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Occurrence of Major Whey Proteins in the pH 4.6 Insoluble Protein Fraction from UHT-Treated Milk

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Supporting Information

ABSTRACT: A clear picture of the protein rearrangement in milk following UHT-treatment was drawn by a comparative analysis of the pH 4.6 soluble protein fraction (SPF) and the pH 4.6 insoluble protein fraction (IPF) recovered from raw and UHT-treated milk samples. The two protein fractions were analyzed by mono- or bidimensional gel electrophoresis under reducing and nonreducing conditions, and protein bands were identified by specific immunostaining. Results showed that bovine serum albumin, β-lactoglobulin, and, to a lesser extent, α-lactalbumin coprecipitated with caseins in UHT-treated milk samples at pH 4.6. These proteins were almost exclusively involved in high molecular weight aggregates held together by disulfide bonds. Partition of α-lactalbumin and bovine serum albumin in the protein fractions obtained upon acidification of milk at pH 4.6 was evaluated by competitive immunoassays. The ELISA-based results suggested the possibility of using pH 4.6 insoluble α-lactalbumin and bovine serum albumin, in addition to pH 4.6 insoluble β-lactoglobulin, as indicators of the intensity of the heat treatment applied to milk.

KEYWORDS: *milk, whey proteins, UHT-treatment, protein aggregates, thermal marker*

INTRODUCTION

Thermal treatment of milk is included in most dairy processes for both hygienic and technological reasons. A number of changes in the protein component of heat-treated milk have long been described. They are of considerable interest to dairy researchers as they can affect nutritional and technological features of milk. Heating can induce many chemical reactions involving specific amino acid residues of single milk proteins, mainly dephosphorylation of phosphoserine residues,[1] lactosylation of ε-amino groups of lysine residues,[2] and deamidation of asparagine residues[3] besides proteolysis.[4] In addition, heating can modify the overall protein organization by inducing the formation of large aggregates of milk proteins.[5] In raw milk, caseins, accounting for about 80% of the total milk protein content, are associated to form supramolecular assemblies, named casein micelles, which are in dynamic equilibrium with the aqueous phase of milk containing soluble globular proteins, essentially β-lactoglobulin (βlg), α-lactalbumin (αla), immunoglobulins, and bovine serum albumin (BSA), collectively called whey proteins. Both caseins and whey proteins are included in milk protein aggregates found in heat-treated milk.[5] Extensive research has been dedicated to the understanding of the heat-induced protein association occurring in differently heated milk or milk fractions as well as in model systems, as recently reviewed in ref 6. Milk protein components in most heat-induced aggregates are linked through intermolecular disulfide bonds. Thermal denaturation of whey proteins is currently regarded as the initial step of the complex series of reactions leading to the formation of these disulfide-linked protein aggregates. Thiol groups of cysteine residues, normally buried within the native conformation of whey proteins, but exposed in thermally unfolded whey proteins, act as building agents on hydrophobically linked intermediate stages via thiol-disulfide exchange reactions. A very effective description of the role played by the free thiol group of βlg in self-aggregation of thermally denatured βlg in water has been provided by analogy with a radical-mediated polymerization reaction.[7] Additional protein covalent association can occur via dehydroalanine formed by heat-induced β-elimination of phosphate from the phosphoserine residues included in caseins.[8] Dehydroalanine can react with other amino acid residues, mainly lysine, but also histidine and cysteine residues, giving rise to further intra- and intermolecular protein cross-linking.[9] Detailed information about heat-induced changes in the protein components of heat-treated milk, mainly concerning lactosylation, deamidation, and disulfide- as well as nondisulfide-mediated covalent protein aggregation has been provided by the analysis of the milk proteome, based on bidimensional gel electrophoresis coupled to mass spectrometry.[10−12] It is worth noting that all of the proteomic studies have been carried out using entire milk samples, which normally retain different amounts of soluble whey proteins depending on the intensity of the heat-treatment applied to milk. However, whey proteins engaged in disulfide-bonded heat-induced protein aggregates have been distinguished from residual soluble whey proteins by comparing bidimensional electrophoresis patterns of the entire milk samples in reducing/nonreducing conditions.[13] By contrast, possible whey proteins hydrophobically associated with heat-induced protein aggregates cannot be selectively recognized in

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entire milk samples using current proteomic tools. Indeed, if whey proteins were included in noncovalent intermediate aggregates in the entire milk samples, they would be released in the denaturing conditions of bidimensional electrophoresis as monomers electrophoretically indistinguishable from residual soluble whey proteins. Involvement of whey proteins in heat-induced protein aggregates can be easily studied by analyzing separately the two protein fractions obtained upon acidification of milk samples at pH 4.6. It is well-known that the amount of pH 4.6 soluble whey proteins decreases while that of pH 4.6 insoluble whey proteins increases as a consequence of heat-induced association of whey proteins with caseins.[14] By analyzing the two protein fractions separated upon acidification of heat-treated milk samples at pH 4.6, a gradual conversion of βlg of the pH 4.6 soluble fraction (SPF) of raw milk into pH 4.6 insoluble forms has been observed by increasing the intensity of milk heating from pasteurization to ultrahigh temperature (UHT)-treatment.[15] The results have shown that βlg content in the pH 4.6 insoluble fraction (IPF) is closely related to the intensity of the heat-treatment. Moreover, βlg partition between the protein fractions has been evaluated by immunochemical tools, and more than 90% of the amount of native βlg of raw milk has been found in IPF of UHT-treated milk.

The present work was directed to check if αla and BSA, similarly to βlg, were included in IPF recovered from UHT-treated milk and to define their bonding pattern. IPF and SPF recovered from raw and UHT-treated milk were separated by mono- and bidimensional gel electrophoresis in reducing/nonreducing conditions, and patterns were specifically immunostained to obtain information about the chemistry of major whey proteins of UHT-treated milk. Moreover, partition of αla and BSA between the two protein fractions obtained by splitting milk proteins on the basis of their solubility at pH 4.6 was estimated by an enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Milk Sampling and Preparation of Protein Fractions. Raw bovine milk was obtained from a private herd and managed according to the sanitary regulations. Five 1 L bottles of UHT-treated milk, produced by different Italian farms, were from commercial sources and legally provided before the expiry date, that is 90 days from the packaging date. A 10 mL aliquot of each milk sample was used as soon as possible to prepare the two protein fractions SPF and IPF. Each milk aliquot was centrifuged at 3940*g* for 10 min at 4 °C using a Labofuge 400R (Heraeus, Germany), and the upper fat was manually removed. The pH of each skimmed milk sample was adjusted to 4.6 by adding 1 M HCl under pH-meter control, and the resulting suspension was centrifuged at 3940*g* for 10 min at 4 °C. A 4 mL aliquot of the supernatant was freeze-dried and dissolved in 2 mL of 50 mM NH_4HCO_3 at pH 8.5. The resulting solution was used as SPF of the milk sample. The precipitate was washed twice with 10 mM sodium acetate at pH 4.6, then suspended in water, and dissolved by bringing the pH to neutrality with 0.2 M NaOH. After an additional centrifugation to remove any residual pellet, the supernatant was lyophilized and then dissolved using 50 mM NH_4HCO_3 at pH 8.5 up to a final volume of 50 mL. The resulting solution was used as IPF of the milk sample. According to this procedure, solutes of SPF were 2-fold-concentrated with respect to starting milk, whereas those of IPF were 5-fold-diluted. Both SPF and IPF recovered from the five UHT-treated milk samples were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS−PAGE). Profiles of SPF from the different UHT-treated milk samples were quite similar as well as those of IPF. Analysis of SPF and IPF from one of these milk samples, chosen at random, was reported in the present paper.

Immunoreagents. Anti-BSA antibody preparation was purchased from Sigma (Saint Louis, MO). Polyclonal antiβlg antibodies were as previously described.[15] Antiαla antiserum was obtained by Primm (Milano, Italy) using electrophoretically pure bovine αla as immunogen in current immunization protocols.[15] The antisera recognizing single caseins were produced by Primm (Milano, Italy) using the antipeptide antibody technology, according to the procedure previously described.[16] In particular, four peptides, reproducing the sequence of mature bovine κ-casein (f92−111), α_{s2}-casein (f193−207), β-casein (f195−209), and α_{s1}-casein (f186−199), with one cysteine residue added to their N-terminus, were chemically synthesized, HPLC purified and linked to ovoalbumin. Each coupled product was used to immunize two rabbits. All of the antisera were filtered on 0.45 μm (Millipore, Bedford, MA), split up into aliquots of 200 μL, and stored at −20 °C.

Electrophoresis and Immunoblotting. Vertical polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS−PAGE) was performed as previously described.[17] To improve resolution of protein aggregates, bidimensional SDS−PAGE was performed according to the procedure reported by Havea et al.[18] Briefly, nonreduced samples were analyzed in duplicate in the first dimension by SDS−PAGE. After the run, the gel was removed from the glass plates and cut so that each strip corresponded to a single electrophoretic profile. One of the two strips relative to each sample was Coomassie-stained to control protein separation. The other strip was incubated in 5 mL of SDS sample buffer containing 1% (v/v) β-mercaptoethanol. After reduction of disulfide bonds, the strip was thoroughly washed with distilled water to remove β-mercaptoethanol, then the strip was placed in a large well of a second SDS−PAGE gel, perpendicular to the spacers. For immunoblotting analysis, SDS−PAGE profiles were electrically transferred (100 V for 30 min at 4 °C) from the gel onto a nitrocellulose paper previously rinsed in 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). The paper was then held in blocking buffer (10% v/v heat-inactivated horse serum in PBS) for at least 1 h at room temperature. Immunodetection was carried out by incubating the paper with the appropriate rabbit antiserum at a 1:1000 dilution in blocking buffer for 1 h at room temperature and, after three washes with blocking buffer, with horseradish peroxidase-labeled goat antirabbit polyclonal antibodies (BioRad, Hercules, CA) at a 1:2000 dilution in blocking buffer for 1 h at room temperature. For protein band detection, the nitrocellulose paper was placed in 10 mM Tris-HCl, pH 7.5, containing 0.5 mg/mL of 3,3-diaminobenzidine (Fluka, Buchs, Switzerland) and 0.06% (w/v) $NiCl_2$ hexahydrate (Fluka). To start the reaction between horseradish peroxidase-labeled goat antirabbit IgG antibodies and 3,3-diaminobenzidine, 100 μL of 30% (v/v) hydrogen peroxide was added to 20 mL of solution. Incubation of the nitrocellulose membrane was performed at room temperature with agitation and examined periodically until the protein bands became suitably dark (1 to 5 mins). The reaction was stopped by rinsing the nitrocellulose membrane with distilled water.

Recovery of Protein Aggregates from the Stacking Gel after SDS−PAGE Analysis. An aliquot of 100 μL of IPF from UHT-treated milk, diluted 1:1 with the SDS−PAGE sample buffer without β-mercaptoethanol, was loaded in the large well of a comb for bidimensional SDS−PAGE (thickness, 0.75 mm). After the electrophoretic run, the stacking gel was cut and transferred to a tube containing 100 μL of 1% (v/v) β-mercaptoethanol in 9 M urea. After 1 h at 37 °C, 100 μL of SDS sample buffer was added, the suspension was accurately mixed, and the liquid phase was recovered. Aliquots of 20 μL of this solution were analyzed by SDS−PAGE in comparison with 5 μL of IPF from both UHT-treated milk and raw milk, diluted 1:1 with the SDS−PAGE sample buffer.

Competitive ELISA. Microtiter plates (cod. 3911, Falcon, Oxnard, CA) were coated using a solution 1 μg/mL of αla (Sigma, Saint Louis, MO) in 50 mM NH_4HCO_3, pH 8.5, for αla immunodetection and a solution 0.4 μg/mL of BSA (Sigma, Saint Louis, MO) in 50 mM

NH_4HCO_3, pH 8.5, for BSA immunodetection. After overnight incubation at 4 °C, the wells were washed twice with PBS 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 utilization.

Each sample was serially diluted ($1:4^n$, n = 1−9) in 50 mM NH_4HCO_3 at pH 8.5, and each dilution was analyzed in duplicate. Analysis of a standard solution was included in each experiment (0.8 mg/mL αla in 50 mM NH_4HCO_3 at pH 8.5 for the αla assay and 0.4 mg/mL BSA in 50 mM NH_4HCO_3 at pH 8.5 for the BSA assay). The 50 μL aliquots of the serial dilutions were introduced in each well, and then 50 μL of a dilution of the antiserum in the blocking buffer was added (1:2000 (v/v) antiαla antiserum for αla assay and 1:10000 (v/v) anti-BSA antiserum for BSA assay). Maximal binding of the antibodies to the plate was determined by adding to the wells 50 μL of 50 mM NH_4HCO_3 at pH 8.5 instead of the antigen solution. Incubation and subsequent steps of washing, antirabbit 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, USA). Net absorbance of each dilution was used for ELISA determinations. It was obtained by subtracting from each mean value of ELISA absorbance the mean ELISA signal given by the standard solution at the highest concentration tested. Data were elaborated using the log/logit curve fit method of Microplate Manager III Data Analysis software (Bio-Rad, Hercules, CA).

RESULTS AND DISCUSSION

Protein Aggregation in Heat-Treated Milk. A descriptive picture of the composition of the two protein fractions recovered upon acidification of raw and UHT-treated milk samples at pH 4.6 was obtained by monodimensional SDS−PAGE electrophoresis as shown in Figure 1. Each sample was analyzed according to conventional SDS−PAGE protocols in both denaturing and reducing conditions (panel A) but also in nonreducing conditions, thus preserving cross-linked protein aggregates held together by disulfide bonds (panel B). As specified in the legend to the Figure 1, equal volumes of SPF and IPF from raw and UHT-treated milk samples were loaded on the gels to compare the protein content of each fraction from the two milk samples. The total amount of whey proteins included in SPF of raw milk (lane 1, panel A) was clearly higher than that found in SPF recovered from UHT-treated milk (lane 2, panel A). This result is in agreement with previous reports about heat-induced coprecipitation of whey proteins with casein micelles,[14] and it was mainly due to heat-induced aggregation of βlg with the casein micelles, as previously determined by ELISA.[15] Analysis by densitometry of the monodimensional SDS−PAGE profiles indicated that significant amounts of the other two minor whey proteins, that is, αla and BSA, in addition to βlg, switched from SPF to IPF following UHT-treatment applied to milk (from lane 1 to lane 4 of panel A). Apart from monomeric caseins migrating as thick bands in the 30−35 kDa range along the profiles of IPF of both milk samples, higher mass bands were detected only in the profile of IPF from the UHT-treated milk sample (lane 4, panel A). Occurrence of β-mercaptoethanol-resistant high molecular weight protein aggregates composed of 50−80-kDa proteins confirmed that other covalent bonds are responsible for heat-induced protein aggregation in milk in addition to disulfide bonds.[8,9] By immunostaining SDS−PAGE profiles with casein-specific polyclonal antibodies, α_{s1}-casein was mainly detected in the protein aggregates resistant to reducing conditions (results shown in Figure S1, Supporting Information). By comparing

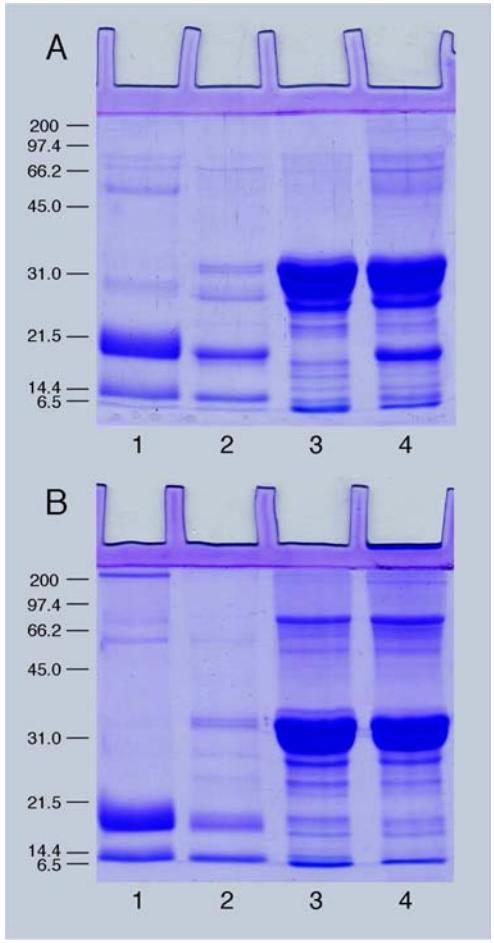

Figure 1. SDS−PAGE analysis of 2 μL of SPF from raw milk (lane 1), UHT-treated milk (lane 2), 10 μL of IPF from raw milk (lane 3), and UHT-treated milk (lane 4) in both denaturing and reducing conditions (panel A) or in denaturing and nonreducing conditions (panel B). SPF and IPF were obtained as described in Materials and Methods. A mixture of eight standard proteins at known molecular weight was run in a separate lane of each gel. The protein bands detected in the electrophoretic profile of this mixture were schematically reported to the side of each panel and labeled with the respective molecular weight (given in kDa). The gels were stained with Coomassie Brilliant Blue R-250.

the gel patterns of A and B panels, some interesting differences were observed. A similar cluster of protein bands in the 50−80 kDa range of the profile of denatured IPF from both raw and UHT-treated milk (lanes 3 and 4, panel B) was observed, but the proteins found in the profile of IPF from raw milk were sensitive to the β-mercaptoethanol reducing action as they were almost missing in the samples analyzed in panel A. Immunoblotting analysis using casein-specific polyclonal antibodies showed that these protein bands were mainly made up of α_{s2}-casein (results shown in Figure S1, Supporting Information), only in partial agreement with previous literature reports. Indeed, homopolymers of both α_{s2}-casein and κ-casein assembled through disulfide bridges have been detected in milk in native conditions and under various environments and heat-treatments.[19] More recently, occurrence of additional disulfide-linked κ-casein and α_{s2}-casein heteropolymers in heat-induced milk protein aggregates has been suggested by proteomic studies.[12] By contrast, κ-casein was poorly immunorecognized in the 50−80 kDa range only along the profile of IPF from

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UHT-treated milk (κ-casein immunostaining of these samples was shown in panel E of Figure 3, lanes 1 to 4). It must be pointed out that κ-casein immunodetection along the SDS−PAGE profiles was based on the use of antipeptide antibodies. As previously observed,[19] protein recognition by antipeptide antibodies is dependent on the protein conformation. It is possible that κ-casein polymerization altered the native antibody-reactive conformation of the protein so that the target sequence stretch became hardly accessible to the antibodies. As already observed in the analysis of reduced samples (lanes 1 and 2 of panel A), the total amount of whey proteins included in SPF from raw milk decreased as a result of the UHT-treatment of milk (lanes 1 and 2 of panel B), but no switch of βlg, αla, and BSA from SPF of raw milk to IPF of UHT-treated milk was observed using nonreduced samples. In compliance with previous reports,[13] immunoglobulins migrated as the sharp protein band at an apparent molecular weight close to 200 kDa in the profile of SPF from the nonreduced raw milk sample (lane 1, panel B). This band disappeared in SPF from UHT-treated milk, and apparently, it did not generate any novel band along the profile of IPF from UHT-treated milk. By contrast, protein aggregates having a molecular weight higher than 200 kDa were stained at the top of the stacking gel of this profile (lane 4, panel B). These aggregates are clearly held together by disulfide bonds, as similar bands did not occur in the profile of reduced IPF from UHT-treated milk samples. Finally, monomeric caseins recovered as pH 4.6 soluble forms in both reduced and nonreduced SPF from UHT-treated milk (lanes 2 of panels A and B) likely raised from a partial heat-induced disruption of casein micelles.

Disulfide Protein Complexes in Heat-Treated Milk. Bidimensional SDS−PAGE analysis, performed according to the procedure reported by Havea et al.,[18] provided additional valuable information about the composition of disulfide-linked protein complexes in UHT-treated milk. The first dimension electrophoretic profile of IPF from the UHT-treated milk sample was incubated with β-mercaptoethanol and loaded onto an additional polyacrylamide gel for the run in the second dimension (Figure 2). The reduced sample analyzed in the first dimension profile was taken as reference for the second dimension electrophoretic run (lane RS). A protein band migrating as a monomeric casein was released from the 50−80 kDa bands of the first dimension SDS−PAGE profile, but residual protein aggregates were still stained in the second dimension SDS−PAGE profile. In the bidimensional SDS−PAGE analysis of IPF from raw milk, only monomeric caseins were obtained from the 50−80 kDa bands (results not shown). With regard to the 50−80 kDa area of the first dimension profile, bidimensional SDS−PAGE analysis confirmed immunoblotting results of the monodimensional SDS−PAGE. By contrast, the βlg monomer was not detected in the bidimensional map of IPF from UHT-treated milk, although the βlg band was clearly stained in the profile of the reference reduced sample. Equally, no band with the apparent molecular weight of αla (14 kDa) was detected, whereas no substantial information about BSA (66 kDa) could be drawn from this analysis because of comigration in the same gel area of the 50−80-kDa casein aggregates. These results clearly demonstrated that IPF from UHT-treated milk included βlg and αla, but neither as free monomers nor as components of protein aggregates smaller than 200 kDa. Seemingly, these whey proteins in nonreducing conditions were tightly packaged into the large aggregates found at the top of the stacking gel of the first dimension SDS−PAGE gel. However, βlg and αla bands were not at all recognized in the bidimensional SDS−PAGE map, although the stacking gel was included in the first dimension electrophoretic profile incubated with β-mercaptoethanol and loaded onto the second dimension gel. Failure to detect βlg and αla in the bidimensional SDS−PAGE map might be due either to a fast reassemblage of the protein aggregates in the first dimension profile after removal of β-mercaptoethanol or to the release of the single components from the protein aggregates during incubation and/or washes of the first dimension profile. To verify the occurrence of major whey proteins in these large aggregates, the protein components recovered from the stacking gel of the nonreduced IPF from UHT-treated milk, after SDS−PAGE and incubation with β-mercaptoethanol, were analyzed by SDS−PAGE and immunoblotting (Figure 3). In addition to antiβlg and antiαla antibodies, anti-BSA antibodies were used to highlight possible involvement of BSA in protein aggregates in UHT-treated milk, thus overcoming problems concerning the recognization of BSA along the Coomassie-stained SDS−PAGE profiles mainly due to the occurrence of the 65−70-kDa casein aggregates. The same immunoblotting analysis was carried out also using polyclonal antibodies against κ-, β-, α_{s1}-, and α_{s2}-casein. As shown in Figure 3, the sample recovered from the stacking gel of the nonreduced IPF from UHT-treated milk included βlg (panel B), αla (panel C), BSA (panel D), and κ-casein (panel E). According to these results, βlg, αla, BSA, and κ-casein are linked to supramolecular complexes higher than 200-kDa exclusively through disulfide bridges. It is worth noting that heat-induced protein aggregates with gradually increasing molecular weight, as presumable intermediate reaction products, were not detected. This fact indicated that once the disulfide-mediated protein association in milk had been triggered by heating, it proceeded quickly as long as all of the proteins carrying at least a free thiol group were involved, similarly to a radical-mediated chain reaction, as suggested for the aggregation of βlg in a model study.[7]

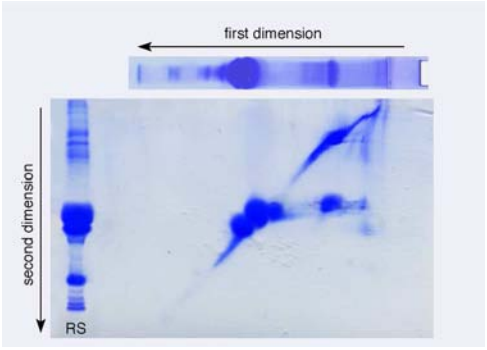

Figure 2. Bidimensional SDS−PAGE analysis of IPF from UHT-treated milk. The sample denatured in nonreducing conditions was set in two wells of a standard SDS−PAGE gel. After electrophoresis, one of these profiles was cut, incubated with β-mercaptoethanol, and then loaded onto an additional SDS−PAGE gel for the second dimension electrophoresis. The other profile of the first run was stained and reported at the top of of this figure to show position and protein band separation of the first dimension profile loaded onto the second gel. The sample denatured in reduced conditions, marked as RS in the figure, was analyzed in a separate lane of the second gel and used as reference for the second run. Proteins were stained with Coomassie Brilliant Blue R-250.

ELISA Detection of αla and BSA. Changes in whey protein composition of SPF and IPF induced by heat treatment

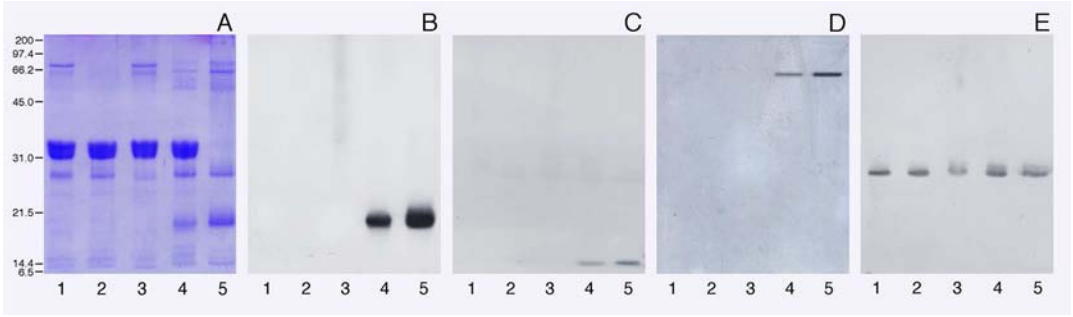

Figure 3. SDS−PAGE and immunoblotting analysis of the protein components recovered from the stacking gel of the first dimension SDS−PAGE profile of nonreduced IPF from UHT-treated milk after β-mercaptoethanol incubation (lane 5). IPF from raw milk, either nonreduced (lane 1) or reduced by preincubating with β-mercaptoethanol (lane 2), and IPF from UHT-treated milk, either nonreduced (lane 3) or reduced by preincubating with β-mercaptoethanol (lane 4), were run as reference samples. The gel was stained with Coomassie Brilliant Blue R-250 (panel A) or transferred onto nitrocellulose and immunostained with antiβlg (panel B), antiαla (panel C), anti-BSA (panel D), and anti-κ-casein (panel E) antibodies.

were clearly shown by SDS−PAGE analysis, but they could not be accurately evaluated by densitometry. Analytical methods able to specifically detect αla and BSA, regardless of the composition of the protein aggregates, were required. Two distinct competitive ELISAs, based on antiαla and anti-BSA antisera as specific reagents, were developed to determine the amounts of both αla and BSA actually included in both SPF and IPF of raw and UHT-treated milk. The concentration of the two proteins in the samples, calculated according to the data analysis performed by Microplate Manager III software, were reported in Tables 1 and 2. The contents of αla and BSA

Table 1. Partition of αla between the Two Fractions Recovered from Raw and UHT-Treated Milk after Precipitation at pH 4.6

milk sample	fraction	αla concn in the fraction[a] (μg/mL)	αla concn in starting milk[b] (mg/mL)
raw milk	SPF	2467.1 ± 220.9	1.234
	IPF	2.5 ± 0.3	0.012
UHT-treated milk	SPF	1750.7 ± 114.4	0.875
	IPF	70.4 ± 5.0	0.352

[a]αla concentration values obtained by ELISA analysis of the fractions prepared as described in Materials and Methods. [b]αla concentration values referred to the starting milk samples.

Table 2. Partition of BSA between the Two Fractions Recovered from Raw and UHT-Treated Milk after Precipitation at pH 4.6

milk sample	fraction	BSA concn in the fraction[a] (μg/mL)	BSA concn in starting milk[b] (mg/mL)
raw milk	SPF	806.1 ± 48.8	0.403
	IPF	1.1 ± 0.1	0.005
UHT-treated milk	SPF	66.6 ± 4.0	0.033
	IPF	72.8 ± 3.7	0.364

[a]BSA concentration values obtained by ELISA analysis of the fractions prepared as described in Materials and Methods. [b]BSA concentration values referred to the starting milk samples.

determined in SPF from raw milk by ELISA were very close to those expected.[20] The trace amounts of αla and BSA detected in IPF from raw milk were likely due to coprecipitation of these whey proteins with isoelectric casein. A different picture of the partition of αla and that of BSA between SPF and IPF recovered from UHT-treated milk was obtained by ELISA. The αla content in SPF from UHT-treated milk was about 70% of that found in SPF from raw milk, and the complementary amount of missing αla was detected in IPF of UHT-treated milk (Table 1). By contrast, ELISA results indicated that about 90% of BSA included in SPF from raw milk moved to IPF because of UHT treatment of milk, similarly to βlg,[15] whereas poor amounts of pH 4.6 soluble BSA were retained in SPF from UHT-treated milk (Table 2). According to the above-reported results, αla and BSA occurring in IPF from UHT-treated milk can be regarded as markers of the heat treatment, similarly to pH 4.6 insoluble βlg,[15] thanks to their heat-induced disulfide-mediated linkage to caseins and their subsequent coprecipitation together with caseins at pH 4.6. These findings were consistent with previous literature reports suggesting that pH 4.6 insoluble whey proteins, the so-called "heat-denatured serum proteins in the casein fraction," can represent markers of heat load applied to milk. All of the analytical methods used, based on different biochemical technologies, ranging from polarography to capillary zone electrophoresis,[21] suffered from poor selectively and/or sensitivity, mainly because relatively small amounts of whey proteins had to be measured in the presence of a large excess of caseins. The immunochemical approach has allowed one to overcome a number of issues related to the analysis of the protein fraction from milk and dairy products.[22] Despite the inherent complexity of this fraction, recognition of a specific component can be easily attained thanks to highly selective and sensitive reagents such as antibodies, without recourse to expensive equipment and/or highly skilled operators. A monoclonal antibody directed toward heat-induced epitopes of bovine alkaline phosphatase has been used to estimate mild heat treatments of milk such as pasteurization.[23] More severe combinations of heating time and temperature have been classified by monitoring lactoferrin denaturation with polyclonal antibodies developed against native lactoferrin.[24] The structural sensitivity of αla to heat stress has been also exploited to evaluate the heat-treatment degree of milk samples. Two distinct monoclonal antibodies have been used to separately quantify the native and heat-denatured forms of αla by a competitive ELISA format and by a biosensor.[25,26] The antiαla antiserum used in the present work was simply developed against native αla, and then it was not able to recognize any novel haptenic structure raised by heating. However, according to the ELISA results obtained using this

antiserum, the amount of pH 4.6 insoluble αla from UHT-treated milk was about 30% of the αla amount currently found in raw milk. The αla denaturation index of direct UHT-treated milk, evaluated by using the two monoclonal antibodies, has been shown to range from 24.2 to 45.4%.[26] The agreement between these findings and those obtained in the present work suggested that the heat-denatured forms of αla specifically recognized by the monoclonal antibody were insoluble at pH 4.6, but they were still reactive to polyclonal antibodies developed against native αla. Moreover, heat-induced association of whey proteins to caseins and their consequent pH 4.6 insolubility seemed to occurr according to their heat sensitivity, increasing in the order αla > βlg > BSA.[21] Indeed, the major part of BSA was found in UHT-treated milk as a component of large protein aggregates insoluble at pH 4.6, as well as the more heat-stable βlg.[15] Taking into account that BSA is more heat-sensitive than βlg, immunodetection of pH 4.6 insoluble BSA should allow one to distinguish among mild heat-treated milk in the realm of pasteurization. Further work is in progress to check the effectiveness of these novel ELISA-based analytical methods for classifying heat load applied to drinking milk. By monitoring simultaneously different protein markers of heated milk, including Amadori compounds,[17] keeping quality can be improved in the processing and control of milk and dairy products.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

SDS−PAGE and immunoblotting analysis of IPF from raw milk and UHT-treated milk. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

βlg, β-lactoglobulin; αla, α-lactalbumin; BSA, bovine serum albumin; IPF, the pH 4.6 insoluble protein fraction recovered from milk; SPF, the pH 4.6 soluble protein fraction recovered from milk; UHT, ultrahigh temperature; 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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