 113–116.

Nakamura, T.; Sado, H.; Syukunobe, Y. Production of low antigenic whey protein hydrolysates by enzymatic hydrolysis and denaturation with high pressure. *Milchwissenschaft* **1993**, *48*, 141–145.

Okamoto, M.; Hayashi, R.; Enomoto, A.; Kaminogawa, S.; Yamauchi, K. High-pressure proteolytic digestion of food proteins: selective elimination of β-lactoglobulin in bovine milk whey concentrate. *Agric. Biol. Chem.* **1991**, *55*, 1253–1257.

Otani, H. Antigenically reactive regions of bovine milk proteins. *Jpn. Agric. Res. Q.* **1988**, *22*, 135–142.

Otani, H.; Hosono, A. Antigenic reactive regions of S-carboxymethylated βLG. *Agric. Biol. Chem.* **1987**, *51*, 531–536.

Otani, H.; Tokita, F. Changes of antigenicity of βLG upon heat treatments in the presence of milk components, L-cystein and N-ethylmaleimide. *Jpn. J. Zootech. Sci.* **1979**, *50*, 623–629.

Otani, H.; Tokita, F. Antigenicities and some properties of β-lactoglobulin heated in the presence of lactose. *Jpn. J. Zootech. Sci.* **1980**, *51*, 711–716.

Otani, H.; Morita, S. I.; Tokita, F. Antigenic reactivity of s-carboxymethylated β-lactoglobulin with antiserum to β-lactoglobulin from UHT processed milk. *Jpn. J. Zootech. Sci.* **1984**, *55*, 287–289.

Ouchterlony, O. Antigen-antibody reactions in gels. *Acta Pathol. Microbiol. Scand.* **1953**, *32*, 231–240.

Pahud, J-J.; Monti, J. C.; Jost, R. Allergenicity of whey protein: its modification by tryptic in vitro hydrolysis of the protein. *J. Pediatr. Gastroenterol. Nutr.* **1985**, *4*, 408–413.

Papiz, M. Z.; Sawyer, L.; Eliopoulos, E. E.; North, A. C. T.; Findlay, J. B. C.; Sivaprasadarao, R.; Jones, T. A.; Newcomer, M. E.; Kraulis, P. J. The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein. *Nature* **1986**, *324*, 383–385.

Park, K. H.; Lund, D. B. Calorimetric study of thermal denaturation of β-lactoglbulin. *J. Dairy Sci.* **1984**, *67*, 1699–1706.

Poulsen, O. M.; Hau, J.; Kollerup, J. Effect of homogenization and pasteurization on the allergenicity of bovine milk analyzed by a murine anaphylactic shock model. *Clin. Allergy* **1987**, *17*, 449–458.

Ratner, B.; Dworetzky, M.; Oguri, S.; Aschheim, L. Studies on the allergenicity of cow's milk. III. Effect of heat treatment on the allergenicity of milk and protein fractions from milk as tested in guinea-pigs by sensitization and challenge by oral route. *Pediatrics* **1958**, *22*, 653–658.

Reddy, M. M. M. K.; Karande, A. A.; Adiga, P. R. A Common epitope of β-lactoglobulin and serum retinol-binding proteins: elucidation of its core sequence using synthetic peptides. *Mol. Immunol.* **1992**, *29*, 511–516.

Sawyer, W. H. Heat denaturation of bovine β-Lactoglobulin and relevance of disulfide aggregation. *J. Dairy Sci.* **1968**, *51*, 323–329.

Schmidt, R. H.; Packard, V. S.; Morris, H. A. Effect of processing on whey protein functionality. *J. Dairy Sci.* **1984**, *67*, 2723–2733.

Shimizu, M. Structure of proteins adsorbed at an emulsified oil surface. In *Food Macromolecules and Colloids*; Dickinson, E., Lorient, D., Eds; The Royal Society of Chemistry: Cambridge, U.K., 1995; pp 34–41.

Spakman, D. H.; Stein, W. H.; Moore, S. Automatic recording apparatus for use in chromatography of amino acids. *Anal. Chem.* **1958**, *30*, 1190–1206.

Takahashi, T.; Yamauchi, K.; Kaminogawa, S. Comparison between the antigenicity of native and unfolded β-lactoglobulin. *Agric. Biol. Chem.* **1990**, 54, 691–697.

Taylor, S. T. Immunologic and allergic properties of cows' milk proteins in humans. *J. Food Prot.* **1986**, *49*, 239–250.

Watanabe, K.; Kyogoku, N.; Matsuda, T.; Nakamura, R. Effect of $FeCl_3$ on heat denaturation of β-lactoglobulin A in acid media. *J. Agric. Food Chem.* **1985**, *33*, 403–405.

Received for review April 3, 1995. Revised manuscript received August 15, 1995. Accepted September 7, 1995.[⊗]

JF9406368

[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1995.