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Food and Agricultural Immunology

ISSN: 0954-0105 (Print) 1465-3443 (Online) Journal homepage: https://www.tandfonline.com/loi/cfai20

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To cite this article: Stephanie Muller-renaud, Didier Dupont & Philippe Dulieu (2003) Quantification of κ-casein in milk by an optical immunosensor, Food and Agricultural Immunology, 15:3-4, 265-277, DOI: 10.1080/09540100400013401

To link to this article: https://doi.org/10.1080/09540100400013401

Published online: 11 Oct 2010.

Food and Agricultural Immunology, Volume 15, Number 3–4
(September–December 2003), pp. 265–277

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Quantification of κ-casein in Milk by an Optical Immunosensor

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(*Original manuscript received 17 March 2004; revised manuscript accepted 23 September 2004*)

κ-casein (κ-CN) plays an important role in the stability and coagulation properties of milk. An immunoassay to quantify κ-casein in milk, using an optical biosensor based on surface plasmon resonance (SPR) measurement, has been developed. The assay consists of a two-step sandwich strategy, with two anti-κ-casein antibodies directed against each extremity of the casein to quantify only native κ-casein. The analysis time per sample was less than ten minutes. The antibody-coated surface could be used for more than 150 determinations. The detection limit was established at 0.45 μg.ml^{-1} and the intra- and inter-assay variation coefficients were 4.28% and 6.8%, respectively. The method was applied to raw milk in order to quantify intact κ-casein, with no pretreatment of the sample. It also allowed the monitoring of κ-CN concentration during milk coagulation after addition of rennet.

Keywords: *κ-casein, milk, surface plasmon resonance*

INTRODUCTION

In cow's milk, caseins (CNs) make up around 76–86% of the total milk protein and represent four gene products named α_{S1}-, α_{S2}-, β- and κ-CN, respectively. The relative concentration of these four CNs in milk can vary with different factors such as the breed (Storry *et al*., 1983), the lactation stage of the cow (Barry & Donnelly, 1980) and the feeding (Sutton, 1989). Furthermore, indigenous milk enzymes can hydrolyze CNs and influence the CN concentrations. These variations can affect the coagulation properties of milk (Okigbo *et al*., 1985), the cheese yield (Montagne *et al*., 1995) and the general quality of cheese. Native CNs play an important role in cheese production, since they will constitute the cheese matrix, whey proteins and degradation products being mainly eliminated in the whey at

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ISSN 0954-0105 (print)/ISSN 1465-3443 (online)
DOI: 10.1080/09540100400013401

draining. So, the determination of the concentration of native CNs seems to be important in achieving an accurate estimation of the quality of milk used for cheesemaking.

Several biochemical methods, such as gel electrophoresis (Ng-Kwai-Hang & Kroeker, 1984; Collin *et al*., 1987; Deshmukh *et al*., 1989), Fast Protein (FPLC) (Andrews *et al*., 1985; Guillou *et al*., 1987; Collin *et al*., 1991) and High Performance Liquid Chromatography (HPLC) (Visser *et al*., 1991; Bobe *et al*., 1998; Mas *et al*., 1999; Bordin *et al*., 2001) and capillary electrophoresis (De Jong *et al*., 1993; Vallejo-Cordoba, 1997) have been developed to quantify the four major CNs in milk. However, none of these methods gave an optimum separation of all major milk proteins. In particular, separation between α_{S1}- and α_{S2}-CN by FPLC, and α_{S2}- and κ-CN by HPLC, were not always achieved (Leonil *et al*., 1995; Bobe, *et al*., 1998). Moreover, these techniques required sample preparation, increasing the time of analysis. Finally, a microparticle-enhanced nephelometric immunoassay has been developed to quantify αS-, β- and κ-CN (Collard-Bovy *et al*., 1991; El-Bari *et al*., 1991) and applied to milk and dairy products (Humbert *et al*., 1991). This technique uses polyclonal antibodies directed against the whole CN, rending impossible the discrimination between native CN and their degradation products.

Biosensor systems, using the detection principle of surface plasmon resonance (SPR) (Jonsson *et al*., 1991), are a recent addition to research and a new analytical tool. The biosensors enable the study of all types of interactions between two molecules (protein-protein, enzyme-substrate, antigen-antibody…) in a real time measurement and without labeling requirements. The SPR detects and measures changes in the refractive index, due to the binding and dissociation of interacting molecules in proximity to the gold surface. The change in refractive index is proportional to the quantity of analyte interacting with the ligand and allows the development of quantitative analysis. Among the optical biosensors commercially available, the Biacore system (Biacore International SA, Uppsala, Sweden) is one of the most commonly used. Indeed, it has already allowed the detection of enterotoxins (Rasooly & Rasooly, 1999; Rasooly, 2001; Homola *et al*., 2002), drug residues (Sternesjo *et al*., 1995; Baxter *et al*., 2001; Gaudin & Maris, 2001; Haasnoot & Verheijen, 2001; Ferguson *et al*., 2002; Crooks *et al*., 2003) or vitamins (Caselunghe & Lindeberg, 2000; Indyk *et al*., 2000), in milk and dairy products. Several analysis kits are now commercialized to quantify contaminants or vitamins in food products.

Among the four CN, κ-CN, which accounts for 10–12% of CN, presents a particular interest in milk and cheesemaking. This CN, soluble in the presence of calcium at all temperatures, allows the stabilization of micelle structures. In fresh raw milk, κ-CN does not seem to be sensitive to hydrolysis by plasmin (Eigel *et al*., 1979), but could be hydrolyzed by cathepsin-D at Phe_{105}-Met_{106}, the bond cleaved by chymosin (McSweeney *et al*., 1995; Larsen *et al*., 1996). The bacterial proteases are able to hydrolyze κ-CN and produce a compound similar to para-κ-casein (f1-105). This proteolysis could induce the destabilization of UHT milk during storage (Nieuwenhuijse & Van Boekel, 2003). κ-CN plays an important role during the first stage of the cheesemaking process. Its hydrolysis by chymosin in rennet at the Phe_{105}–Met_{106}, produces the para-κ-CN (f1-105) and caseinomacropetide (f106–169) and allows the destabilization of the micelle structures and the coagulation of milk.

This paper describes the development of an immunoassay to quantify κ-CN using Biacore. The method needs to be able to quantify specifically native κ-CN only and not its degradation products. Therefore, the strategy used is a sandwich immunoassay, taking the protein to be quantified between two antibodies directed against its N- and C-terminal extremities (Figure 1). This method was developed and validated on milk and applied to study the coagulation of milk.

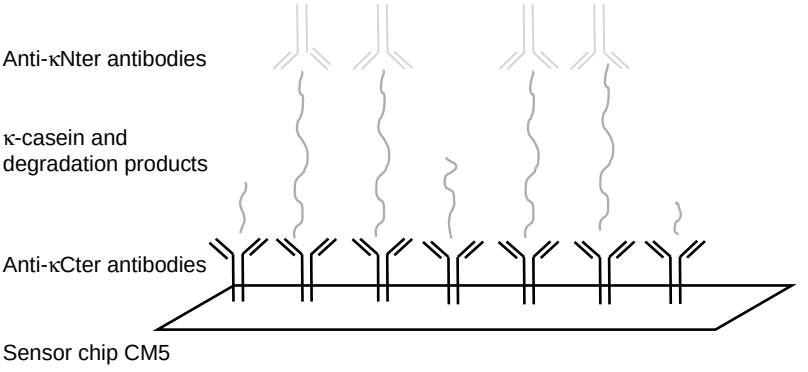

FIG. 1. Strategy used to quantify κ-casein.

MATERIALS AND METHODS

Reagents

β-CN was purified as described previously (Senocq *et al*., 2001b). α_{S1}-, α_{S2}- and K-CN, α-lactalbumin, β-lactoglobulin, Immunoglobuline G (IgG) and Bovine Serum Albumin were obtained from Sigma-Aldrich (Sigma-Aldrich, St Quentin Fallavier, France).

Milk Samples

Milk powder. Skimmed low heat milk powder (SRTAL-INRA, Poligny, France) was used for biosensor calibration. Milk was reconstituted at 12% (w/v) in $CaCl_2$ 10 mM. The concentration of each CN in milk powder had been determined by reverse phase HPLC (RP-HPLC) (Nutrinov, Vezin-le-Coquet, France).

Milk samples. Six herds (three Montbeliarde and three Holstein) of ten cows each, were milked four times over a three month period, for a total number of 48 milk samples. Samples were aliquoted and stored at -20°C. Before analysis, the top fat layer was removed by centrifugation (1800 *g*, 4°C, 15 min).

Biosensor Assay

Apparatus. The optical biosensor used to perform the assay was a Biacore 3000 (Biacore International SA, Uppsala, Sweden).

Detection principle. The BIA-technology allows real-time measurement of molecule interactions using Surface Plasmon Resonance (SPR) detection (Jonsson *et al*., 1991). One of the reactants is immobilized onto an extended carboxymethyldextran matrix layered on a gold surface (Sensor chip CM5), and the other is introduced, in a continuous flow over the sensor surface. The SPR detects and measures changes in refractive index due to the binding and dissociation of interacting molecules in proximity to the gold surface. The change in refractive index is proportional to the quantity of analyte interacting with the ligand and causes a shift in the angle of index at which the SPR phenomenon occurs. These shifts are monitored continuously over time, shown as sensorgrams and expressed in resonance units (RU). The integration of SPR detection, a microfluidic system and operator designed sensor surfaces into one automated analytical system provides quantitative analysis of biospecific interactions.

Peptide Immunogens

The peptides were chosen in the κ-CN sequence and corresponded to the N-and C-terminal extremities of the CN. They were called K-Nter and K-Cter respectively. The sequence -QEQNQEQPIRCEKDER*C*-, corresponding to the fragment 1–16 of κ-CN, constituted the peptide κ-Nter. The sequence -*C*EVIESPPEINTVQVTSTAV-, corresponding to fragment 150–169 of κ-CN, constituted the peptide κ-Cter.

The peptides were chemically synthesized by Synt:em (Nîmes, France), with a minimum purity of 85%. In the κ-Nter peptide, the cysteine residue was added at the C-terminal extremity of the peptide and modified by C-terminal amidation. In the κ-Cter peptide, a cysteine residue was added at the N-terminal extremity of the peptide.

The peptides were conjugated 2:1 w/w to Keyhole Limpet Hemocyanin (KLH) to increase their immunogenicity.

Production of Polyclonal Antibodies

Polyclonal antibodies (Pab) directed against β-, α_{S1}- and α_{S2}-CN were produced according to the procedure described above (Senocq *et al*., 2001a).

Production of Monoclonal Antibodies

Female BALB/c mice (IFFA-CREDO, St-Germain sur l′Arbresle, France) were immunized with a mixture of the two conjugated peptides (5 μg of each) in Complete Freund Adjuvant (Difco laboratories, Detroit, MI, USA) distributed equally into the rear foot pads. After 14 days, mice were immunized with 5 μg of each conjugated peptides in Incomplete Freund Adjuvant (IFFA-CREDO), using the same procedure. On day 17, draining lymph popliteal nodes were removed and pooled. The polyclonal serum of mice was collected to be tested. Fusion of lymphocytes and myeloma cells X63/Ag 8.653 (Kearny *et al*., 1979) was carried out with 1ml of 45% PEG 1000 (VWR International, Fontenay-sous-Bois, France) following the procedure described by Köhler and Milstein (1975). Supernatants of hybrid clones were assayed by antigen coated on plate enzyme linked immunosorbent assay (ACP-ELISA) (see below).

Ascites from interesting Mabs were obtained according to the procedure of Jones *et al*. (1990).

Mabs were purified from ascitic fluid by affinity chromatography using a HiTrap NHS-activated HP column (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturers recommendations. Briefly, 10 mg of κ-CN in 1 ml of coupling buffer (0.2 M $NaHCO_3$, 0.5 M) were covalently immobilized on the column. Any excess active groups were deactivated by washing with 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3 and the non-specifically bound ligands were washed out with 0.1 M acetate, 0.5 M NaCl pH 4. Ascitic fluid (1 ml) diluted in 4 ml of running buffer (75 mM Tris-HCl pH 8), was then injected. The column was washed with running buffer. Mabs were eluted with elution buffer (100 mM glycine-HCl, 0.5 M NaCl pH 2.7) and the eluted fraction was neutralized by addition of ¼ volume of 1 M Tris-HCl pH 9. The purified antibodies were aliquoted and stored at −20°C.

Characterization of Monoclonal Antibodies

By ACP ELISA. This method was used to test the hybrid clones against peptides κ-Nter, κ-Cter and κ-CN. Briefly, 100 μl of each antigen at 0.5 μg ml^{-1} in 0.1 M bicarbonate buffer pH 9.6 were coated onto a microtiter plate (NUNC F96 Maxisorp, Dominique Dutscher, Brumath, France) and incubated 1 h 30 min at 37°C. The remaining binding sites were blocked by incubating 250 μl gelatin (VWR International, Fontenay-sous-bois, France) at 10 $g.l^{-1}$ in phosphate-buffered saline, 0.05% Tween 20 (PBS-T) for 1 h at 37°C in the wells. Culture supernatants (50 μl) were diluted 1:2 in PBS-T and incubated 1 h at 37°C. Bound Igs

were detected by incubating 100 µl of donkey anti mouse immunoglobulin alkaline phosphatase conjugate (Jackson Immunoresearch, Interchim; Montluçon, France) diluted 1/3000 in PBS-T for 1 h at 37°C. Wells were rinsed between each incubation step for 15 s with four changes of 250 µl PBS-T. After the final rinsing, 100 µl *p*-nitrophenyl phosphate (Sigma-Aldrich, St Quentin Fallavier, France) at 1 g l^{-1} in 1 M-diethanolamine-HCl, 1 mm-$MgCl_2$, 0.1 mm Zinc acetate were incubated in the wells. After 30 min, the absorbance at 405 nm was read against a blank, and corrected for background signal in the absence of antigen.

Isotyping of Mabs. In standard screening conditions, light and heavy chains of bound Ig were specifically detected using anti-isotypic secondary antibodies peaked from Kit 37502 from Pierce (Interchim, Montlucon, France).

By Biacore. Mabs recognizing κ-Nter or κ-Cter peptides and κ-CN were tested in Biacore to evaluate their capacity to capture κ-CN.

Preparation of the sensor surface. RAM were immobilized covalently on a carboxymethyl 5 sensor chip, CM5 (Biacore International SA, Uppsala, Sweden) by amine coupling, as described previously (Jonsson *et al*., 1991). Briefly, a continuous flow of 10 mM HEPES pH 7.4 containing 150 mM NaCl, 3.4 mM EDTA and 0.005% Surfactant P20 (HBS-EP, Biacore) over the sensor surface at 5 µl min^{-1} was maintained. The CM5 matrix was activated by injection of a solution containing 0.05 M N-hydroxysuccinimide (NHS), 0.2 M N-ethyl-N′-(3-di-ethylaminopropyl) carbodiimide (EDC) (Biacore). Next, 35 µl of RAM (30 mg l^{-1} in 10 mM 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 RAM. RAM were immobilized on flow channel Fc 2, with Fc1 being used as reference cell.

Assay principle. A continuous flow was maintained at 10 µl min^{-1}. 20 µl of hybrid clones' supernatant recognizing κ-Nter or κ-Cter and κ-CN were injected onto the RAM sensor chip, followed by the injection of 20 µl of κ-CN at 20 µg ml in HBS-EP. Regeneration was realized with 10 µl of NaOH 50 mM.

Biosensor Immunoassay

Assay principle. Anti-κCter antibodies (1/20 in Acetate pH 4.5) were immobilized on the sensor surface as described previously. A continuous flow was maintained at 30 µl min^{-1}. 30 µl of sample were injected, followed by 30 µl of anti-κNter monoclonal antibodies (diluted 1/100 in HBS-EP buffer). The regeneration of the sensor surface was realized by two 15 µl injections of 5 mM NaOH.

Calibration curve. Calibration curve was realized with serial dilutions (1/6000 to 1/300) of milk powder of known κ-CN concentration in HBS-EP buffer. The calibration curve was established with the response corresponding to the binding of anti-κNter antibodies on the native κ-CN (Figure 2).

Specificity of the assay. The specificity was assessed by replacing Mab anti-κNter by Pab directed against β-, α_{S1}- and α_{S2}-CN as detector.

Quantification in milk samples. Milk samples were diluted 1/1200 in HBS-EP buffer. Each sample was analyzed in duplicate for determination of the κ-CN concentration.

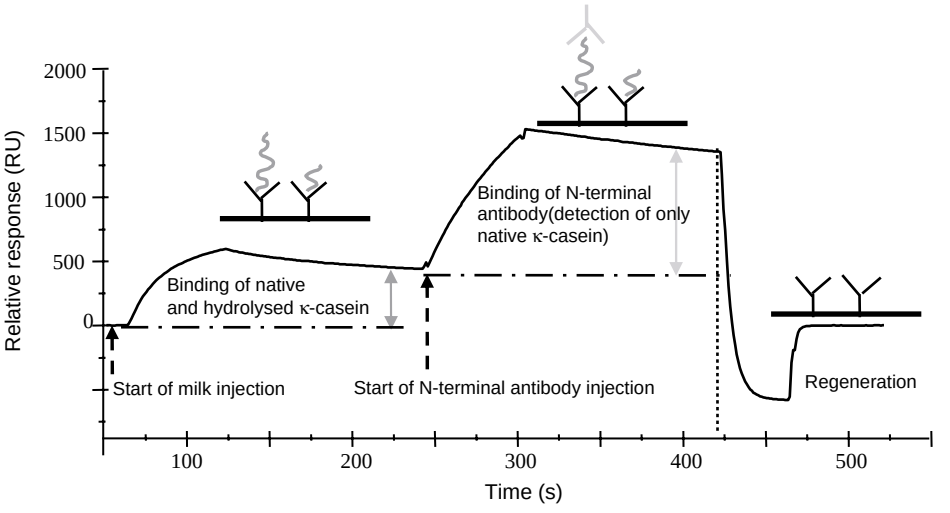

FIG. 2. Sensorgram obtained with a milk sample.

Assay validation. The detection limit was calculated from the mean of the measured content of a representative blank sample ($n = 20$) plus 3 times the standard deviation (s) of the mean (mean + 3s).

Assay precision was defined by determining *intra-assay* (within run) and *inter-assay* (between runs) variation. Intra-assay variation was determined by ten successive analyses of the same sample at three different dilutions (1/5000, 1/2000 and 1/1200). Inter-assay variation was evaluated with the same samples in 10 different runs.

To determine the accuracy, the recovery after addition of exogenous κ-CN was calculated. Four different quantities of milk powder corresponding to 0.5, 1.25, 1.75 and 2.5 mg ml^{-1} of κ-CN were added to a raw milk sample. Each sample analyzed was diluted 1/1200. Responses were the mean of two independent determinations.

Monitoring of Milk Coagulation

A rennet containing chymosin (513 mg l^{-1}) and pepsin (326 mg l^{-1}) was used for coagulation experiment. One ml of rennet diluted 1/1000 was added to 10 ml of reconstituted milk powder and incubated at 20°C. An aliquot of 50 µl was taken at different times (0, 10, 20, 30, 36, 38, 40, 42, 44, 47, 60 min), diluted 1/600 in HBS-EP and analyzed.

RESULTS

Production and Characterization of Antibodies

One fusion experiment yielded 370 hybridoma. Among those, 12 clones producing κ-CN specific Mabs were obtained. Using ACP-ELISA, ten reacted with κ-Cter peptides, two with κ-Nter peptides. These Mabs were tested in Biacore via a RAM sensor chip to see if they were able to capture κ-CN. Four Mabs directed against the C-terminal end of κ-CN recognized κ-CN after fixation on the RAM sensor chip. The Mab presenting the best fixation of κ-CN was chosen as capture antibody for the immunoassay and was called anti-κCter (data not shown).

The secondary antibody was chosen among the Mabs recognizing the N-terminal extremity of κ-CN and called anti-κNter.

The different anti-κCter Mabs belong to the IgG subclasses. The Mab chosen as capture antibody is an IgG1. The two anti-κNter Mabs were from the IgM isotype.

Characteristics of the Assay

Specificity of the assay. The specificity of the assay was assessed in two different ways. Firstly, the specificity of the anti-KCter antibodies immobilized on the chip was tested against the principal milk proteins (α_{S2}-, β-, κ-CN, α-lactalbumin, β-lactoglobulin, immunoglobulin G and bovine serum albumin). The different proteins were injected onto the anti-KCter coated sensor chip at 10 μg ml. No cross-reaction was observed. Then, the anti-KNter was replaced, after milk injection, by specific Pabs from α_{S1}-, α_{S2}-, or β-CN. No binding of these probes on the captured κ-CN was observed.

Time of analysis. The analysis for one sample, including the regeneration time, was around 10 minutes.

Stability. The regeneration conditions were optimal by addition of 5 mM NaOH. A sensor surface could be used for more than 200 cycles without any significant decrease in baseline. However, a new calibration may be necessary after 100 cycles.

Standard curve. The curve has been established with terminal antibodies response to κ-CN concentrations in reconstituted milk ranging from 0 to 20 μg ml^{-1} (Figure 3).

Detection limit. The detection limit, determined from the mean of the measured content of a representative blank sample ($n=20$; mean + 3 ds), was established at 0.45 μg ml^{-1}.

Reproducibility. The reproducibility, determined with different concentrations of a raw milk sample, established for intra- and inter-assay, was 4.28% and 6.8%, respectively.

Accuracy. Between 103 and 108% of exogenous additions of four known κ-CN concentrations in a raw milk sample were recovered (Table 1).

Analysis of Milk Samples

Forty-eight milk samples from three herds of Holstein cows (herds 1, 2, 3) and three herds of Montbeliarde cow (herds 4, 5, 6) collected at eight different milking times, were analyzed. The concentrations of intact κ-CN obtained for milk samples ranged from 3.1 to 4.7 mg ml^{-1} (Figure 4).

Analysis of Milk Coagulation

The milk coagulation has been studied by monitoring the concentration of intact κ-CN after addition of rennet to reconstituted milk at 20°C (Figure 5). The κ-CN concentration was stable for 30 min, then decreased rapidly between 30 and 45 min, to be practically nil after 60 min.

DISCUSSION

The development of an immunoassay for the quantification of κ-CN in milk using Biacore is presented in this study. The strategy used was a sandwich assay with two monoclonal antibodies directed against each extremity of the protein. Thus, only intact κ-CN could be quantified and not its degradation products. To develop this assay, the immunological tools

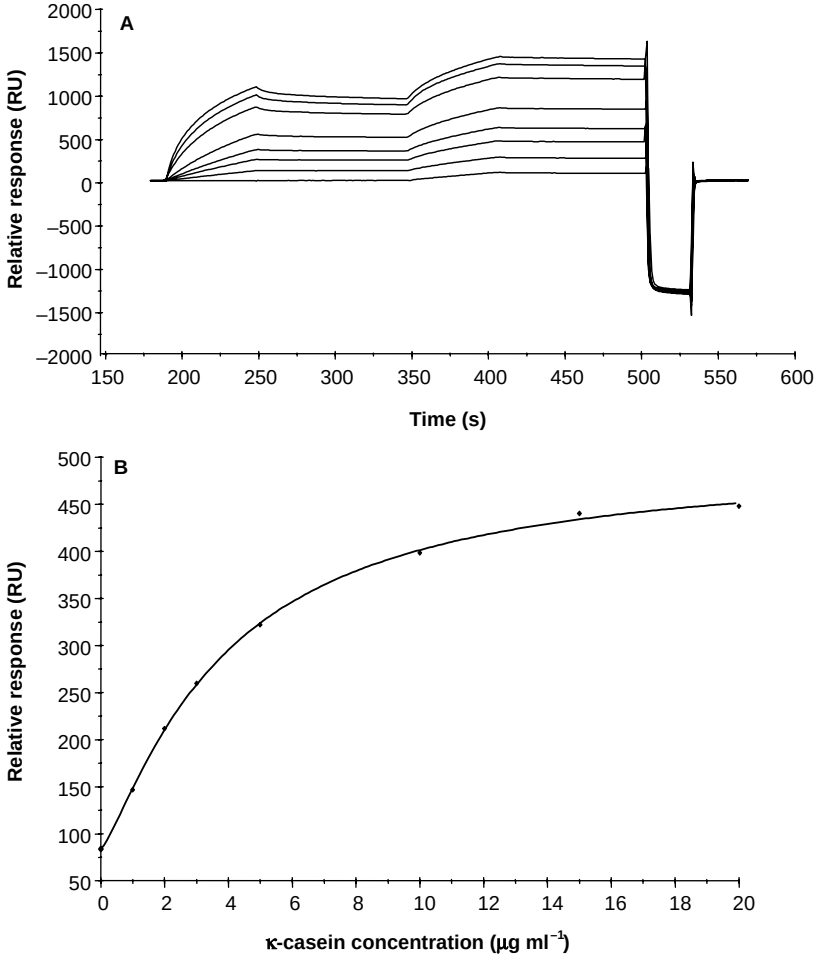

FIG. 3. Determination of a standard curve. A: sensorgram obtained for κ-casein concentration ranging from 0 to 20 μg ml^{-1}. B: Resulting standard curve calculated with N-terminal antibody response.

TABLE 1. Determination of recovery after exogenous addition of κ-casein concentration in a raw milk sample. Four different quantities of reconstituted milk powder corresponding to 0.5, 1.25, 1.75 and 2.5 mg ml^{-1} of κ-casein were added to a raw milk sample. Each sample analysed was diluted 1/1200. Responses were the mean of two determinations

	Theoretical κ-CN concentration (mg ml^{-1})	κ-CN concentration obtained (mg ml^{-1})	Recovery (%)
Milk		4.85	
Milk + 0.5 mg ml^{-1}	5.35	5.83	108
Milk + 1.25 mg ml^{-1}	6.1	6.45	106
Milk + 1.75 mg ml^{-1}	6.6	6.83	103
Milk + 2.5 mg ml^{-1}	7.35	7.58	103

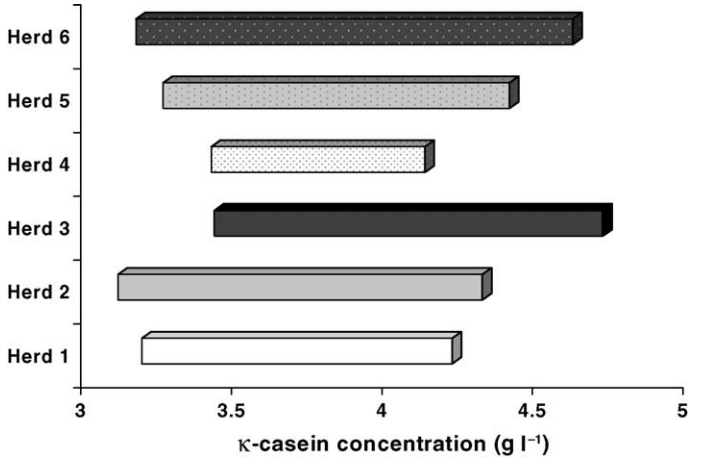

FIG. 4. κ-CN concentration calculated in 48 samples of raw milk. Six herds, three Holstein (herds 1. 2. 3) and three Montbeliarde (herds 4, 5, 6) of ten cows each, were milked height times over a three month period.

have been produced from synthetic peptides. These tool immunogens were used to target the specificity of antibodies against the interesting part of the CN. Two peptides were chosen from each extremity of the κ-CN. The strategy relied on the hypothesis that the peptide mimicked the antigenic determinant exposed by the CN. A first selection of the antibodies produced was carried out using ACP-ELISA test. Among the 370 hybridoma obtained in the fusion experiment, only 12 recognized the peptides and κ-CN. This low rate could be explained by the use of a carrier protein. Among these antibodies, a second selection was carried on Biacore to choose the antibodies which were then immobilized on the sensor chip and used to capture κ-CN. The choice was made following two criteria. The first was the ratio between the fixation level of the antibody on the RAM sensor chip and the fixation of

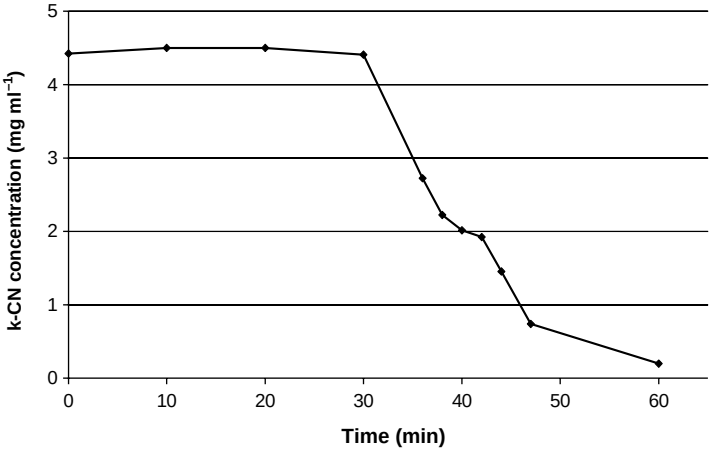

FIG. 5. Quantification of intact κ-CN in reconstituted milk after rennet addition. 1 ml of rennet diluted 1/1000 was added in 10 ml of reconstituted milk and incubated at 20°C. An aliquot of 50 µl was taken at different times, diluted 1/600 in HBS-EP and analyzed.

κ-CN on this antibody. The second was the stability of the antibody-antigen association. The two specific Mabs from the N-terminal extremity belong to IgM class of antibodies. Since it is difficult to purify Ig M, the choice of capture antibodies has been made among the Mabs recognizing the C-terminal extremity of the CN, which belong to IgG classes.

To quantify κ-CN in milk, a calibration curve was established with a reference milk powder of known κ-CN concentration to reduce the matrix effect. In milk, 95% CN are organized in a micellar structure in association with calcium and phosphate. The micelles therefore have to be disrupted for the quantification of single CN. The HBS-EP buffer used for sample dilution contains EDTA, which is known to chelate with calcium and so destabilize the CN association. To verify if the response observed with milk or milk powder was due to the binding of a CN complex and not only κ-CN, the secondary antibodies antiκ-Nter were replaced by specific Pabs from β-, α_{S1}- or α_{S2}-CN. No binding of these probes on the immobilized antigen has been observed demonstrating that only κ-CN had been captured by the anti-κCter.

This technique was applied to quantify κ-CN in 48 milk samples. The κ-CN concentrations for these samples ranged from 3.1 to 4.7 mg ml^{-1} (Figure 4). The values obtained in this study varied significantly, with a large range of concentrations. These important variations could be explained by the size of the herd. The limited size of the herds was deliberately chosen in order to increase the variability in the concentrations. The results did not show a significant influence of the breed, the feeding and the lactation stage on the κ-CN concentration of milk. The primary response obtained after milk injection corresponded to a higher κ-CN concentration than the secondary response obtained after the binding of the N-ter antibodies (data not shown). The primary response corresponds to the fixation of κ-CN and C-terminal fragments of κ-CN onto the chip whereas the secondary response corresponds to the detection of the entire protein only by the specific antibodies from the N-extremity of κ-CN. The difference in concentration could be due to the proteolysis of a part of κ-CN. Effectively, although κ-CN seems to be insensitive to degradation by plasmin (Eigel, 1977), it is known to be hydrolyzed by cathepsin-D (McSweeney *et al*., 1995; Larsen *et al*., 1996) and bacterial proteinases from populations superior to 10^4 cfu/ml (Fairbairn & Law, 1986).

During cheesemaking, milk coagulation is due to the cleavage of κ-CN by rennet chymosin. Several methods have been developed to measure the proteolysis of κ-CN by chymosin. Most of them quantify the CMP by Far UV spectroscopy, HPLC (Van Hooydonk & Olieman, 1982), ELISA (Picard *et al*., 1994) or nephelometric methods (Prin *et al*., 1996). The biosensor technique developed in this paper permits to quantify the native κ-CN and was applied to follow intact κ-CN concentration in milk after addition of rennet chymosin. The results (Figure 5) show three phases: a first phase during the first 30 min where the κ-CN concentration was stable and no milk destabilization was observed. During the second phase, between 30 and 45 min, a rapid decrease in the κ-CN concentration and the beginning of milk flocculation were observed. And a third phase after 45 min where the milk was completely coagulated. The milk coagulation is conveniently considered as a three step process: an enzymatic phase with proteolysis of κ-CN, followed by a secondary phase during which the destabilized micelles begin to aggregate and a third phase that involves changes in the coagulum structure (Hyslop, 2003). The results presented here confirm that, experimentally, the first two steps were not separable.

Since the analysis of one sample on Biacore requires 10 min, the technique will be of great interest for monitoring milk coagulation during the manufacture of fresh and blue cheeses. Indeed, for these two types of cheese, clotting time is over an hour. However, for soft, hard and semi-hard cheeses, the Biacore technique will not be useful, as the coagulation time is too short for carrying out several analyses during this period. The time of analysis could be shortened by injecting the antigen and the N-ter antibodies at the same time, rather than injecting these two molecules sequentially. Finally, it has been shown that the stability of

sterilized milks could be affected by proteolysis phenomena (Nieuwenhuijse & Van Boekel, 2003), and more precisely by the cleavage of κ-CN peptide bond Phe_{105}-Met_{106} by thermoresistant psychrotrophic bacteria proteases. The Biacore technique could be of help to the producers of milk for drinking to monitor the evolution of milk batch stability throughout storage.

The immunoassay developed here is able to quantify native κ-CN in milk. It is fast ($\approx$ 10 min per analysis), repeatable, sensitive, automated and requires no pretreatment of the sample, apart from dilution in the course buffer. It could therefore be of great interest in routine analysis. The same strategy will be applied to quantify the other CN (β-, α_{S1}- and α_{S2}-CN) using specific antibodies on the same sensor surface. This technique could constitute a progress in the characterization of the quality of milk and could be used in diverse applications in cheese.

ACKNOWLEDGEMENTS

The authors wish to thank Biacore International SA for its financial support and Dr Helen Lamprell for revising the English.

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