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Detection of pH 4.6 Insoluble β-Lactoglobulin in Heat-Treated Milk and Mozzarella Cheese

C. MANZO,[†] R. PIZZANO,*[,†] AND F. ADDEO[†,‡]

Consiglio Nazionale delle Ricerche (CNR), Istituto di Scienze dell'Alimentazione, via Roma 52 A-C, I-83100 Avellino, Italy, and Dipartimento di Scienza degli Alimenti, Università degli Studi di Napoli Federico II, via Università 100, Parco Gussone, I-80055 Portici, Italy

Different protein aggregates including β-lactoglobulin (βlg) were detected in the pH 4.6 insoluble fraction recovered from actual heat-treated milk samples by gel electrophoresis and immunoblotting. A competitive enzyme-linked immunosorbent assay (ELISA) using anti-βlg polyclonal antibodies was developed to analyze the βlg partition in the protein fractions obtained upon acidification of both milk and Mozzarella cheese at pH 4.6. According to ELISA determinations, nearly 90% of the pH 4.6 soluble βlg included in raw milk was found in the pH 4.6 insoluble fraction of ultrahigh temperature (UHT)-treated milk. As concerns Mozzarella cheese analysis, ELISA results indicated that about 36% of the total βlg milk content was transferred from pasteurized milk to Mozzarella cheese, whereas less than 0.5% was transferred from raw milk. The pH 4.6 insoluble βlg proved to be a suitable indicator of the intensity of the heat treatment applied to milk. The ELISA-based detection of this parameter was suggested for quality control of both drinking milk and raw milk cheese.

KEYWORDS: β-Lactoglobulin; heat treatment; milk; Mozzarella cheese; thermal marker

INTRODUCTION

Thermal treatments of milk are included in most dairy processes either to ensure food safety, to extend the shelf life of the final products, or to improve functional properties of the protein components (*1*). In addition to heat-induced changes involving amino acid residues of milk proteins, mainly dephosphorylation (*2*), formation of lysinoalanine (*3*), and lactosylation (*4*), a range of large heterogeneous protein aggregates has long been recognized in heat-treated milk. The extent of protein association has been found markedly dependent upon a number of factors, including pH, protein concentration, milk composition (whey proteins/caseins ratio, salts concentration), besides time, temperature, and rate of heating (*5*). The aggregation kinetics, the co-polymer stoichiometry, and bonds involved in protein association have been intensively studied in heated model systems and in reconstituted skim milk (*6, 7*). On the basis of these reports, thermal denaturation of the major whey proteins is currently regarded as the initial step leading to the heat-induced aggregation of milk proteins. At temperatures above about 60 °C, unfolding of whey proteins results in the exposure of reactive amino acid side groups, normally buried within the native conformation. Surfacing of chemically reactive groups in thermally denatured whey proteins combined with salt bridges and other hydrophobic interactions are responsible for self-aggregation of whey proteins as well as for association of both whey proteins and their aggregates with casein micelles (*8*) and, to a lesser extent, fat globules (*9*) in heat-treated milk. As a consequence of association of whey proteins with casein micelles, the amount of whey proteins recovered from the pH 4.6 soluble fraction of milk decreases, whereas that associated with isoelectric casein increases (*10*). Thiol−disulfide exchange reactions are largely responsible for heat-induced aggregation of milk proteins, and many different thiol−disulfide interaction pathways are possible according to the content of cysteine residues of each milk protein. Heat-induced association of β-lactoglobulin (βlg), the major whey protein in bovine milk, to κ-casein through intermolecular disulfide bonds has been well-recognized (*6*), but disulfide bonding patterns in model and milk systems are different, as recently reported (*11*).

This work was focused on the βlg−casein aggregates formed in actual heat-treated milk samples to find a relationship between the amount of the pH 4.6 insoluble βlg and severity of heat treatment applied to milk. The protein aggregates included in the pH 4.6 insoluble protein fraction (IPF) recovered from commercial raw, pasteurized, and ultrahigh temperature (UHT)-treated milk samples were analyzed by gel electrophoresis and immunoblotting to identify both the main protein components and the types of bonding involved in protein association. The amount of βlg included in IPF from commercial milks was evaluated using polyclonal antibodies directed against βlg in a competitive enzyme-linked immunosorbent assay (ELISA) format. βlg content was also determined in IPF from experimental samples of Mozzarella cheese to test the reliability of the developed ELISA-based method for quality control of milk used in cheese-making.

* To whom correspondence should be addressed. Telephone: +39-825-299561. Fax: +39-825-781585. E-mail: rpizzano@isa.cnr.it.

[†] Consiglio Nazionale delle Ricerche (CNR).

[‡] Università degli Studi di Napoli Federico II.

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MATERIALS AND METHODS

Milk Sampling and Casein Preparation. Raw bovine milk was drawn from a private herd, managed according to the sanitary regulations, and treated as soon as possible at a laboratory for casein preparation. Pasteurized and UHT-treated milk were from commercial sources. Four 500 mL bottles of pasteurized milk and five 1 L bottles of UHT-treated milk, by different local farms, were used. Milk samples were centrifuged at 3939*g* for 10 min at 4 °C using a Labofuge 400R equipped with a swinging bucket rotor 4 × 180 mL, cod. 8179 (Heraeus, Germany), and the upper fat layer was manually removed. The whole casein was obtained from defatted milk by adjusting the pH to 4.6 with 1 M HCl under pHmeter control. The pellet was washed twice with 10 mM CH_3COONH_4 at pH 4.6, and then it was suspended in water. The pH was brought to neutrality with 0.2 M NaOH, and the casein solution was lyophilized. The sodium caseinate was weighed and dissolved at the desired concentration either in the sample buffer for the electrophoresis analysis or in 50 mM NH_4HCO_3 at pH 8.5 for ELISA assaying. By a preliminar vertical polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS−PAGE) analysis (see below), the four pasteurized milk samples were quite similar, as well as the five UHT-treated milk samples. One of the pasteurized milk samples and one of the UHT-treated milk samples were chosen at random and used as examples of their respective heat-treated groups in the subsequent work.

Partition of *β*lg from UHT-Treated Milk. A 4 mL aliquot of UHT-treated milk was used in the experiments reported in **Table 2**. After fat removal, the pH of the liquid phase was adjusted to 4.6 with 1 M HCl under pHmeter control. A 2 mL aliquot of supernatant was dried and dissolved in 1 mL of 50 mM NH_4HCO_3 at pH 8.5. The precipitate was washed twice with 5 mL of 10 mM ammonium acetate at pH 4.6, freeze-dried (about 120 mg were recovered), and then dissolved in 20 mL of 50 mM NH_4HCO_3 at pH 8.5. According to these procedures, solutes of the pH 4.6 soluble fraction were 2-fold-concentrated with respect to starting milk, whereas those of IPF were 5-fold-diluted.

Preparation of *β*lg. Electrophoretically pure bovine *β*lg and water buffalo *β*lg were obtained by fast protein liquid chromatography (FPLC). Whey proteins were recovered from skimmed raw milk and fractionated on a Q-Sepharose Fast Flow anion exchanger (Pharmacia, Uppsala, Sweden), according to the method described by Andrews et al. (*12*).

Production of anti-*β*lg Polyclonal Antibodies. FPLC-purified bovine *β*lg was used in Freund's adjuvant to immunize two rabbits according to current protocols (*13*). The serum collected from rabbit bleeding was filtered on 0.45 *μ*m (Millipore, Bedford, MA), split up into aliquots of 200 *μ*L, and stored at −20 °C.

Preparation of Protein Fractions from Mozzarella Cheese Samples. Regular samples of water buffalo Mozzarella cheese were supplied by a local factory (Vannulo, Capaccio Scalo, Italy). Experimental bovine Mozzarella cheese samples were from a set of samples specifically prepared as reference samples to gain recognition for the addition of dried proteins to stretched cheeses within the research project named GEFORPASTA, financed by Italian MIUR (*14*). In particular, two Mozzarella cheese samples adulterated with dried milk products were assayed. The first sample was made by adding 2.3 kg of a commercial preparation of anhydrous milk proteins to 100 L of pasteurized bovine milk, whereas the second sample was made by adding 1 kg of a commercial preparation of calcium caseinate to 100 L of pasteurized bovine milk. Taking into account that 100 L of bovine milk yielded about 12.5 kg of Mozzarella cheese, the adulterating ingredients were expected to constitute respectively 15.5% (w/w) and 7.4% (w/w) of the corresponding Mozzarella cheese samples. Cheese samples (1 g) was suspended in 100 mL of 50 mM NH_4HCO_3 at pH 8.5 and homogenized with an Ultra-Turrax (model T25, IKA Labortechnik, Staufen, Germany) at 12 000 rpm for 2 min at room temperature. After gentle mixing in a rotating device (rotating plate stirrer 712, ASAL s.r.l., Cernusco sul Naviglio, Italy) for 10−12 h at 4 °C, each suspension was centrifuged at 3939*g* for 10 min at 4 °C using a Labofuge 400R equipped with a swinging bucket rotor 4 × 180 mL, cod. 8179 (Heraeus, Germany), and fat was manually removed from the cheese solution surface. The pH 4.6 soluble and pH 4.6 insoluble cheese protein fractions were obtained by adjusting the pH of the defatted sample solutions to 4.6 with 1 M HCl under pHmeter control. The two protein fractions were freeze-dried and used for electrophoretic and ELISA analysis.

ELISA. *β*lg immunodetection was performed according to the ELISA format classified as "indirect antibody capture assay" with the antigen competition variation (*15*). A 2 *μ*g/mL solution of FPLC-purified *β*lg in 50 mM NH_4HCO_3 at pH 8.5 was used to coat microtiter plates (cod. 3911, Falcon, Oxnard, CA). After overnight incubation at 4 °C, the wells were washed twice with phosphate-buffered saline solution (PBS: 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136 mM NaCl, and 2.7 mM KCl at pH 7.4) and filled up with blocking buffer [10% (v/v) heat-inactivated horse serum in PBS] to saturate the remaining sites for protein binding. Microplates were placed at 37 °C for at least 1 h in a humid atmosphere. They were then emptied and kept at −20 °C, ready for immediate use.

Each sample was serially diluted ($1{:}4^n$, $n = 1-6$) in 50 mM NH_4HCO_3 at pH 8.5, and each dilution was analyzed in triplicate. Analysis of a standard solution of 2 mg/mL *β*lg in 50 mM NH_4HCO_3 at pH 8.5 was included in each experiment. The 50 *μ*L aliquots of the serial dilutions were introduced in each well, and then 50 *μ*L of a 1:20 000 (v/v) dilution of the antiserum in the blocking buffer was added. Maximal binding of the antibodies to the plate was determined by adding 50 *μ*L of 50 mM NH_4HCO_3 at pH 8.5 to the wells instead of antigen solution. Incubation and subsequent steps of washing, anti-rabbit enzyme-linked antibody reaction, and revelation were as previously described (*16*). The ELISA signals were recorded at 450 nm with a Benchmark microplate reader (Bio-Rad, Hercules, CA). Net absorbance of each dilution was used for *β*lg determination. It was obtained by subtracting from each mean value of ELISA absorbance the mean ELISA signal given by the *β*lg standard at the highest concentration tested (2 mg/mL). Data were elaborated using the log/logit curve fit method of Microplate Manager III Data Analysis software (Bio-Rad, Hercules, CA). The percent inhibition values plotted in **Figure 3** were obtained by applying the following formula: $(E_{max} - x) \times 100/E_{max}$, where E_{max} was the net signal obtained by testing the NH_4HCO_3 buffer and x was the net absorbance of the sample dilution.

Detection of Water Buffalo *β*lg by ELISA. The ELISA response of FPLC-purified water buffalo *β*lg was determined in comparison to that of FPLC-purified bovine *β*lg. In detail, each *β*lg sample, prepared at 2 mg/mL in 50 mM NH_4HCO_3 at pH 8.5, was serially diluted ($1{:}4^n$, $n = 1-8$) in 50 mM NH_4HCO_3 at pH 8.5. Each dilution was analyzed in triplicate using the ELISA format described above, and data were elaborated according to the log/logit curve fit method already mentioned. The ELISA curves relative to the two *β*lg samples displayed quite similar parameters (concentration and slope at inflection point, asymptote values), thus showing that water buffalo *β*lg and bovine *β*lg were equally detected by ELISA using anti-bovine *β*lg polyclonal antibodies as immunoreagents.

SDS−PAGE Analysis: Electrophoresis and Immunoblotting. SDS−PAGE was performed as described by Di Luccia et al. (*17*). For immunoblotting analysis, after electrophoresis, the proteins were electrically transferred from the gel onto nitrocellulose paper as described by Towbin et al. (*18*). Immunodetection was carried out using rabbit anti-*β*lg antiserum as the primary reagent and horseradish peroxidase-labeled goat anti-rabbit IgG polyclonal antibodies as the secondary reagent (BioRad, Hercules, CA). The procedure for bands immunostaining was essentially that already described (*19*).

RESULTS AND DISCUSSION

Protein Aggregates Including *β*lg in Heat-Treated Milk: An Overview. Upon heating milk above 60 °C, the whey proteins increasingly lose their globular conformation, associate with the casein micelles through covalent or hydrophobic interactions, and precipitate together with the caseins upon acidification to pH 4.6. SDS−PAGE analysis of covalently linked protein aggregates included in IPF recovered from different heat-treated milks was shown in **Figure 1**. In addition to the conventional incubation in both denaturing and reducing

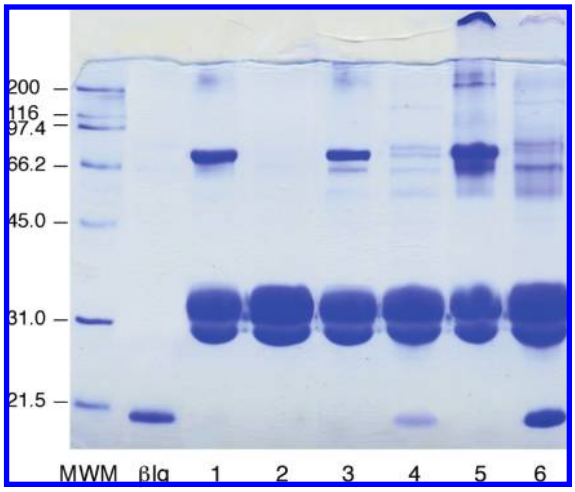

Figure 1. SDS−PAGE analysis of IPF recovered from raw, pasteurized, and UHT-treated milk and incubated in the sample buffer lacking β-mercaptoethanol (lanes 1, 3, and 5, respectively) or in the conventional sample buffer including β-mercaptoethanol (lanes 2, 4, and 6, respectively). A mixture of proteins at known molecular weight (lane MWM) and FPLC-purified βlg (lane βlg) were used as reference. The molecular weight of markers was given in kilodaltons. The gel was stained with Coomassie Brilliant Blue R-250.

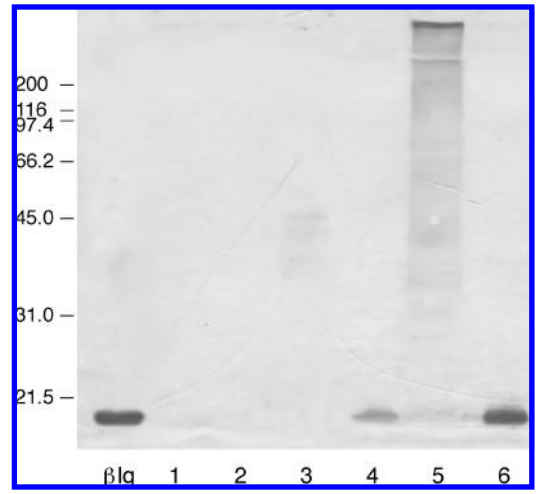

Figure 2. Immunodetection of βlg along the SDS−PAGE gel shown in **Figure 1**. Purified βlg (lane βlg) and IPF recovered from raw without (lane 1) or after (lane 2) reduction with β-mercaptoethanol, from pasteurized milk without (lane 3) or after (lane 4) reduction and from UHT-treated milk without (lane 5) or after (lane 6) reduction were analyzed. Immunostaining was carried out using 1:5000 (v/v) diluted rabbit antiserum raised against bovine βlg as a primary reagent and 1:2000 (v/v) diluted horseradish-peroxidase-labeled goat anti-rabbit IgG antibodies as a secondary reagent.

conditions, each sample was incubated in a modified sample buffer lacking β-mercaptoethanol to preserve protein aggregates held by disulphide bonds. The monomer caseins migrated by SDS−PAGE as thick bands in the 30−35 kDa range. A major band migrating as a protein of about 70 kDa was detected in denatured IPF from raw milk (lane 1), but it disappeared by incubating the sample with β-mercaptoethanol (lane 2) without generating any novel band along the profile. This finding was consistent with previous literature reports indicating that κ-casein and α_{s2}-casein can assemble in raw milk through disulfide bridges (*20*). Protein components with an apparent molecular weight higher than those of casein monomers were also detected along the electrophoretic profiles of pasteurized milk (lane 3), and, to a higher extent, of UHT-treated milk (lane 5). These protein aggregates were partly sensitive to β-mercaptoethanol-reducing action (lanes 4 and 6), thus confirming that other covalent bonds are responsible for protein aggregation in heated milk in addition to disulfide bonds (*21*). By pre-incubating samples with β-mercaptoethanol, IPF of pasteurized milk (lane 4) and, to a higher extent, IPF of UHT-treated milk (lane 6) released a 18 kDa protein component displaying similar mobility of βlg purified by FPLC from bovine milk (lane βlg). Immunoblotting analysis using anti-βlg polyclonal antibodies confirmed that these 18 kDa protein bands corresponded to monomeric βlg, as shown in **Figure 2** (lanes βlg, 4, and 6). These βlg bands clearly originated from the protein aggregates detected by anti-βlg polyclonal antibodies along the electrophoretic profile of nonreduced IPF from pasteurized and UHT-treated milk (lanes 3 and 5, respectively). Apart from the different total βlg amount recovered by reducing the heat-induced protein aggregates included in IPF from pasteurized and UHT-treated milk, composition of these aggregates in the two nonreduced samples appeared quite different. Faint bands migrating in the 50−70 kDa area were immunostained in the profile relative to pasteurized milk, whereas a major signal of βlg immunodetection was obtained at the top of the stacking gel of the profile relative to the UHT-treated milk sample, thus showing that βlg was mostly associated to protein complexes having a molecular weight higher than 200 kDa (lane 5). By

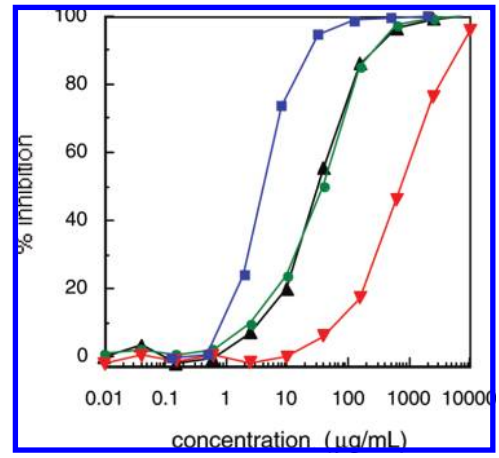

Figure 3. Competitive ELISA inhibition curve of IPF recovered from raw (■) and UHT-treated milk (▲). A not previously centrifuged sample from UHT-treated milk was also tested (●) for a step-by-step control of the sample preparation (see the text). All of the samples were dissolved at 10 mg/mL in 50 mM NH_4HCO_3 at pH 8.5, and $1:4^n$ serial dilutions of each sample solution were assayed in duplicate. Native βlg was used as a positive reference (▼). In this experiment, 2 mg/mL βLg was considered as blank (ELISA signal = 0.035 ± 0.004), and E_{max}, obtained by testing the buffer alone, was 1.526 ± 0.020. The experimental procedure was described under the Materials and Methods. Concentration values of βlg were calculated using the Microplate Manager III software and reported in **Table 1**.

contrast, no protein band containing βlg was immunostained in IPF from raw milk, neither in the nonreduced sample (lane 1) nor in the reduced sample (lane 2). On the whole, these results indicated that βlg was almost exclusively found associated via disulfide bridges to casein aggregates in actual heat-treated milk, giving rise to supramolecular complexes displaying up to an apparent molecular weight higher than 200 kDa in UHT-treated milk. Both the amount and the composition of the βlg−casein aggregates were heavily dependent upon severity of the heat treatment applied to milk. It was worthy of remark that disulfide-

Table 1. βlg Concentration in the 10 mg/mL IPF Solutions Assayed in the Competitive ELISA Reported in **Figure 3**

sample	βlg concentration (μg/mL)
raw milk	41.5 ± 1.4
UHT-treated milk	974.9 ± 32.9
UHT-treated milk without centrifugation	974.6 ± 33.0

Table 2. Partition of βlg between the Two Fractions Recovered from UHT-Treated Milk after Precipitation at pH 4.6[a]

sample	βlg concentration in the sample solution (μg/mL)	βlg concentration in starting milk (mg/mL)
pH 4.6 soluble fraction	412 ± 31	0.206
pH 4.6 insoluble fraction	580 ± 43	2.900

[a] βlg concentration values obtained by ELISA analysis of the samples prepared as described in the Materials and Methods are shown in the second column, while βlg concentration values referred to starting UHT-treated milk are in the third column.

bonded products including βlg were different not only in a model system with respect to a milk system, as previously reported (*11*), but also in differently heat-treated milk systems.

ELISA Detection of the pH 4.6 Insoluble βlg in UHT-Treated Milk. In compliance with the results reported above, the pH 4.6 insoluble βlg could be used as a thermal marker in processed milk, but an analytical method able to detect specifically βlg, regardless of the composition of the protein aggregate to which it was linked, was required. Therefore, a competitive ELISA using anti-βlg polyclonal antibodies as reagent was developed, and IPF recovered from UHT-treated milk was assayed in comparison to IPF from raw milk. FPLC-purified βlg was assayed as a positive reference and used as a standard for ELISA determinations. The dose−response ELISA inhibition curves were reported in **Figure 3**, and the concentration of βlg in the samples calculated according to the data analysis performed by Microplate Manager III software was reported in **Table 1**. The trace amounts of βlg found in the sample from raw milk was likely due to co-precipitation with isoelectric casein. However, such an amount was so low that it was not detected in the electrophoresis and immunoblotting experiments reported in **Figures 1** and **2**. A significant amount of βlg, nearly corresponding to its total content in milk, was found in IPF recovered from UHT-treated milk. Indeed, it is currently acknowledged that βlg accounts for about 10% of the total milk protein content, that is an average of a 3.2 mg/mL βlg is expected in milk containing an average of 33 mg/mL total proteins (*22*). According to the present results, βlg content was slightly lower than 10% of IPF recovered from a milk sample, that is, 0.975 mg/mL with respect to 10 mg/mL. It is worth noting that IPF recovered from milk samples had been dissolved in 50 mM NH_4HCO_3 at pH 8.5 and routinely centrifuged before assaying, as described in the Materials and Methods. In principle, this procedure was adopted to avoid any interference because of possible microprecipitates dispersed in the pH 8.5 solution. However, the occurrence of any βlg-including microprecipitate was definitively excluded because similar results were obtained for the sample from UHT-treated milk, regardless of centrifugation (**Figure 3** and **Table 1**). The βlg partition between the two protein fractions obtained by precipitating skimmed UHT-treated milk at pH 4.6 was more accurately evaluated by ELISA (**Table 2**). Only 6.4% of the βlg amount currently found in the soluble fraction from raw milk was recovered in the soluble fraction from UHT-treated milk, whereas the major part of βlg (more than 90%) moved to IPF because of UHT treatment of milk, as previously assessed. These figures of residual pH 4.6 soluble βlg in processed milk were in close agreement with the values recently reported (*23*).

Table 3. Amount of βlg Detected by ELISA in IPF Recovered from 1 g of Mozzarella Cheese Produced from Raw and Different Pasteurized Milks

starting milk	addition to milk before cheese-making	βlg amount (mg)
raw bovine milk	none	0.098 ± 0.007
pasteurized bovine milk	none	9.096 ± 0.392
	2.3 kg of A in 100 L of milk	11.947 ± 0.516
pasteurized bovine milk	none	9.449 ± 0.480
	1 kg of B in 100 L of milk	8.413 ± 0.350
raw water buffalo milk	none	0.121 ± 0.004

(A) Anhydrous milk proteins containing 12.55% (w/w) of βlg (1.255 ± 0.171 mg/mL of βlg were determined in a 10 mg/mL solution). (B) Calcium caseinate containing 0.27% (w/w) of βlg (27 ± 3 μg/mL of βlg were determined in a 10 mg/mL solution).

ELISA Detection of the pH 4.6 Insoluble βlg in Mozzarella Cheese Samples. According to the above-reported results, βlg occurring in IPF from UHT-treated milk might be regarded as a marker of the heat treatment. Further work is in progress to validate this novel ELISA-based analytical procedure for evaluating heat-treatment degree of drinking milk. Because βlg resulted to be linked to caseins in heat-treated milk, it was expected to be included in IPF of cheese produced from heat-treated milk. Mozzarella cheese was used as a model cheese system to evaluate the pH 4.6 insoluble βlg content (**Table 3**). A large set of experimental bovine Mozzarella cheese samples (see the Materials and Methods) was assayed. The pH 4.6 insoluble βlg content of bovine Mozzarella cheese produced from raw milk was determined by ELISA and compared to that of different experimental Mozzarella cheese samples from pasteurized bovine milk. Results reported in **Table 3** indicated that even pasteurization produced a significant increase, e.g., 93−96 times, of the pH 4.6 insoluble βlg content of cheese with respect to that from raw milk. Taking into account that 100 L of bovine milk yielded 12.5 kg of Mozzarella cheese, percentages as high as 35−37% (w/w) of βlg included in pasteurized milk were transferred to cheese. Responsiveness of the present analytical procedure was checked by assaying additional Mozzarella cheese samples obtained from pasteurized milk spiked with two different dried milk products, one consisting of anhydrous milk proteins and the other consisting of calcium caseinate, as specified in the footnotes to **Table 3**. The βlg content in the adulterated Mozzarella cheese samples, as determined by ELISA, was consistent with the amount and the composition of the dried milk product added to milk.

In addition, commercially available water buffalo Mozzarella cheese, registered as a product of designated origin (PDO), produced in compliance with standard production regulation (*24*), was assayed as an actual raw milk cheese reference. The same ELISA procedure could be used, because polyclonal antibodies raised against bovine βlg were able to recognize water buffalo βlg as well as bovine βlg (see the Materials and Methods), likely thanks to the strict homology existing between the two βlg amino acid sequences (*25*). The βlg content in the Mozzarella cheese sample from raw water buffalo milk was very much like that determined in the cheese sample from raw bovine milk.

At last, a nearly similar βlg amount, accounting for about 0.1% (w/w) of cheese (e.g., 1.065 ± 0.061 mg of βlg was detected in 1 g of the regular sample obtained from water buffalo

raw milk), was found in the pH 4.6 soluble fraction of Mozzarella cheese samples. Most likely this amount could be regarded as the residual βlg derived from 4% partition of soluble proteins occurring at separation of curd and whey during cheese manufacture (*26*).

On the whole, these results indicated that ELISA detection of βlg linked to caseins via disulfide links might be appropriate for evaluating *a posteriori* intensity of thermal treatments applied to both drinking milk and milk processed in Mozzarella cheesemaking. In principle, such a method could be used for quality control of all of cheeses traditionally made from raw milk, representing a significant proportion of cheeses produced in Southern Europe (e.g., Swiss, Cheddar, Manchego, Raclette, St. Paulin, in addition to water buffalo Mozzarella cheese, used as a model of raw milk cheese in this work). It is worth noting that even milk pasteurization, altering indigenous milk microflora, can affect the specific sensory characteristics of traditional raw milk cheeses, as reviewed by Grappin and Beuvier (*27*).

The analytical approach proposed in this paper was based on differences in milk protein trim because of heat treatments, as demonstrated by electrophoretic and immunoblotting results. In all respects, the pH 4.6 insoluble βlg can be considered a marker of early protein modifications occurring in milk following thermal treatments. It proved to be a marker especially valuable because it allowed us to distinguish between raw and pasteurized milk, whereas novel haptenic structures rising from non-enzymatic lactosylation of milk proteins according to the early Maillard reaction did not (*16*). After all, the test consists of imunochemical detection of pH 4.6 insoluble βlg. It is easy to use and can be performed in any laboratory basically equipped. Highly purified βlg used as a standard and anti-βlg polyclonal antibody preparations used as a reagent are commercially available. In addition, sample preparation requires only recovery of the pH 4.6 precipitate fraction from either milk or cheese samples.

ABBREVIATIONS USED

βlg, β-lactoglobulin; IPF, pH 4.6 insoluble protein fraction recovered from milk; UHT, ultrahigh temperature; FPLC, fast protein liquid chromatography; ELISA, enzyme-linked immunosorbent assay; SDS−PAGE, vertical polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; PBS, phosphate-buffered saline solution, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136 mM NaCl, and 2.7 mM KCl at pH 7.4.

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