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Determination of the Heat Treatment Undergone by Milk by Following the Denaturation of α-Lactalbumin with a Biosensor

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Dairy industries are interested in knowing the heat treatment undergone by milk so as to control the quality of drinking milks or to control their heating systems. Among the different techniques available to characterize the heat treatment of milk, estimation of the denaturation of proteins has been widely used. However, because the concentration of the proteins in raw milk can fluctuate significantly, determining only the concentration of a native protein without knowing its concentration in the raw milk before undergoing heat treatment can lead to significant imprecision. The objective of this study was to develop, on Biacore 3000, a biosensor assay for determining the denaturation index of α-lactalbumin by quantifying separately the native and "heat-denatured" forms of α-lactalbumin with specific monoclonal antibodies. α-Lactalbumin denaturation index is independent of the concentration of α-lactalbumin in the original raw milk. The technique developed is discriminating, fast, repeatable, fully automated, and requires no pretreatment of the milk sample.

KEYWORDS: Biosensor; milk; α-lactalbumin; heat treatment; denaturation; antibodies

INTRODUCTION

Before or during processing, milk is usually submitted to heat treatment. For process control or regulation purposes, there is a need to know the severity of the heat treatment milk has undergone.

Few techniques allow accurate determination of the rate of denaturation of milk proteins (*1*). Alkaline phosphatase and peroxidase determinations have been used for many years to assess the completeness of dairy products' pasteurization (*2*) and to evaluate the severity of milk thermization (*3*). In contrast, lactulose determination is a suitable technique used for strongly heated milks, such as UHT and sterilized milks (*4*). Unfortunately, none of these techniques allow the study of all types of heat treatment. Furthermore, they are based on the determination of a bio-indicator concentration that can fluctuate among milks, rendering the determination of the heat treatment undergone by a milk, without the reference of the original raw milk, difficult (*3*). Lactulose concentration, for instance, has been shown to fluctuate in milk during storage due to evolution of phosphate, citrate, and calcium concentrations that play a role in the formation of lactulose (*5*).

Among the major whey proteins, α-lactalbumin (14.2 kDa) is the most resistant to heat-denaturation in milk (*6*) and remains at measurable concentration, even after UHT-treatment (*7*). Because UHT milks represent around 90% of the drinking milks commercialized in France, α-lactalbumin was chosen as thermal bio-indicator for the present study. Additionally, it has been shown that denaturation of α-lactalbumin causes unfolding of the molecule (*8*). These conformational modifications may induce the appearance of new epitopes at the surface of the molecule or the disappearance of some epitopes present in the native form of α-lactalbumin. Thus, immunochemical techniques could be an attractive alternative for following heat denaturation of this protein because of their specificity and sensitivity. An enzyme-linked immunosorbent assay (ELISA) was previously developed to quantify native α-lactalbumin (NAL) in heat treated milks (*7*). This technique, which used a rabbit polyclonal serum specific to NAL, showed a decrease of the NAL concentration proportional to the intensity of the heat treatment. However, its application is limited because it is necessary to know the concentration of NAL prior to heat treatment. Moreover, it has been demonstrated that the concentration of NAL in individual raw milk fluctuates significantly with the stage of lactation and the casein and *β*-lactoglobulin phenotypes (*9*). Variation of NAL concentration is probably more limited in bulk than in individual milk, but may remain significant. Recently, we succeeded in producing two monoclonal antibodies (Mabs), one NAL specific (Mab 20) and the other specific to the "heat denatured" form (Mab 130) of α-lactalbumin (HDAL) (*10*). By use of these probes, two ELISAs for specific quantification of NAL and HDAL were developed. Results obtained with this method showed that classification of milks according to the heat treatment undergone was possible, without having to know the α-lactalbumin concentration of the original raw milk. However, this method, based on the use of two ELISAs, showed poor repeatability and was extremely cumbersome (4 to 8 samples analyzed per day). Therefore, its use as

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a control method for routine analysis of series of milk samples was impossible.

Consequently, using the same immunological probes, the present study shows the development of a technique using the generic biosensor Biacore 3000 device. Biacore 3000 is an optical biosensor that allows the progress of biomolecular interactions to be monitored in real time. The technique developed allows the denaturation index of α-lactalbumin on milk to be determined within 4 min. It is fully automated, and no pretreatment of the milk sample apart from dilution in the course buffer is required.

MATERIALS AND METHODS

Chemicals. NAL and HDAL were purified as previously described (*10*). Mab 20 and Mab 130 monoclonal antibodies were raised on mice as previously described (*10*). Ascites were obtained according to the procedure of Jones et al. (*11*). They were purified by affinity chromatography using NAL or HDAL covalently immobilized onto Hi-Trap NHS-activated HP columns (Amersham pharmacia biotech, Uppsala, Sweden) following the method proposed by the manufacturer.

Milk Samples. A total of 24 pasteurized, 10 highly pasteurized, 16 direct UHT, 29 indirect UHT, and 8 sterilized milks were collected, by Arilait Recherches, in French factories during the winter and summer periods. All the milk samples were analyzed directly after treatment. Direct UHT, indirect UHT, and sterilized milks were stored for a further 90 days at either 25 or 35 °C and analyzed again after storage.

ELISA. Inhibition ELISAs were performed for NAL or HDAL quantification in milk. For NAL quantification, flat-bottomed ELISA plates were coated with 1 μg/mL NAL in bicarbonate buffer 0.1 M pH 9.6 (100 μL per well) and incubated for 1 h 30 at 37 °C. Blocking of the remaining binding sites was performed with phosphate-buffered saline/0.05% Tween 20 pH 7.2 (PBS-T). Serial dilutions of NAL (0−1000 ng/mL, 75 μL) in 0.4 M trisodium citrate, 75 mM EDTA, 0.05% Tween 20 pH 6.3 (TSCT-EDTA) were used as standards. Milk samples diluted in TSCT-EDTA (four dilutions from 1/1000 to 1/5000, 75 μL), or NAL standards, were incubated in test tubes with 75 μL of 1/1500 dilution of Mab 20 ascites for NAL quantification for 1 h 30 at 37 °C. A 100-μL sample of the mixture was then added to each ELISA plate well and further incubated for 1 h 30 at 37 °C. The reaction was revealed with 100 μL of donkey anti-mouse-immunoglobulin−alkaline phosphatase conjugate (Immunoresearch Laboratories Inc., West Grove, PA) diluted 1/5000 in PBS-T and incubated 1h at 37 °C. A 100-μL sample of *p*-nitrophenyl phosphate at 1 mg/mL (Sigma-Aldrich, St Quentin Fallavier, France) was used as substrate. Absorbance was measured at 405 nm using a Ceres 900 microplate reader (Bio-Tek Instruments Inc., Winooski, VT). The same procedure was followed for HDAL quantification, except that NAL was replaced by HDAL and Mab 20 ascites replaced by Mab 130 diluted 1/500 000. Each NAL or HDAL quantification was made in triplicate. Results were expressed by calculating the mean α-lactalbumin denaturation index ((HDAL/(NAL + HDAL)) × 100).

Biacore. Biacore technology (Biacore International SA, Uppsala, Sweden) uses Surface Plasmonic Resonance (SPR) to monitor the reaction between antibody and antigen. SPR detects changes in the refractive index close to a metal surface. One of the reactants is immobilized, and the other is introduced in solution flowing over the sensor surface. The sensor surface consists of a dextran matrix coupled to a thin gold film. This matrix extends out from the sensor surface and permits immobilization of biomolecules through amine groups. The reaction between immobilized ligands and injected analyte takes place in the hydrophilic environment defined by the dextran matrix. When immobilized ligands bind the analyte, the angle at which SPR occurs is changed. Changes in the angle are expressed in arbitrary units called resonance units (RU).

The reaction is monitored continuously, and the binding curve is directly visualized on a computer screen. The integration of SPR detection, a microfluidic system and operator designed sensor surfaces in one automated analytical system provides easy, flexible, and quantitative analysis of biospecific interactions.

Immobilization Procedure. Monoclonal antibodies 20 and 130 were immobilized covalently on a CM5 sensorchip by amine coupling as described previously (*12*). All the chemicals used for antibody immobilization were from Biacore International SA. Briefly, a continuous flow of HBS-EP (10 mM Hepes, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20) pH 7.4, over the sensor surface at 5 μL/min, was maintained. The CM5 carboxymethylated dextran matrix was activated by the injection of 35 μL of a solution containing 0.2 M *N*-ethyl-*N*′-(3-diethyl-aminopropyl)-carbodiimide (EDC) and 0.05 M *N*-hydroxysuccinimide (NHS). Next, 35 μL of Mab 20 or 130 1/10 in sodium acetate pH 4.5 were injected, followed by 35 μL of 1 M ethanolamine to block remaining NHS-ester groups. The immobilization level was 10 000 RU corresponding to 10 ng/mm^2 of Mab. Mab 20 was immobilized on flow channel (Fc) 2, with Fc1 being used as a reference cell. Mab 130 was immobilized on Fc4, with Fc3 being used as a reference cell.

Biacore Assay. Whole milk samples (10 μL) diluted 1/1000 in HBS-EP were injected on Fc1 and Fc2 and on Fc3 and Fc4 for NAL and HDAL determinations, respectively. Fc1−Fc2 and Fc3−Fc4 were regenerated with 5 μL of 10 mM Glycine−HCl pH 2.7 and 5 μL of 15 mM NaOH, respectively. Each NAL or HDAL quantification was made in triplicate. Results were expressed by calculating the mean α-lactalbumin denaturation index ((HDAL/(NAL + HDAL)) × 100).

Repeatability of the Biacore assay was assessed by determining the relative repeatability standard deviation (RSDr) after quantification in triplicate of NAL and HDAL in the 87 commercial milk samples supplied by Arilait Recherches. Accuracy of the method was assessed by comparison of the denaturation index obtained using Biacore on these 87 commercial milk samples with those obtained by inhibition ELISA.

Finally, the possible influence of EDTA on the structure of native α-lactalbumin was checked by comparing the results obtained on milk samples diluted in HBS-EP or in HBS-P (same buffer without EDTA) from Biacore.

Statistical Analysis. Data obtained with the Biacore assay on 193 milk samples were processed by factorial discriminant analysis (FDA) using the DISCRIM procedure on SAS (*13*). Five classes of milk samples (pasteurized, highly pasteurized, direct UHT, indirect UHT, and sterilized) were proposed and validated by FDA. Results were expressed as percent correctly classified into each group.

RESULTS AND DISCUSSION

Analytical Characteristics of the Biacore Assay. No particular influence of the EDTA contained in the HBS-EP buffer used to dilute milk samples was observed when the same samples were injected with an HBS buffer containing no EDTA (data not shown). Sensorgrams obtained by injecting raw, pasteurized, highly pasteurized, direct UHT, indirect UHT, and sterilized milk samples on sensorchips coated with Mab 20 and Mab 130 as capturing antibodies are represented **Figure 1**, parts **A** and **B**, respectively. For NAL determination, the intensity of the response increases as the intensity of the heat treatment decreases (raw > pasteurization > high pasteurization ≥ direct UHT > indirect UHT > sterilization). In contrast, for HDAL determination, the intensity of the response increases as the intensity of the heat treatment increases.

For NAL determination, regeneration occurred by using one 5 μL injection of 10 mM Glycine−HCl, pH 2.7 onto the sensorchip. In contrast, for HDAL determination, regeneration occurred by using one 5 μL injection of 15 mM sodium hydroxide onto the sensorchip.

The technique was shown to be highly repeatable (intraCV = 1.5 and 2.4% for NAL and HDAL determination, respectively), fast, fully automated, and required only 2 μL of milk sample and no particular pretreatment of the sample apart from dilution in the course buffer. Approximately 100 samples can be analyzed on the sensorchip before the binding capacity of the two monoclonal antibodies is altered. Analysis in parallel

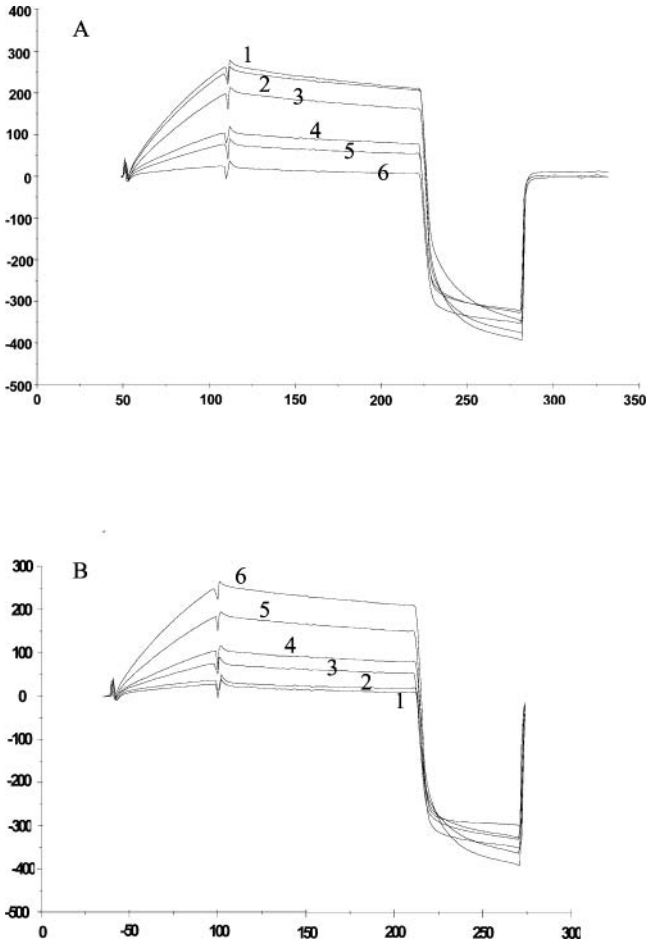

Figure 1. Overlay plot of binding to the antibody 20 (A) and 130 (B) of commercial raw (**1**), pasteurized (**2**), highly pasteurized (**3**), direct UHT (**4**), indirect UHT (**5**) and sterilized milks (**6**).

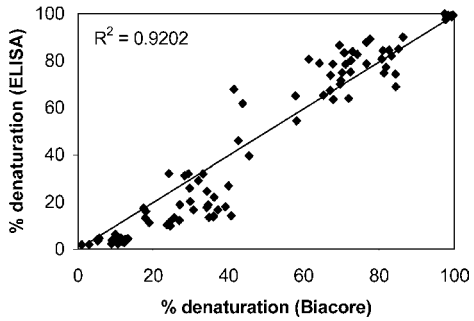

Figure 2. Correlation between the percentage of denaturation of α-lactalbumin as determined by ELISA and Biacore.

of 87 commercial milk samples on Biacore and by ELISA showed a satisfactory correlation between the two techniques ($r^2 = 0.92$) (**Figure 2**)

Discrimination on Biacore of the Heat Treatment of Milk after Manufacture. Figure 3 represents, for each type of heat treatment, the minimal and maximal α-lactalbumin denaturation index observed using Biacore. Denaturation index ranged from 5.2 to 18.9% for pasteurized milks, from 23.7 to 40.7% for highly pasteurized milks, from 24.2 to 45.4% for direct UHT milks, from 57.8 to 86.4% for indirect UHT milks, and from 97.2 to 99.7% for sterilized milks. **Figure 3** shows that the different heat treatments studied were easily discriminated, except for the high pasteurization and direct UHT treatments that were found to be extremely close. Usually, the range of the time/temperature couples used for these two heat treatments

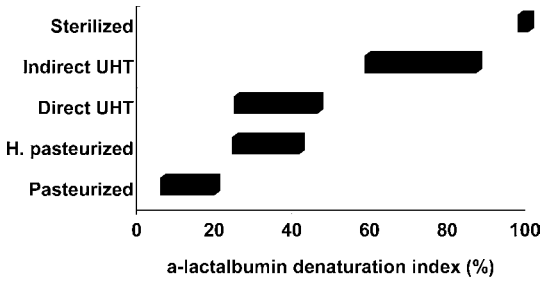

Figure 3. Minimal and maximal α-lactalbumin denaturation index determined using Biacore on 24 pasteurized, 10 highly pasteurized, 16 direct UHT, 29 indirect UHT, and 8 sterilized commercial milks directly after manufacture.

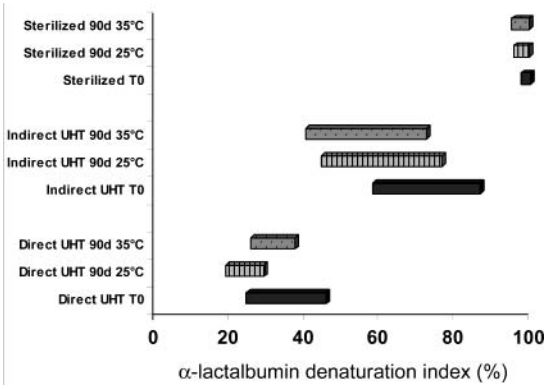

Figure 4. Effect of storage on the evolution of α-lactalbumin denaturation index in commercial direct UHT, indirect UHT, and sterilized milks analyzed directly after manufacture (solid bar) and after 90 days at 25 °C (bar with lines) or 35 °C (bar with dots).

in France is 95−110 °C/3s-1min and 140−148 °C/2s-10s for high pasteurization and direct UHT, respectively. It is thus probable that these two treatments have similar denaturing effects on milk proteins.

Effect of Milk Storage on α-Lactalbumin Denaturation Index Determined by Biacore. The evolution of α-lactalbumin denaturation index, as determined by Biacore during milk storage, was further studied. For this purpose, direct UHT, indirect UHT, and sterilized milks that were analyzed directly after manufacture were stored for a further 90 days at 25 or 35 °C. Results obtained showed that the denaturation index of α-lactalbumin decreased significantly after storage, at both temperatures (**Figure 4**). This decrease was shown to be the result of a decrease in the response obtained for HDAL, the response corresponding to NAL being quite stable (data not shown). This decrease in the response obtained with HDAL after storage does not seem to be the result of further proteolytic degradation of HDAL by residual heat-resistant proteases. Indeed, no significant proteolysis of α-lactalbumin was detected, after 90 days storage at 25 or 35 °C, using Western-blotting and revelation of the reaction with α-lactalbumin specific antibodies (data not shown). However, a possible explanation is that Maillard reaction may occur during storage and modify some epitopes on α-lactalbumin rendering their recognition by Mab 130 difficult.

Although we observed that the denaturation index of α-lactalbumin was not stable during milk storage (**Figure 4**), the different types of milk samples were still discriminated when results were plotted on a two-dimensional graph with NAL response on the *X*-axis and HDAL response on the *Y*-axis (**Figure 5**). FDA statistical analysis determined that only 6 milk samples out of the 193 analyzed on Biacore were not correctly

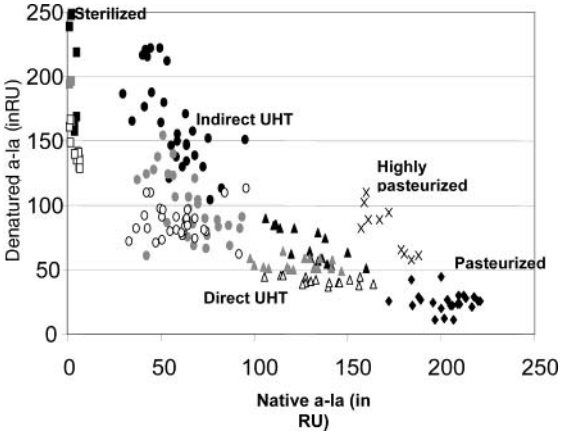

Figure 5. NAL/HDAL response (in resonance units) determined by Biacore on commercially pasteurized (◆), highly pasteurized (×), and direct UHT analyzed after manufacture (▲), 90 days at 25 °C (gray triangle) or 90 days at 35 °C (△),; indirect UHT analyzed after manufacture (●), 90 days at 25 °C (gray circle), or 90 days at 35 °C (○); and sterilized milks analyzed after manufacture (■), 90 days at 25 °C (gray square), or 90 days at 35 °C (□).

classified. Indeed, 100% of the highly pasteurized and the sterilized milks were correctly classified when analyzed using the Biacore assay. In contrast, among the 24 pasteurized milks analyzed on Biacore, 2 were wrongly classified as highly pasteurized milks. Three out of the 48 direct UHT milks were wrongly classified as highly pasteurized milks. Finally, only 1 out of the 87 Indirect UHT milk samples analyzed on Biacore was incorrectly clustered as a sterilized milk.

In this study, the development of an optical immunosensor for the determination of the heat treatment a milk has been subjected to is presented. The assay is based on the quantification of the native and the heat-denatured form of a protein marker, α-lactalbumin, using two specific monoclonal antibodies. Until now, most of the authors expressed α-lactalbumin denaturation in milk as the loss in percentage of NAL, taking the original raw milk as a reference (*7*, *14*). The NAL concentration varies significantly in raw milk, therefore these techniques cannot be applied to milk of unknown origin. On the contrary, the quantification of both NAL and HDAL in milk, together with expression of the results as the percentage of denatured α-lactalbumin, allowed determination of the heat treatment the sample was submitted to, without knowing the α-lactalbumin concentration of the original raw milk.

The assay is fast, repeatable, sensitive, automated, and requires no pretreatment of the sample apart from a dilution in the course buffer. It could therefore be of great interest for routine analysis. However, the Biacore device used to detect the antigen−antibody interactions is a tool for research applications that remains expensive.

Using this technique, we demonstrated that discrimination of pasteurized, direct UHT, indirect UHT, and sterilized milks was easy when the samples were analyzed directly after manufacture. Furthermore, statistical analysis of the data obtained on commercial milks analyzed on Biacore before and after a 90d storage at 25 or 35 °C showed that only 6 out of the 193 milks analyzed were incorrectly classified. These results reinforce the idea that the assay could be of great interest for manufacturer in controlling their heating process.

However, a 3-month storage at either 25 or 35 °C resulted in a significant decrease in the HDAL concentration in milk. Only a few studies have been carried out on the evolution of proteins during the storage of milk. Proteolysis is susceptible to occur during storage of UHT-milks and is caused mainly by either indigenous or bacterial proteases. In particular, plasmin and its zymogen plasminogen have been found to be resistant to heat inactivation (*15*), and psychrotrophic bacteria are known to produce extracellular proteinases partly resistant to UHT treatment (*16*). Therefore, could the proteolysis phenomenon be responsible for the decrease observed in HDAL in milk upon storage? Probably not. In fact, no significant proteolysis of α-lactalbumin was detected, after 90 days storage at 25 or 35 °C, using Western-blotting and revelation of the reaction with α-lactalbumin specific antibodies (data not shown).

It is therefore more probable that the decrease in HDAL observed during storage of UHT and sterilized milk is the result of increased Maillard reactions causing a decrease in the accessibility of the epitope recognized on HDAL by monoclonal antibody 130. This hypothesis is supported by the fact that during storage at 25 or 35 °C of UHT or sterilized milk, the furosine concentration of the milk samples significantly increased (*17*).

Finally, characterization of HDAL and the epitope recognized by the monoclonal antibody according to the conformation of the molecule could help in understanding the biochemical processes that occur during storage of UHT and sterilized milks. Such work is currently in progress.

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