Microparticle-Enhanced Nephelometric Immunoassay.

1. Measurement of $\alpha_s$-Casein and $\kappa$-Casein

C. COLLARD-BOVY, E. MARCHAL, G. HUMBERT, and G. LINDEN
Laboratory of Applied Biochemistry
Associated with Institut National de la Recherche Agronomique
Faculty of Sciences
BP 239, 54506 Vandoeuvre les Nancy, France

P. MONTAGNE, N. EL BARI, and J. DUHEILLE
Laboratory of Immunology
Faculty of Medicine
BP 184, 54525 Vandoeuvre les Nancy, France

P. VARCIN
Sanofi Research
Montpellier, France

ABSTRACT

$\alpha_s$-Casein and $\kappa$-casein were measured in milk and curd by a microparticle-enhanced nephelometric immunoassay (NEPHELIA®). Specifically designed microspheres were coated with antigen ($\alpha_s$-casein and $\kappa$-casein) and then agglutinated by specific antibodies. The light scattered by the agglutinates was quantified with a nephelometer. Antigen-coated microsphere agglutination was inhibited by free antigen solution, allowing its measurement. Calibration curves for $\alpha_s$-casein and $\kappa$-casein in milk and curd, performed with a low heat milk powder as standard, largely covered the usual concentrations of caseins. Accuracy (average ratios of recovery were 98.7 and 104.3%) and precision (coefficients of variation from 1.9 to 7.4%) assessed the fidelity of the method. NEPHELIA®, applied to casein determination, offers many advantages over the classical methods of milk protein measurement: high dilution of the reagents, no pretreatment of the samples, enhanced sensitivity (few micrograms per liter), short reaction time (1 h), and easy use (no washing or phase separation).

(Key words: nephelometric immunoassay, $\alpha_s$-casein, $\kappa$-casein)

Abbreviation key: BSA = bovine serum albumin, CN = casein ($\alpha_s$, $\beta$, $\kappa$), LA = lactalbumin, LG = lactoglobulin, MS = microspheres, MS-$\alpha_s$-CN = $\alpha_s$-casein-coated microspheres, MS-$\kappa$-CN = $\kappa$-casein-coated microspheres.

INTRODUCTION

Milk is a complex, rich, and main nutrient-balanced mixture. It is a milk fat emulsion, a protein suspension, and a solution of protein, lactose, salt, vitamins, and enzymes. The two groups of milk proteins—caseins and whey proteins—are important to the nutritional quality and the stability of milk.

The dairy industry is confronted with problems with regard to milk proteins in milk composition (selection of breeds, change in composition) and technological treatments (denaturation of milk, decreased cheese-making yield). The gradual decrease in the concentration of proteins in milk during the last 20 yr in many countries of the world caused decreased cheese yield and reduced organoleptic qualities of milk and dairy products (16).

Chemical methods for measuring milk proteins are available. Procedures such as the Kjeldahl method or infrared spectrometry (15)
only measure the total protein content of milk. Gel electrophoresis (17), HPLC, and fast protein liquid chromatography (1, 4) are more sensitive and discriminant techniques, but they are not developed in the dairy industry. Immunological methods, including immunoelectrophoresis (9, 10), radioimmunoassay (3), ELISA (13, 21), and conventional immunonephelometry (7), have been improved. The previously reported application of conventional immunonephelometry to casein measurement (7) not only demonstrated the attributes of nephelometric assays (easy to perform assays) but also identified limitations of these methods (poor sensitivity and necessary clarifying pretreatment of samples).

We describe herein a new microparticle-enhanced nephelometric immunoassay and its application for the measurement of \( \alpha_v \)-casein (CN) and \( \kappa \)-CN in bovine milk. This microparticle-enhanced nephelometric immunoassay (NEPHELIA\textsuperscript{®}, Diagnostics Pasteur, Marnes la Coquette, FR) is based on the ability of antigen-coated microspheres (MS) to agglutinate in the presence of corresponding antibodies. The agglutination builds large MS clusters and induces turbidity, scattering the light of an incident monochromatic beam. The scattered light is measured with a specifically designed nephelometer. The competition for the antibody sites between the antigen bound to the MS and the free antigen inhibits the MS agglutination and, thus, allows the nephelometric measurement of the free antigen (5, 19).

MATERIALS AND METHODS

Reagents

Chemical reagents of analytical reagent grade were supplied by Merck, Darmstadt, Germany (Triton X-100, boric acid, disodium hydrogenphosphate, sodium dihydrogenphosphate, sodium azide, EDTA, acrolein, 2-hydroxyethylmethacrylate, methacrylic acid, 1,4-dithiothreitol, iodoacetamide, and glycine); Eastman Kodak Co., Rochester, NY (N,N'-methylene-diacrylamide); Prolabo-Rhône-Poulenc, Paris, France (SDS, tris-hydroxymethylaminomethane, acetic acid, 2-aminoethanol, sucrose, urea, 2-mercaptoethanol, trisodium citrate, sodium chloride, sodium hydroxide, and hydrochloric acid); Touzart et Matignon, Paris, France (polyethylene glycol 6000); Pharmacia, Upsala, Sweden (Sephadex G-75); and Whatman, London, England (DEAE-cellulose DE 22).

Bovine serum albumin (BSA) was from IBF (Biotechnics, Industrie Biologique Française, Villeneuve-la-Garenne, France). \( \alpha \)-Lactalbumin (LA) and \( \beta \)-lactoglobulin (LG) were prepared as reported (14). \( \beta \)-Casein was obtained following the method of Aschaffenburg (2) modified by Garnier et al. (8). Freund's incomplete and complete adjuvants were purchased from Hoechst Behring, Marburg, Germany.

Fresh raw milks were collected from November 1985 to March 1987 from nine herds with an average of 28 cows (Friesian and Red and White French breeds) as described (11). Bulk raw milk (5000 L), stored from 24 to 48 h at 4°C, was obtained every week by the mixture of four successive milkings. A part of the milk of each collection was pasteurized (72°C, 15 s) and fat standardized (25 g/L) by partial skimming and then used in an experimental pilot-plant cheese making of soft cheese (Camembert-type cheese) and pressed cheese (Saint Paulin-type cheese). In both classical cheese-making processes, rennet was used to coagulate the standardized milk, which was stocked for maturing during 45 min at 37°C with calcium chloride and several starters (11).

A low heat milk powder was obtained as follows: pasteurization (72°C, 15 s), evaporation (45°C) to 50% of dry extract, filtration (63°C), and atomization (spray process at 170°C). The dried milk was dissolved (1.2 g of powder in 10 g of distilled water) by stirring for 15 min followed by storage for 2 to 6 h in darkness at room temperature. The concentrations of \( \alpha_v \)-CN and \( \kappa \)-CN in the solution (11.98 g/L and 2.88 g/L, respectively) were measured by fast protein liquid chromatography (ion exchange on Mono Q, Pharmacia, Uppsala, Sweden). The use of the low heat milk powder as the standard for nephelometric assays of \( \alpha_v \)-CN and \( \kappa \)-CN was evaluated prior to the experiment by comparative study of 260 fresh milk samples (data not shown).

Preparation of Caseins

Whole casein was purified according to Nitschmann and Lehmann (20). Skim milk was acidified to pH 4.6 by addition of 12N...
Preparation of Antisera

α₅-Casein or κ-CN were separately dissolved in .14 M NaCl to 10 g/L, mixed volume to volume (1 ml) with complete Freund’s adjuvant, and injected subcutaneously into five rabbits for each protein. Booster injections with incomplete Freund’s adjuvant were administered monthly for 8 mo. Blood samples (30 to 40 ml) were collected 7 d after the booster injections. Antisera were separated by centrifugation (2000 × g, 10 min), clarified by a 1000 × g for 10 min, and finally split into aliquots and frozen at -20°C.

Reactivity and specificity of the antisera were controlled by conventional immunonephelometry (7). Reactivity was tested by mixing threefold diluted antiserum with nine dilutions of antigen solution (.65 to 166.66 mg/L) in .14 M NaCl. Antiserum titer was defined as the amount of antigen precipitated by 1 ml of pure antiserum. Specificity was assessed against the main milk proteins according to the same protocol by mixing antigens in the following way: anti-κ-CN antiserum with α-LA, β-LG, BSA, α₅-CN, and β-CN and anti-α₅-CN antiserum with α-LA, β-LG, BSA, κ-CN, and β-CN.

Preparation of α₅-CN-Coated and κ-CN-Coated MS

Polyfunctional hydrophilic MS were synthesized as previously described (6), by copolymerization with gamma irradiation (⁶⁰Co, 75 krad/cm², 3 h) of acrylic monomers [acrolein, 47% (vol/vol), 2-hydroxyethylmethacrylate, 49.7%, methacrylic acid, 2%, and N,N'-methylenediacrylamide, 1.3% (wt/vol), total concentration, 120 g/L] in the presence of SDS (.9 g/L) as surfactant.

α₅-Casein and κ-CN were separately bound to these MS by mixing 2 × 10⁻⁸ mol of each purified casein with 10 mg of MS in 1 ml of .05 M borate buffer, pH 8, for 2 h at room temperature and 18 h at 4°C. Any remaining free sites of MS were blocked by primary amino functions of glycine (.04 M in the binding mixture) by stirring for 2 h at room temperature. Then, unbound ligands were separated from coated MS by centrifugation (1 h, 5000 × g) on a sucrose discontinuous gradient (200 to 800 g/L in .05 M borate buffer, pH 7.2). The κ-CN-coated MS (MS-κ-CN) were stored at 4°C with 30 mM NaN₃, and α₅-CN-coated MS (MS-α₅-CN) were frozen at -20°C.

Immunonephelometric Assays for α₅-CN and κ-CN

Immunonephelometric assay of coated MS were performed by mixing coated MS (25 mg/L) with serial dilutions of antisera (1:50 to 1:6400) for agglutinations and, for inhibitions, by mixing coated MS and 800-fold diluted anti-α₅-CN or 400-fold diluted anti-κ-CN antiserum with free α₅-CN or κ-CN (0 to 5 mg/L).

The experimental procedure for assays in bovine milk consisted of mixing antisera (800-fold diluted anti-α₅-CN, 480-fold diluted anti-κ-CN) with unknown sample (or six serial dilutions of the standard for calibration curves). After incubation for 30 min at room temperature, MS-α₅-CN or MS-κ-CN was added 60 min prior to nephelometric measurements with the Diagnostics Pasteur nephelometer (NEPHELIA® N 600). Milk samples needed no pretreatment and were used 36,000-fold (for α₅-CN) and 72,000-fold diluted (for κ-CN) in the reaction mixture. Curd samples from soft cheese (Camembert-type cheese) and pressed cheese (Saint Paulin-type cheese) were suspended (3.33 g/L) in .04 M citrate buffer, pH 8, and then 750-fold diluted in the reaction buffer. All dilutions were performed with an automatic dilutor (Hamilton, Bonaduz, Switzerland) in the milk nephelometry buffer (.05 M borate, pH 8, containing 1.5 mM EDTA, 30 mM NaN₃, 2 g/L of Triton X-100, and 30 g/L of polyethylene glycol 6000). This buffer was previously determined to optimize physicochemical conditions.

RESULTS AND DISCUSSION

Control of the Obtained Antisera

Electrophoretic patterns of $\alpha_s$-CN, $\beta$-CN, and $\kappa$-CN preparation are shown in Figure 1. These purified proteins were used as immunogen in the preparation of the antisera, to coat MS, in the immunonephelometric study of coated MS, and for evaluation of the assays.

The titer of the antisera did not increase after the third booster injection. Antisera titers ranged from 125 to 500 µg of casein precipitated by 1 ml of antiserum for three and four rabbits immunized with $\alpha_s$-CN and $\kappa$-CN, respectively. Because the purified $\alpha_s$-CN was composed of $\alpha_{s1}$- and $\alpha_{s2}$-CN, the anti-$\alpha_s$-CN antisera reacted with the total $\alpha_s$ fraction. Specificity studies showed that weak cross-reactions were observed between the anti-$\alpha_s$-CN antisera (1%), the anti-$\kappa$-CN antisera (1 to 4%), and $\beta$-CN preparation. These reactions were probably due to the contamination, observed by electrophoresis, of the $\beta$-CN preparation by $\alpha_s$-CN and $\kappa$-CN. One of the anti-$\kappa$-CN antisera reacted with $\alpha_s$-CN (8%) and would require purification on immunoadsorbent prior to use. All other specificity assays showed no measurable cross-reaction.

Immunonephelometric Study of Coated MS

Microspheres of 235 nm (SD = 6 nm, $n = 26$) dry diameter were produced by copolymerization of acrylic monomers with a polymerization yield of 63%. $\alpha_s$-CN and $\kappa$-CN were covalently bound to these MS via the formation of imine bonds between aldehyde groups of the MS and primary amino functions of the protein. The MS-$\alpha_s$-CN and MS-$\kappa$-CN had an excellent long-term stability when stored as previously described (6).

The reactivity study of the MS-$\alpha_s$-CN and MS-$\kappa$-CN with the antisera allowed the determination of their immunonephelometric performance. They were agglutinated, respectively, by serial dilutions of anti-$\alpha_s$-CN and anti-$\kappa$-CN antiserum, and the agglutinations were quantified using the nephelometer after 30 min and 1 h of reaction (Figure 2). Antiserum dilutions used for the inhibition assays were selected to ensure that excess antigen was present. Respective agglutination of the MS-$\alpha_s$-CN or MS-$\kappa$-CN was fully inhibited by the purified $\alpha_s$-CN (1.5 mg/L) or $\kappa$-CN (5 mg/L).
Figure 2. Agglutinations of \( \alpha_s \)-casein-coated microspheres (MS-\( \alpha_s \)-CN) and \( \kappa \)-casein-coated microspheres (MS-\( \kappa \)-CN) by serial dilutions of anti-\( \alpha_s \)-casein (\( \alpha_s \)-CN) and anti-\( \kappa \)-casein (\( \kappa \)-CN) antiserum: MS-\( \alpha_s \)-CN and anti-\( \alpha_s \)-CN antiserum (\( \bullet \)), reaction time 1 h, MS-\( \kappa \)-CN and anti-\( \kappa \)-CN antiserum (\( \square \)), reaction time 30 min. Symbols drawn aside correspond to coated MS alone (25 mg/L).

Assays of \( \alpha_s \)-CN and \( \kappa \)-CN in Milk and \( \alpha_s \)-CN in Curd

The calibration range (Figure 4) for \( \alpha_s \)-CN assay in milk was from 1.68 to 53.91 g/L with use of 36,000-fold diluted samples and from 10.52 to 336.93 g/kg for the \( \alpha_s \)-CN assays in curd. For the \( \kappa \)-CN assay in milk (72,000-fold diluted samples) the standard curve included concentration ranges from 0.40 to 12.96 g/L.

As can be seen in Table 1, linear recovery profiles were observed for all assays on large ranges of casein concentration: \( \alpha_s \)-CN in fresh milk between 4.0 and 34.3 g/L, \( \alpha_s \)-CN in preparation used for the MS coating, demonstrating the specificity of the reaction (Figure 3). About 100 and 300 \( \mu \)g/L of \( \alpha_s \)-CN and \( \kappa \)-CN, respectively, in the reaction mixtures were able to produce 50% of inhibition, and a wide range was observed. The MS-casein conjugates were thus usable for sensitive measurement of \( \alpha_s \)-CN and \( \kappa \)-CN by microparticle-enhanced nephelometric immunoassay.

Figure 3. Inhibition of \( \alpha_s \)-casein-coated microspheres (MS-\( \alpha_s \)-CN) and \( \kappa \)-casein-coated microspheres (MS-\( \kappa \)-CN) agglutinations by free \( \alpha_s \)-casein (\( \alpha_s \)-CN) and free \( \kappa \)-casein (\( \kappa \)-CN): MS-\( \alpha_s \)-CN and 800-fold diluted anti-\( \alpha_s \) antiserum (\( \bigcirc \)), MS-\( \kappa \)-CN and 400-fold diluted anti-\( \kappa \)-CN antiserum (\( \bigstar \)). Symbols drawn aside correspond to agglutinations of coated MS (25 mg/L) without free casein. Reaction time 1 h.

Figure 4. Calibration curves for \( \alpha_s \)-casein (\( \bigcirc \)) and \( \kappa \)-casein (\( \bigstar \)) quantitation in bovine milk.
Camembert-type and Saint Paulin-type cheese curd (between 21.5 and 137.2 g/kg and between 35.5 and 117.4 g/kg, respectively), and K-CN in milk from 1.3 to 3.4 g/L. Mean recovery was between 98.7 and 104.3%.

Results of the precision studies are given in Table 2 for the assays of \( \alpha_\text{S}-\text{CN} \) and K-CN in milk. Coefficients of variation for within and between assays were between 1.9 and 5.6% and assessed the reproducibility of the method in spite of the great dilutions of samples. These dilutions were possible because of the sensitivity of the assay and provided the possibility for assays without a sample blank or sample pretreatment by skimming or clarification. The coefficients of variation were 7.4 and 5.2% (n = 21) for \( \alpha_\text{S}-\text{CN} \) determination in curd from soft cheese (mean = 59.88 g/kg, SD = 4.46 g/kg) and pressed cheese (mean = 76.31 g/kg, SD = 3.99 g/kg), respectively.

CONCLUSIONS

Microparticle-enhanced nephelometric immunoassay thus appears as a new and suitable method to measure caseins in bovine milk and curd. This technique is accurate and simple in procedure: dilution of samples (milk, curd, or standard), addition of reagents (antiserum and coated MS), automated measurement without washing or phase separation, and editing of results. Reaction time is short, and up to 480 specimens can be assayed in 1 h.

Compared with conventional immunonephelometry, this new method offers the advantages of a greater security in the results (no error in case of antigen excess because of the chosen inhibition mode), an economy of used antiserum (very diluted), and an absence of sample pretreatment by using high dilutions due to the greater sensitivity. Applied to caseins, this microparticle-enhanced nephelometric immunoassay needs a fitted procedure for each assayed protein (\( \alpha_\text{S}-\text{CN} \) and K-CN) and sample type (milk and curd). Other reports describe the application of this method to measure whey proteins \( \alpha\text{-LA} \) and \( \beta\text{-LG} \) (14) and to follow the evolution of these parameters during 1 yr of an experimental cheese making (11).

### REFERENCES


Nephelometric immunoassay of caseins