Specific Detection of the Amadori Compounds in Milk by Using Polyclonal Antibodies Raised against a Lactosylated Peptide[§]

Rosa Pizzano,*[,†] Maria Adalgisa Nicolai,[†] Rosa Siciliano,[‡] and Francesco Addeo[†,‡]

Dipartimento di Scienza degli Alimenti, Università degli Studi di Napoli Federico II, via Università 100, Parco Gussone, I-80055 Portici, Italy, and CNR, Istituto di Scienze dell'Alimentazione, via Roma, I-83100 Avellino, Italy

Polyclonal antibodies raised against a synthetic lactosylated peptide were found to be specifically directed against the carbohydrate moiety of the immunogen, recognizing, in addition to the Amadori compound, lactulose and, to a lesser extent, lactose and galactose. These antibodies were effective in detecting lactosylated proteins both on a model system containing β-casein and on milk subjected to different thermal treatments. Widespread lactosylation of the protein components was found in UHT-treated and sterilized milk, whereas it was very limited in raw and pasteurized milk. The index of lactosylation determined by competitive ELISA tests was linearly correlated to the furosine content of milk samples treated within 60 and 100 °C. This ELISA-based procedure is recommended for rapid monitoring of raw milk processing and extension.

Keywords: *Maillard reaction; Amadori compound; polyclonal antibodies; lactosylated peptides; milk; ELISA; immunoblotting*

INTRODUCTION

During milk processing and storage free amino groups of proteins are nonenzymatically glycated by lactose according to the Maillard reaction (Mauron, 1981). The early stable products, named Amadori compounds, are N-substituted 1-amino-1-deoxy-2-ketoses, resulting from the Amadori rearrangement of the primary adducts. Although no browning or flavor changes are observed in milk in the early stage of the Maillard reaction, protein quality is reduced owing to the conversion of the lysine residues into biologically unavailable forms (Finot et al., 1981). Although such damage is generally of poor nutritional significance in a healthy adult mixed diet, it has to be taken into consideration in specific cases, for instance, in infant foods (Hurrell, 1990).

Interest in the evaluation of the Maillard reaction products in milk and cheese is not limited to evaluating the impact of the technological processes on quality. In some countries there are legal restrictions preventing the milk and cheese extension with milk or components in powder due to the lower cost of reconstituted milk with respect to raw milk. Furosine, namely ϵ-*N*-(2-furoylmethyl)-L-lysine, obtained by acid hydrolysis of lactosylated lysine residues, has been proven to be a suitable indicator of the presence of Amadori compounds in milk and dairy products (Erbesdobler et al., 1987). However, evaluation of furosine content requires a previous step of a 24 h acid hydrolysis, and therefore even the most recently proposed HPLC procedures (Henle et al., 1995) prove to be too cumbersome and time-consuming for on-line routine control.

Nowadays, immunochemical methods are widely applied to detect specific components in complex mixtures. A monoclonal antibody, produced against lactose-treated ovoalbumin, was used to estimate the lactose−protein Maillard adducts in variously processed and stored milk products (Kato et al., 1994; Matsuda and Kato, 1996). However, this monoclonal antibody was unable to distinguish pasteurized from UHT-treated milk, thus indicating that its binding specificity was not directed toward Amadori lactose−protein adducts.

In this work polyclonal antibodies, raised against a lactosylated synthetic peptide, were used to specifically detect the Amadori compounds in milk. The antibodies' binding specificity was investigated by testing the native peptide and different mono- and disaccharides in competitive ELISA assays. The ability of the polyclonal antibodies to detect Amadori adducts formed by protein−lactose reaction was assayed by immunoblotting and ELISA using bovine β-casein in a lactose-containing model system. The reactivity of the antisera toward lactose-bound proteins of pasteurized, UHT-treated, and in-bottle-sterilized milk with respect to raw milk was evaluated by using immunoblotting. The ELISA response of the polyclonal antibodies toward whole bovine casein samples obtained from differently heat-treated milk samples was compared with the furosine content.

MATERIALS AND METHODS

Adduct Formation between a Synthetic Peptide and Lactose. The peptide RPKPQQFFGLM (P) was chemically synthesized by Neosystem S.A. (Strasbourg, France). The purity grade and chemical structure of the synthetic peptide were verified by RP-HPLC followed by electrospray mass spectrometry (ES/MS) analysis according to the procedures reported below. The peptide was eluted from the RP-HPLC column as a single sharp peak at a retention time (RT) of 15.3 min, giving, by ES/MS analysis, a signal at molecular mass 1347.8 ± 0.1 Da, consistent with the theoretical mass of the peptide, that is, 1347.75 Da. A peptide sample (25 mg) was incubated with a 1:10 (w/w) excess of lactose (Sigma, St. Louis, MO) in 5 mL of 50 mM sodium−phosphate buffer, pH 7.8.

* Author to whom correspondence should be addressed (telephone +39 81 7762121; fax +39 81 7755153; e-mail pizzano@unina.it).

§ In memory of Prof. Giacomino Randasso.

† Dipartimento di Scienza degli Alimenti.

‡ CNR.

10.1021/jf980493f CCC: $15.00
Published on Web 11/21/1998

After an overnight reaction at 60 °C, the reaction mixture was dried down in a Speed-Vac centrifuge (Savant, Farmingdale, NY). The solid residue was dissolved in 2 mL of 0.1% trifluoroacetic acid and loaded on a prepacked Sep-Pak C_{18} cartridge (Waters, Milford, MA), and salts were eluted by washing with 10 mL of 0.1% trifluoroacetic acid. The peptide fraction was then eluted with 5 mL of 70% acetonitrile. A blank reaction was carried out by incubating synthetic peptide in conditions similar to that for lactosylation, apart from the presence of lactose.

RP-HPLC Analysis. Analysis of the synthetic peptide and its lactose adducts was carried out by RP-HPLC using a Vydac C_{18} column (0.46 × 25 cm, 5 μm) attached to a Waters HPLC System (Datasystem Millenium, HPLC pumps Waters 510, Detector Waters 486) using 0.1% trifluoroacetic acid in water (solvent A) and 0.07% trifluoroacetic acid in acetonitrile (solvent B). Aliquots of 20 μg of samples were dried down in a Speed-Vac centrifuge (Savant), dissolved in 50 μL of solvent A, loaded onto a column equilibrated with 25% B, and eluted by means of a linear gradient from 25 to 55% of B over 30 min at a flow rate of 1 mL/min. The elution was monitored at 220 nm, and HPLC peaks were manually collected, dried down, and redissolved in 1% acetic acid 50% acetonitrile in water for the further ES/MS analysis (Fenn et al., 1989; Mann et al., 1989).

ES/MS Analysis. ES/MS spectra were recorded on a PLATFORM single-quadrupole instrument, equipped with an electrospray ion source (Micromass, Manchester, U.K.). Samples were injected directly into the ion source (kept at 80 °C) via a loop injection at a flow rate of 10 μL/min. The quadrupole was scanned over m/z 400−2000 at 10 s/scan, and the spectra were acquired and elaborated using MASS-LINX software (Micromass). Calibration was performed by a separate injection of myoglobin (16951.5 Da). All mass values are reported as average masses.

Conjugation of the Lactose-Treated Peptide to the Carrier and Production of the Antisera. The whole peptide fraction recovered from the lactose-incubated synthetic peptide was linked to keyhole limpet hemocyanin (KLH) according to the procedure reported by Bauminger and Wilchek (1980), using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimmide hydrochloride as coupling agent. Finally, the KLH-conjugated lactose-treated peptide was used to immunize two rabbits. The obtained antiserum, called anti-LAP as it had been raised against the lactosylated adducts of the peptide, was filtered on a 0.45 μm filter (Millipore, Bedford, MA), aliquoted, and stored at −20 °C.

Milk Sampling and Casein Preparation. Raw cows' milk was drawn from a private herd, managed according to EU health regulations, and treated as soon as possible at a laboratory for casein preparation and heating. Pasteurized, UHT-treated, and sterile bottled full-fat milks were from a commercial source. Experimental heated milk samples were obtained by incubating 500 μL aliquots of freshly collected raw milk in sealed capillary glass tubes by immersion at temperatures from 70 to 100 °C for 1, 10, and 30 min. Milk samples were manually defatted by centrifugation at 4500 rpm for 10 min at 4 °C, and whole casein was obtained from skimmed milk by precipitation at pH 4.6, according to the procedure described by Aschaffenburg and Drewry (1959). The pellet was suspended in water, the pH was brought to neutrality with 0.2 M NaOH, and the casein solution was lyophilized.

Preparation of Bovine β-Casein. Whole casein from skimmed raw milk was fractionated on a Q-Sepharose Fast Flow anion exchanger (Pharmacia) equilibrated in 20 mM Tris-Cl, 4.5 M urea, and 0.8 mM DTT, pH 7, by using a linear NaCl gradient from 0 to 0.35 M, as already reported (Pizzano et al., 1998). To monitor the elution of the casein components, an aliquot of each chromatographic fraction was analyzed by isoelectric focusing onto a PhastSystem apparatus (Pharmacia, Uppsala, Sweden) according to the procedure reported by Moio et al. (1989). Fractions containing pure β-casein, as resulting from electrophoretic analysis of the eluate, were pooled, extensively dyalized at 4 °C against 50 mM NH_4HCO_3, pH 8.5, and freeze-dried.

Kinetics of β-Casein Lactosylation. Separately, lactose and β-casein, purified as described, were dissolved in 50 mM NH_4HCO_3, pH 8.5 (lactose, 1.25 g in 15 mL; β-casein, 250 mg in 10 mL) and equilibrated at 37 °C. The solutions were mixed and incubated at 37 °C. At fixed times, 3 mL was withdrawn from the incubation mixture, and immediately the pH of the samples was lowered to 4.6 by adding 0.6 mL of a 1:1 mixture of 1 M sodium acetate and 10% (v/v) acetic acid to eliminate lactose. The pellets were then dissolved in 3 mL of 50 mM NH_4HCO_3, pH 8.5, and the solutions were lyophilized. Lactosylated β-casein samples were stored at −20 °C until analysis.

Determination of Furosine. Furosine content in experimental heated milk samples was determined according to the procedure reported by Resmini et al. (1990).

SDS−PAGE Analysis: Electrophoresis and Immunoblotting. Vertical polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS−PAGE) was performed as described by Di Luccia et al. (1994). After electrophoresis, the proteins were electrically transferred from the gel onto nitrocellulose paper as described by Towbin et al. (1979). Immunodetection was carried out using rabbit antisera against the lactosylated synthetic peptide as primary antibodies and horseradish peroxidase-labeled goat anti-rabbit IgG polyclonal antibodies as secondary reagent (Bio-Rad, Hercules, CA). The procedure was essentially that already described by Chianese et al. (1992), but the blocking solution was replaced with PBS containing heat-inactivated horse serum at 10% in volume.

PAGIF Analysis: Electrophoresis and Immunoblotting. Isoelectric focusing on thin-layer polyacrylamide gel (PAGIF) was carried out as described by Trieu-Cuot and Gripon (1981). The pH gradient in the range 2.5−6.5 was obtained by mixing Ampholine (Pharmacia) 2.5−5, 4.5−5.4, and 4−6.5 in the ratio 1.6:1.4:1 (v/v/v). The gel was stained with Coomassie Brilliant Blue R-250 according to the procedure described by Blakesley and Boezi (1977). After gel electrophoresis, the proteins were transferred by capillary diffusion from the gel onto nitrocellulose paper. The immunostaining procedure was identical to that already described for SDS−PAGE analysis.

Competitive ELISA Assay. Desalted lactosylated peptide mixture or whole bovine casein from UHT-treated milk (50 or 2 μg/mL in 50 mM NH_4HCO_3, pH 8.5, respectively), as specified in the figure legends, 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 (PBS, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136 mM NaCl, 2.7 mM KCl, pH 7.4) and then filled with blocking buffer (10% v/v heat-inactivated horse serum in PBS). Microplates were placed at 37 °C for at least 1 h in a humid atmosphere, being subsequently emptied and kept at −20 °C, ready for immediate utilization.

To detect the antigen in a sample, 50 μL aliquots of serial dilutions of the sample were introduced in each well and then 50 μL of a 1:250 (v/v) dilution of the antiserum in the blocking buffer was added. Analysis of each dilution of the samples was performed at least in triplicate, and mean values were reported. Maximal binding of the antibodies to the plate was determined by adding to the wells 50 μL of 50 mM NH_4HCO_3, pH 8.5, instead of antigen solution.

After 1 h at 37 °C in a humid atmosphere, the plates were emptied and washed five times with PBS. One hundred microliter aliquots of a 1:2000 (v/v) dilution of horseradish peroxidase-labeled goat anti-rabbit IgG polyclonal antibodies (Bio-Rad) were then added. Following incubation for 1 h at 37 °C in a humid atmosphere and five washings with PBS, 100 μL amounts of a solution of tetramethylbenzidine (Sigma), 0.1 mg/mL in 50 mM citrate−phosphate buffer, and dimethyl sulfoxide 10% (v/v), pH 5.0, containing 0.01% (v/v) hydrogen peroxide were added to each microtiter well. After 10−15 min at room temperature, the reaction was stopped by adding 100 μL of 1 M H_2SO_4. The resulting absorbance was measured at 450 nm with a model 450 microplate Reader (Bio-Rad).

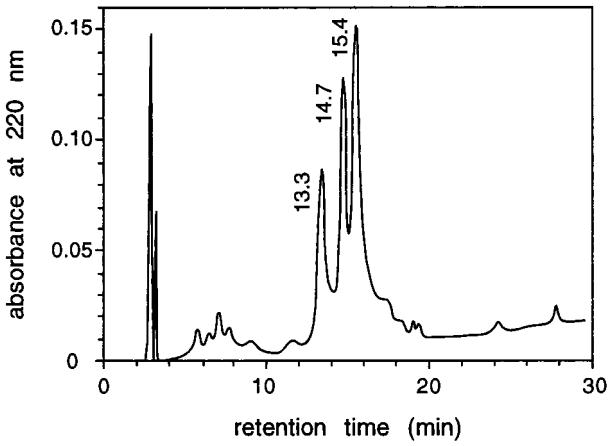

Figure 1. RP-HPLC purification on a Vydac C_{18} column of the synthetic peptide−lactose crude mixture incubated at 60 °C overnight. Experimental conditions were described under Materials and Methods. The peaks at retention times 13.3, 14.7, and 15.4 min were manually collected and analyzed by ES/MS.

RESULTS AND DISCUSSION

Production of Lactosylated Peptide Antigens. The synthetic peptide RPKPQQFFGLM was chosen as lactosylation model substrate because the free amino group of its N terminus and the ϵ-amino group of the lysine residue at position 3 of its amino acid sequence can simulate the two main glycation sites of proteins. The kinetics of peptide lactosylation was monitored by RP-HPLC analysis. The overnight incubation mixture contained three peaks at RT 13.3, 14.7, and 15.4 min, as shown in Figure 1. The ES/MS spectra of these three main RP-HPLC fractions, transformed on a real mass scale, are shown in Figure 2. The unmodified peptide was eluted at RT 15.4 min, as confirmed by ES/MS analysis (Figure 2A). The spectrum of the fraction eluted at 14.7 min presented a signal at mass 1671.6 ± 0.1 Da, corresponding to a 324 mass shift with respect to the synthetic peptide (Figure 2B). The mass increase was in full agreement with the addition of one lactose unit to the peptide. Finally, ES/MS analysis of the fraction at RT 13.3 min gave a mass signal at 1995.8 ± 0.1 Da (Figure 2C) with a 648 mass unit shift with respect to the native peptide, corresponding to the addition of two lactose molecules to the two putative lactosylation sites of the peptide.

Binding Specificity of the Polyclonal Antibodies Raised against Lactosylated Antigens. The specificity of the anti-LAP antisera, raised against the desalted reaction mixture, was defined through competitive ELISA experiments. The dose-dependent inhibition curves relative to the lactosylated peptide mixture, the synthetic peptide, and six free mono- and disaccharides are shown in Figure 3. The unmodified peptide was unable to interact with anti-LAP, even at concentrations 3 orders of magnitude higher than those at which the lactosylated peptide mixture gave the maximal response. This indicates that the sugar moiety of the Amadori compounds used as immunogen has a crucial role in determining the immunological response. As concerns free sugars, only lactulose and, to a lesser extent, lactose and galactose acted as competitors for antibodies binding to the antigen coated on the plate well. Indeed, anti-LAP reactivity toward lactulose can be explained by the fact that the immunological response was raised against Amadori compounds formed

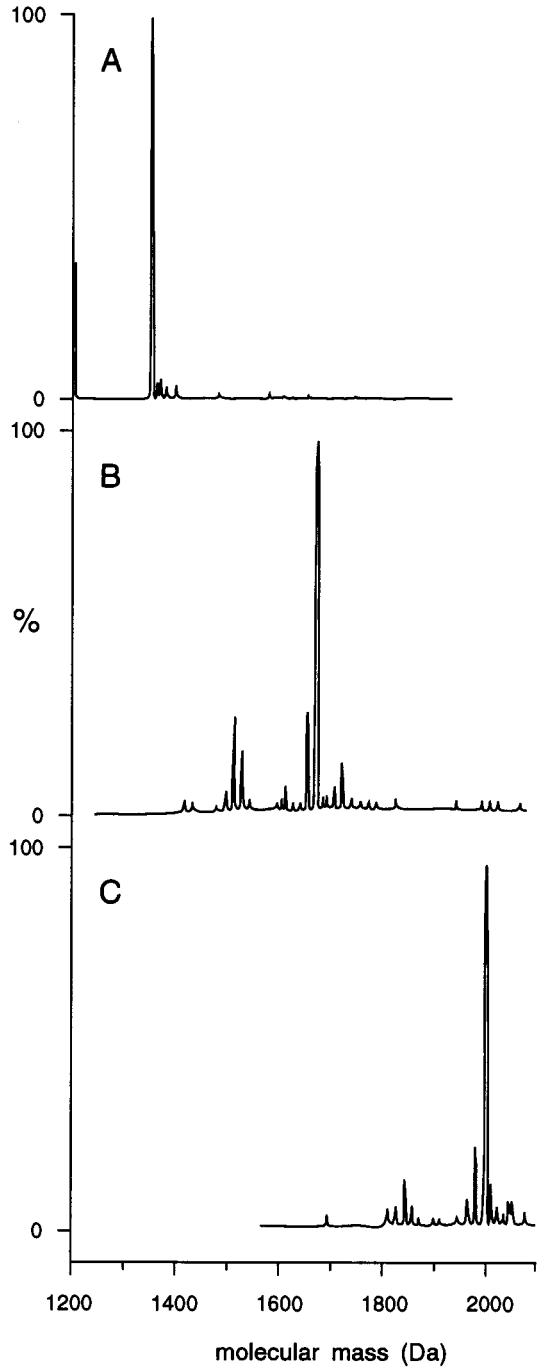

Figure 2. ES/MS spectrum transformed on a real mass scale of the RP-HPLC peak eluted at RT (A) 15.4 min, (B) 14.7 min, and (C) 13.3 min. The measured molecular mass values of the components (mean ± standard error) were (A) 1347.7 ± 0.1, (B) 1671.6 ± 0.1, and (C) 1995.8 ± 0.1, corresponding to the peptide under native form and that containing one or two lactose units, respectively.

from lactose, which were actually N-substituted lactulosyl derivatives of the peptide. Moreover, free galactose, being the nonreducing unit of lactulose, proved to be still able to interact with antibodies. Most likely the lactose recognition by antibodies is also due to its terminal galactosyl residue. In contrast, glucose, that is, the C-4 epimer of galactose, displayed no effect in the competitive ELISA assays, thus showing that antibodies selectively recognized only carbohydrates with specific structural features. No competitive activity was shown by free fructose, even if the reducing glucide moiety of Amadori compounds from lactose and

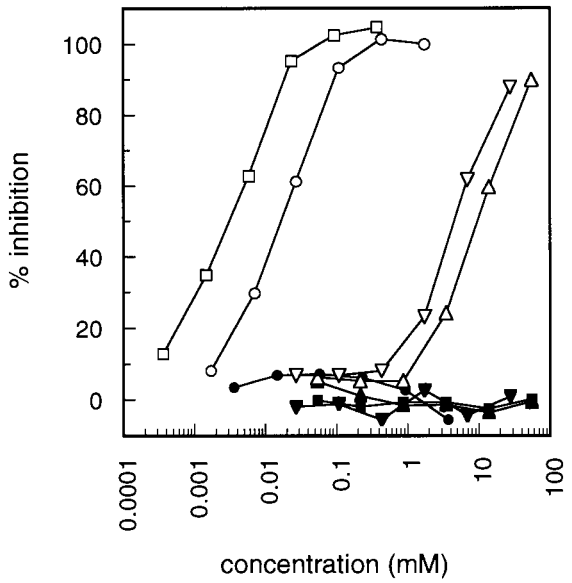

Figure 3. Competitive ELISA assays of the unmodified peptide (●), lactulose (○), lactose (▽), galactose (△), glucose (■), maltose (▼), and fructose (▲). As positive reference sample the lactosylated peptide was assayed (□). The ELISA assay procedure was described under Materials and Methods. Analysis of each sample was performed in triplicate, and the mean values of the obtained ELISA signals were considered. Net absorbance was calculated by subtraction from the actual ELISA signals the signal obtained by testing the lactosylated peptide at the highest concentration (374 μM, ELISA absorbance = 0.13 ± 0.01). % inhibition = (max − x) × 100/max, where max is the net signal obtained by testing the PBS buffer (ELISA value = 0.75 ± 0.02) and x is the net absorbance of the sample.

amines is a 1-amino-1-deoxyfructosyl residue. Finally, because maltose failed to interact with anti-LAP, any effect can be excluded due to a suitable fitting of a disaccharide in the antibodies combining sites.

Detection of an in Vitro Lactosylated Model Milk Protein. To test the ability of the antisera in recognizing early Maillard adducts of proteins in milk, bovine β-casein was allowed to react with lactose at 37 °C using concentrations of both reagents similar to those occurring in milk. The isoelectric protein, recovered from aliquots of the reaction mixture withdrawn at fixed interval times, was analyzed by SDS−PAGE. The electrophoretic patterns of the samples presented a single β-casein band, irrespective of incubation time (Figure 4A). On staining with anti-LAP, the SDS−PAGE profiles revealed a band comigrating with β-casein, the intensity of which gradually increased with incubation time (Figure 4B). No difference between the apparent molecular weight of the lactosylated and native β-casein was detected by SDS−PAGE analysis, even at the longest incubation time. Matsuda et al. (1992) reported that lactosylated β-lactoglobulin was noticeably heavier than native protein, most likely resulting from prolonged incubations at 50 °C and 65% relative humidity.

A reliable procedure for monitoring the β-casein lactosylation consists of testing isoelectric protein in competitive ELISA assays. As shown in Figure 5, titration curves of the protein at increasing incubation times gradually moved toward lower concentrations, owing to the recognition of the progressively lactosylated protein by anti-LAP.

This means that glycation of β-casein by lactose occurred even at physiological conditions and the involved structural modifications could be easily evidenced by anti-LAP antiserum through immunoblotting and ELISA assays.

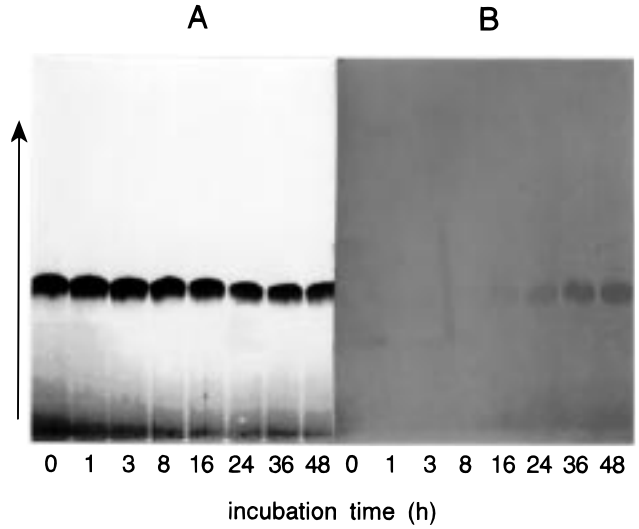

Figure 4. SDS−PAGE analysis of 50 μg amounts of bovine β-casein treated at 37 °C with lactose for the incubation times reported below the corresponding profiles: (A) Coomassie Brilliant Blue R-250 stained gel; (B) immunodetection using 1:500 (v/v) diluted anti-LAP as primary antibodies and 1:2000 (v/v) diluted horseradish peroxidase labeled goat anti-rabbit IgG antibodies as secondary antibodies. Untreated bovine β-casein (lane 0) was also included.

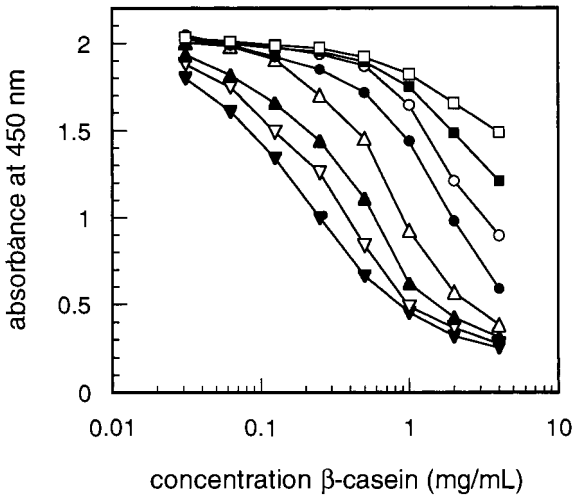

Figure 5. Competitive ELISA assays of bovine β-casein incubated at 37 °C with lactose for 1 h (■), 3 h (○), 8 h (●), 16 h (△), 24 h (▲), 36 h (▽), and 48 h (▼). Untreated β-casein (□) was also assayed. Three aliquots of 50 μL of serial dilutions ($1{:}2^n$, $n = 1-8$) of native or lactose-treated β-casein were tested, starting from 8 mg/mL in 50 mM NH_4HCO_3, pH 8.5, and the mean values of ELISA absorbance were reported. The anti-LAP was used at a final dilution of 1:500 (v/v). The ELISA assay procedure was described under Materials and Methods.

Anti-LAP Reactivity toward Proteins from Differently Heated Milks. To verify the effectiveness of the anti-LAP in distinguishing between commercial drinking milk, pasteurized, UHT-treated, and in-bottle-sterilized milk, samples were analyzed either by SDS−PAGE or by PAGIF, each followed by immunoblotting, in comparison with a raw milk sample. The SDS−PAGE profiles stained with Coomassie Brilliant Blue R-250 and anti-LAP are compared in Figure 6. Faintly lactosylated casein bands were detected by the anti-LAP in raw and pasteurized samples, whereas a large

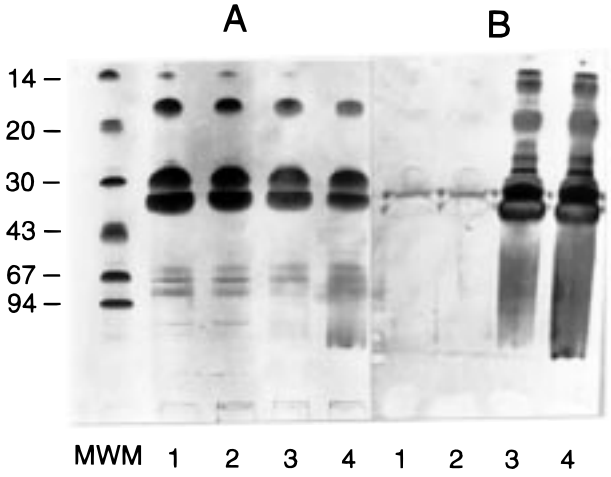

Figure 6. SDS−PAGE analysis of 50 μg amounts of proteins from raw (lane 1), pasteurized (lane 2), UHT-treated (lane 3), and in-bottle-sterilized (lane 4) milk samples: (A) Coomassie Brilliant Blue R-250 stained gel; (B) immunodetection using 1:500 (v/v) diluted anti-LAP as primary antibodies and secondary antibodies as indicated for Figure 4. Molecular weight markers (lane MWM) are given in kilodaltons.

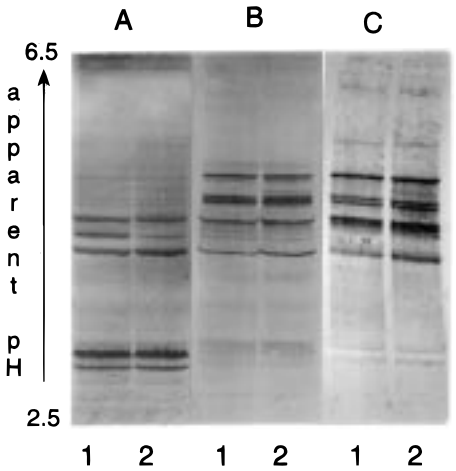

Figure 7. PAGIF analysis of 100 μg amounts of proteins from raw (lane 1) and pasteurized (lane 2) milk samples: (A) Coomassie Brilliant Blue G-250 stained gel; immunodetection using as primary antibodies (B) 1:200 (v/v) diluted anti-LAP and (C) 1:1000 (v/v) diluted anti-α_{s2}-casein serum and secondary antibodies as indicated for Figure 4.

number of intense anti-LAP reactive milk components including β-lactoglobulin, α-lactalbumin, and caseins were detected in UHT-treated and in-bottle-sterilized samples. In addition to this, severe heating had caused a tailing of high molecular mass whey proteins bands such as immunoglobulins, bovine serum albumin, and lactoferrin, owing to the simultaneous denaturation, glycation, and polymerization of single protein species.

Widespread marked antibody recognition was also observed in the immunoblotting of the PAGIF profiles of UHT-treated and sterilized milks, meaning an extensive charge modification of proteins in severely treated milks (results not shown). By contrast, few distinct bands were detected by anti-LAP (Figure 7B) along the PAGIF profile of both raw and pasteurized milk samples stained by Coomassie Brilliant Blue G-250 (Figure 7A). To identify the proteins specifically detected by anti-LAP, polyclonal antibodies against β-, α_{s1}-, κ-, and α_{s2}- casein, β-lactoglobulin and α-lactalbumin were used to stain the PAGIF profiles, according to the procedure proposed by Chianese et al. (1992), and the resulting patterns were compared with that obtained using anti-LAP. The anti-LAP detected bands overlapped strictly with those of α_{s2}-casein components, as shown in Figure 7C, whereas no correspondence with the other considered milk proteins could be found. These results indicate that, among milk proteins, α_{s2}-casein is primarily involved in the Maillard reaction with lactose. Moreover, the finding that Amadori compounds are already contained in freshly secreted milk is in agreement with the furosine content observed in raw milk (Resmini et al., 1992). It is worth noting that, at immunoblotting analysis, lactosylated components of pasteurized milk were quite similar to those occurring in raw milk.

The marked difference in antibody reactivity toward the two sets of milk samples, namely raw and pasteurized milk, on the one hand, and UHT-treated and sterilized milk, on the other, was also found by using indirect ELISA assays (results not shown).

Competitive ELISA assay is one of the most effective immunoassay systems for the quantitative detection of antigenic species contained in a sample (Harlow and Lane, 1988). Unfortunately, milk was not directly analyzable through competitive ELISA assays. In fact, lactose contained in milk, and both lactulose and galactose occurring in heated milk (Berg and van Boekel, 1994), are able to interact with anti-LAP, as shown in Figure 3, so that they may affect the ELISA determinations of lactosylated proteins in milk samples. Nevertheless, the clear reactivity difference of antibodies toward antigens occurring in low- and high-heat milk samples, already evidenced by the immunoblotting experiments, was confirmed by testing whole casein instead of milk samples in competitive ELISA assays (results not shown).

Relationship between ELISA Index and Furosine Content. A procedure for identifying an ELISA index of lactosylation of milk proteins was developed. Whole casein from milk samples heated at different intensities was tested in competitive ELISA assays at six serial concentrations. In correspondence of each casein sample a sigmoid dose−response titration curve was obtained. The concentration giving the ELISA response equal to half the maximal signal was determined by nonlinear regression analysis of each titration curve and reported in Table 1 as $\text{concn}_{Amax/2}$ of the corresponding casein sample. The value of $\text{concn}_{Amax/2}$ was greatly affected by the extent of heat treatment. In fact, the $\text{concn}_{Amax/2}$ relative to raw milk was 3 orders of magnitude higher than that relative to the most severely heated milk sample. $\text{Concn}_{Amax/2}$ proved inversely related to the content of antigenic species: the fewer the adducts in a casein sample, the more concentrated the solution required to obtain a 50% inhibitory effect. Thus, the ratio between the $\text{concn}_{Amax/2}$ of casein from raw milk and that resulting from a sample indicates how many times the concentration of lactose−casein adducts is higher in the heated sample with respect to raw milk. Therefore, we assumed this ratio as an ELISA index of the glycation of milk proteins induced by heating. The ELISA index, calculated from the figures reported in Table 1, and the furosine content of the samples, determined according to the method of Resmini et al. (1990), were correlated, giving the linear relationship shown in Figure 8. This suggested that milk heating may be described either through a direct evaluation of the lactose-derived protein adducts by

Table 1. Effect of Heating on the Formation of Amadori Compound in Milk Measured by Using Competitive ELISA Assays[a]

incubation conditions	$concn_{Amax/2}$[b] (μg/mL)	incubation conditions	$concn_{Amax/2}$[b] (μg/mL)
70 °C, 1 min	1.91×10^4	90 °C, 1 min	4.73×10^2
70 °C, 10 min	3.48×10^3	90 °C, 10 min	1.80×10^2
70 °C, 30 min	8.50×10^2	90 °C, 30 min	9.20×10^1
80 °C, 1 min	1.53×10^3	100 °C, 1 min	2.16×10^2
80 °C, 10 min	4.64×10^2	100 °C, 10 min	7.65×10^1
80 °C, 30 min	3.21×10^2	100 °C, 30 min	1.53×10^1
raw milk	1.39×10^5		

[a] Experimental details: Whole casein was obtained from 10 mL aliquots of raw milk after the incubations at time × temperature specified in the "incubation conditions" column. Triplicates of serial dilution ($1:4^n$, $n = 1-6$) in PBS of 8 mg/mL whole casein were tested in competitive ELISA assays. Plates were coated with 2 μg/mL whole casein from UHT-treated milk, and a 1:500 (v/v) dilution of antiserum was used. The detailed experimental procedure of the assay was as described under Materials and Methods. The mean absorbance value obtained by testing 1 mg/mL whole casein from UHT-treated milk, 0.150 ± 0.007, was assumed as the background ELISA signal. Net ELISA values, calculated by subtracting the background signal from the mean values of the triplicates, were considered for data analysis. In correspondence to each sample a linear relationship was found between the ELISA absorbance and the logarithm of casein concentration. [b] $Concn_{Amax/2}$ was the casein concentration giving half the maximal ELISA signal. It was calculated according to each regression equation using, as ELISA value, half A_{max}, where A_{max} was the net ELISA absorbance obtained by testing the PBS buffer (ELISA value = 1.322 ± 0.027).

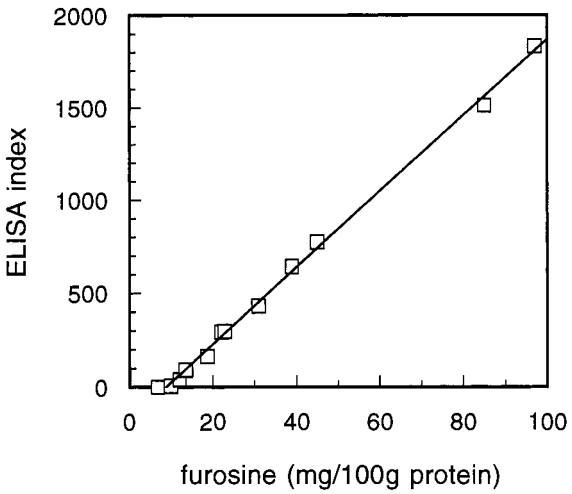

Figure 8. Correlation between ELISA indices of thermal stress and the furosine contents. As ELISA indices of thermal damage, the ratios of the $concn_{Amax/2}$ of casein from raw milk and the $concn_{Amax/2}$ of each sample were reported. The absolute values of the $concn_{Amax/2}$ were from Table 1. Furosine contents were determined as described under Materials and Methods. The ELISA index (y) and the furosine content (x) of the samples were related according to the following linear equation: $y = 20.451x - 178.623$; $r^2 = 0.997$.

ELISA assays, provided that antisera specific for the sugar antigen are available, or by indirect evaluation of these components through furosine determination. Thus, competitive ELISA assays using antipeptide antibodies raised against the lactosylated peptide mixture might represent an alternative analytical method for the quality control of commercial milk and dairy products. In fact, all of the advantages of ELISA assays, that is, high sensitivity, the possibility of testing many samples simultaneously, the very reduced time for each analysis, and the low cost per assay, are well-combined with the fact that no sample pretreatment is required. The validation of this analytical approach for evaluating the lactosylation level of milk samples of different origins and treatments is in progress, mainly concerning the selection of suitable reference products.

ABBREVIATIONS USED

PBS, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136 mM NaCl, 2.7 mM KCl, pH 7.4; P, the peptide RPKPQQFFGLM; ES/MS, electrospray mass spectrometry; RP-HPLC, reverse-phase high-performance liquid chromatography; RT, retention time; UHT, ultrahigh temperature; KLH, keyhole limpet hemocyanin; anti-LAP, antiserum raised against the lactosylated adducts of the peptide conjugated to KLH; ELISA, enzyme-linked immunosorbent assay; SDS−PAGE, vertical polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; MWM, molecular weight markers; PAGIF, polyacrylamide gel isoelectric focusing.

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Received for review May 12, 1998. Revised manuscript received September 24, 1998. Accepted October 2, 1998.

JF980493F