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Development of a Microparticle-enhanced Nephelometric Immunoassay for the Quantification of Beta-casein in Milk

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Beta-casein determination has been performed in cow's milk by a microparticle-enhanced nephelometric immunoassay. Hydrophilic monodisperse beta-casein coated microspheres, specifically conceived as an antigen-antibody reaction support, are agglutinated by anti-beta-casein antiserum. Free beta-casein inhibits this immunoagglutination, allowing its fast (30 min) and sensitive ($1 \mu\text{g l}^{-1}$) quantification with accuracy and precision, by nephelometric measurement of the light scattered by the beta-casein coated microsphere agglutinates. This immunoassay of beta-casein is easy to perform in milk (no washing, no phase separation, nor sample pretreatment). Microparticle-enhanced nephelometric immunoassay appears as an alternative method to measure a large variety of molecules in dairy analysis.

Keywords: Particle immunoassay, immunonephelometry, beta-casein

INTRODUCTION

Microparticle-based immunoassays have been widely used for several years in clinical chemistry to measure many analytes (Kricka, 1985; Bangs, 1990). Preliminary studies (Montagne *et al.*, 1980; Duheille *et al.*, 1984; Bessou *et al.*, 1985a, b) have shown great interest of nephelometric immunoassays using hydrophilic, polyfunctional and monodisperse microspheres (MS) specially synthesized as an antigen-antibody reaction support (Duheille *et al.*, 1982) and covalently coated with protein (MS-protein conjugate). These MS-protein conjugates are agglutinated by specific antibodies and their agglutination can be inhibited by free protein, allowing its quantification by measurement with a nephelometer of the light scattered by MS-protein conjugate

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clusters. The progress in particle synthesis has led to the development of a micro-particle-enhanced nephelometric immunoassay named Nephelia® (Diagnostics Pasteur, France) for clinical analysis (Montagne *et al.*, 1991; Cuilliere *et al.*, 1991).

Bovine beta-casein (β -CN) (Eigel *et al.*, 1984), a major component of milk caseins, is a phosphoprotein of 209 amino acid residues (MW 24 000) (Ribadeau-Dumas *et al.*, 1972). Its proteolysis by the enzyme plasmin (Humbert & Alais, 1979) produces three hydrophobic C-terminal peptides (cleavage at residues 28/29, 105/106 and 107/108) and the complementary N-terminal hydrophilic peptides (Eigel, 1977; Andrews & Alichanidis, 1983). These fragments of denatured β -CN coexist with native β -CN in cow's milk and thus form an intricate system (Eigel *et al.*, 1979).

We report here the development of a microparticle-enhanced nephelometric immunoassay for the measurement of β -CN in cow's milk. Such an example allows us to foresee the applicability of this method to other immunological analyses in the dairy industry.

MATERIALS AND METHODS

Chemical Reagents

Acrolein, 2-hydroxyethylmethacrylate, methacrylic acid, polyoxyethylene ether Triton® X-100 and disodium salt of ethylenediaminetetraacetic acid (EDTA- Na_2) were obtained from Merck (Darmstadt, Germany). *N,N'*-methylene diacrylamide from Eastman Kodak Co. (Rochester, NY). Sodium dodecyl sulphate, hydroquinone, 2-aminoethanol, sucrose, boric acid, sodium azide and polyethylene glycol (PEG) 6000 were analytical grade reagents purchased from Prolabo Rhône Poulenc (Paris, France). Immunonephelometric assays were carried out with a 0.05 M borate buffer, pH 8.0, containing 1.5 mM EDTA- Na_2 , 30 mM NaN_3 , Triton X-100 2 g l⁻¹, and 30 g l⁻¹ of PEG 6000 (milk nephelometry buffer).

Biochemical Reagents

Most bovine caseins and whey proteins were extracted from a pool of fresh raw milks: β -CN according to the method of Aschaffenburg (1963) modified by Garnier *et al.* (1964); alpha-casein (α_s -CN) by the method of Mercier *et al.* (1968); alpha-lactalbumin (α -LA) and beta-lactoglobulin (β -LG) according to Armstrong *et al.* (1967) and Fox *et al.* (1967) respectively. K-casein (K-CN) was a Sigma Chemical Co. (St Louis, MO) product (80% pure). Fragments of denatured β -CN were obtained following the method of Reimerdes & Herlitz (1979) from milk stored on 14 days at 30°C (mixture of C-terminal peptides: β -CN f(29–209), f(106–209) and f(108–209)), and according to Aschaffenburg (1946) from skimmed milk heated up to 95°C for 30 min (complementary N-terminal fragments: β -CN f(1–28), f(1–105, 1–107) and f(29–105, 29–107)). Polyacrylamide gel electrophoresis (Laemmli & Favre, 1973; Hillier, 1976; Basch *et al.*, 1985) and amino acid analysis (Moore & Stein, 1963) (Technicon NC II P) were performed for all protein preparations.

Anti- β -CN antisera were obtained by immunizing rabbits with intradermal injections of purified β -CN (10 mg in 1 ml of 0.14 M NaCl) emulsified with 1 ml of Freund's Complete Adjuvant (Behring, Marburg, FRG) for the first injection and incomplete adjuvant for following booster injections (after 13, 36, 65 and 240 days). Antisera were collected seven days after each booster injection.

A 'low heat' milk powder (pasteurization below 80°C, evaporation to 50% dry extract and atomization) was used as standard for β -CN determination. After redissolving (1.2 g in 10 g of distilled water) and equilibration (2 h in darkness), its β -CN concentration was 7.5 g l⁻¹.

Synthesis of MS

Distilled acrylic monomers (2-hydroxyethyl methacrylate 44.78 g, acrolein 42.30 g, methacrylic acid 1.8 g, *N,N*-methylene diacrylamide 1.13 g) and sodium dodecyl sulphate (0.68 g) were mixed under argon in deionized water (final volume: 0.75 l). The mixture was gamma irradiated (^{60}Co , 60 krad cm^{-2} , 3 h) in a 1.5 l borosilicated glass vessel with rotating agitation. MS suspension thus obtained was stored at 4°C with hydroquinone (1 g l^{-1}) for several years. MS shape, size, dispersion and concentration were determined as previously reported (Duhelle *et al.*, 1982).

Preparation of MS- β -CN Conjugate

MS (10 mg) and purified β -CN (0.5 mg) were incubated for 2 h at room temperature and 18 h at 4°C in 0.05 M borate buffer pH 8 (1 ml). 50 μl of 2.4 M 2-aminoethanol solution, adjusted to pH 8 with acetic acid, were added to the binding mixture before recovery of MS- β -CN conjugate by centrifugation (1 h, 12 000g, 4°C) on a discontinuous sucrose gradient (200/800 g l^{-1} in 0.05 M borate buffer, pH 8). The MS- β -CN conjugate was stored at 4°C in 0.05 M borate buffer, pH 8, 0.03 M NaN_3 .

TABLE 1. Procedure for quantification of β -CN in bovine milk

Reagent	Reaction volume (μl)	Reagent dilution or concentration in reaction cuvette
Anti- β -CN antiserum	30	1/1000
Six serial dilutions of standard milk or milk sample	15	0.0750 to 0.0023 mg l^{-1} 1/400 000
Nephelometry buffer	195	
MS- β -CN conjugate	60	50 mg l^{-1}
Nephelometric measurement after 30 min		

Immunonephelometric study of MS- β -CN Conjugate

This was performed by nephelometric measurement during the reagent's reaction in the milk nephelometry buffer, with anti- β -CN antisera and with or without known amounts of the main milk proteins (β -CN, α_s -CN, K-CN, β -LG and α -LA).

Nephelometric measurements were carried out with the Diagnostics Pasteur nephelometer (Nephelia N 600). A disposable microcuvette (Nephelia cuvette, Diagnostics Pasteur) which contained the reaction mixture was illuminated by the incident light of an He-Ne laser (632.8 nm, 2 mW) and the intensity of scattered light was measured at an angle of 10° with a light sensitive silicon diode.

MS- β -CN Conjugate as a Reagent for Quantification of β -CN in Milk

Microparticle enhanced nephelometric immunoassay was a one-step assay: anti- β -CN antiserum, milk sample and MS- β -CN conjugate were mixed in a disposable microcuvette according to the procedure described in Table 1. All dilutions were performed with a Hamilton (Bonaduz, Switzerland) dilutor in the milk nephelometry buffer. For the calibration curve, serial dilutions of the milk powder solution used as the standard was put in the place of the milk sample.

The precision of the assay was studied by measuring β -CN concentration in three milks (low, middle and high) 10 times in the same run. Recovery studies were performed by comparing results of β -CN determination in six serial dilutions of a milk

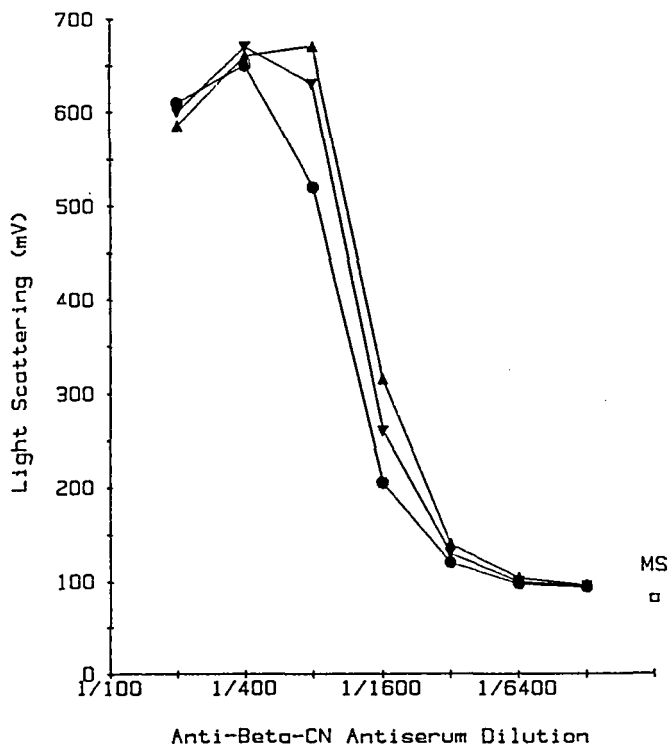


FIG. 1. Intensity of light scattered, as a function of anti- β -CN antiserum dilution, during immunological agglutination of MS- β -CN conjugate (50 mg l^{-1}). Reaction time: 30 min (●); 1 h (▼); 2 h (▲). MS: intensity of light scattered by the MS- β -CN conjugate alone.

with known β -CN concentration (9.3 g l^{-1}) with those of β -CN determination in a milk spiked with seven increasing amounts of purified β -CN (1.43 to 9.50 g l^{-1}). Interference of the fragments of denatured β -CN was assayed by spiking a milk with 2, 4 and 6 g l^{-1} of N-terminal and C-terminal peptides mixtures.

RESULTS

MS- β -CN Conjugate

Hydrophilic and polyfunctional MS (mean dry diameter = 220 nm , $\text{SD} = 7 \text{ nm}$, $n = 49$) were produced by copolymerization of acrylic monomers with a synthesis yield of 60%. Binding of β -CN (electrophoretically 90% pure) to these MS yielded the MS- β -CN conjugate, stable for several months without reduction of the imine bonds formed between aldehyde functions of the MS and primary amino groups of the β -CN (Duheille *et al.*, 1982).

Immunophelometric Study of MS- β -CM Conjugate

MS- β -CN conjugate (50 mg l^{-1}) was agglutinated by serial dilutions ($1/200$ to $1/6400$), of an anti- β -CN antiserum. The light scattered during their agglutination was quantified by the nephelometer as early as 30 min after initiation of the reaction (Figure 1): it increased up to a maximum (670 mV), variable with the reaction time, then decreased to the level of the light scattered by the MS- β -CN conjugate alone (80 mV).

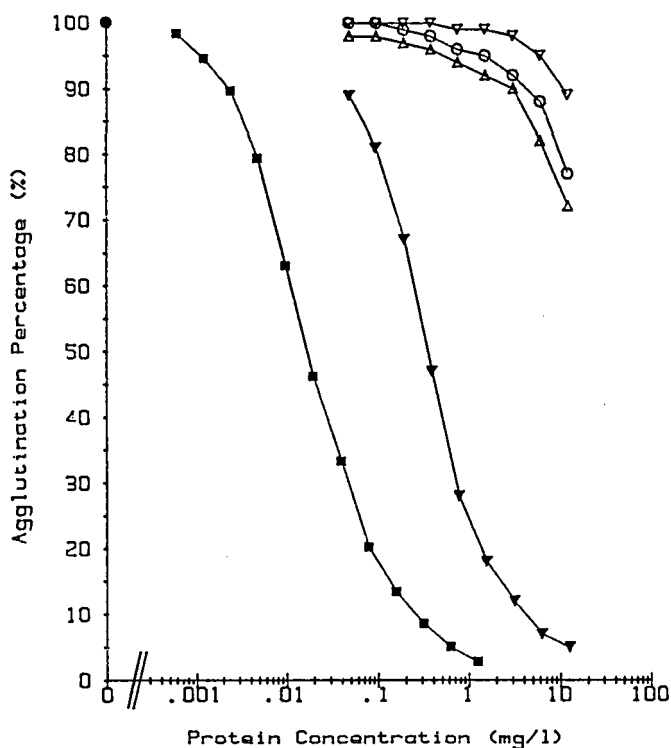


FIG. 2. Variation of agglutination percentage with free protein concentration during inhibition of MS- β -CN agglutination. β -CN (■); K-CN (▼); α_5 -CN (Δ), β -LG (O), α -LA (▽), 100% agglutination without free protein (●). MS- β -CN concentration: 50 mg l⁻¹. Anti- β -CN antiserum 1000-fold diluted. Reaction time: 30 min.

When MS- β -CN conjugate and 1000-fold diluted anti- β -CN antiserum were mixed with various concentrations of free purified β -CN, the MS- β -CN agglutination was completely inhibited by 1.2 mg l⁻¹ of β -CN in the reaction mixture (Figure 2). Fifty per cent inhibition was induced by 17 μ g l⁻¹ of β -CN and 1 μ g l⁻¹ of β -CN was detectable in the reaction mixture as giving an intensity of scattered light significantly lower (35D) than the intensity of light scattered without β -CN.

Figure 2 shows that free α_5 -CN, β -LG and α -LA did not interfere in the MS- β -CN immunoagglutination (cross-reaction lower than 0.1%). However, an inhibition curve was observed with K-CN. Its shape was comparable with that obtained for β -CN but 390 μ g ml⁻¹ of K-CN preparation were necessary to produce 50% inhibition. Electrophoretical study of this commercial K-CN preparation has shown its contamination of β -CN to be about 5%.

Quantification of β -CN in milk

The calibration curve for β -CN determination in bovine milk can be seen in Figure 3. The calibration range extended from 0.94 to 30 g l⁻¹, with a good reproducibility (coefficients of variation (CVs) lower than 4.5% for 10 consecutive assays) for milk sample diluted 400 000-fold in the reaction mixture.

Fidelity of the assay was assessed by the results of the within-assay precision study: $n=10$; mean=4.10 g l⁻¹, SD=0.09 g l⁻¹, CV=2.2%; mean=6.38 g l⁻¹, SD=0.09 g l⁻¹, CV=1.4%; mean=8.89 g l⁻¹, SD=0.16 g l⁻¹, CV=1.8%.

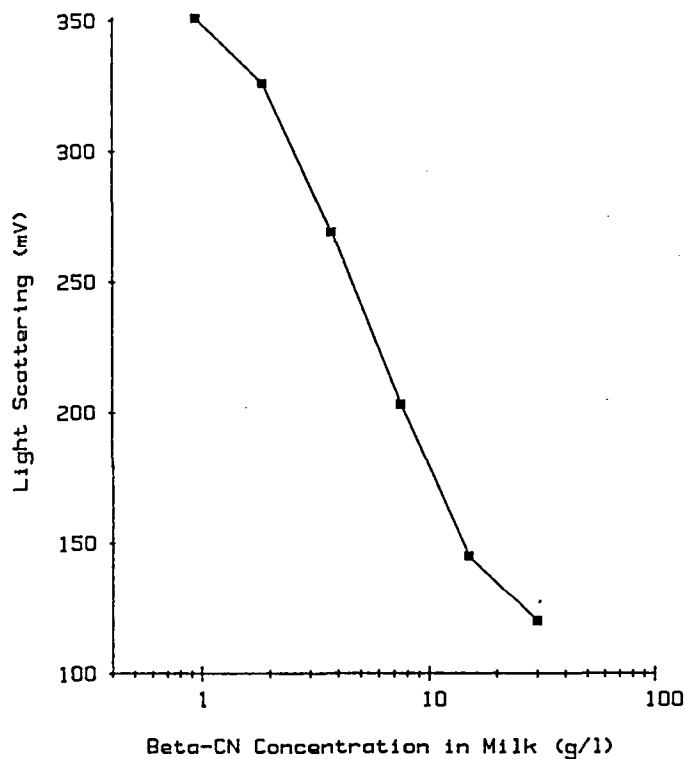


FIG. 3. Calibration curve for Nephelia of β -CN in bovine milk. Assay procedure given in Table 1.

A linear profile can be seen (Figure 4) for analytical recovery from 0.3 g l^{-1} to 9.5 g l^{-1} of β -CN (recovered β -CN = $-0.179 + 1.064 \text{ added } \beta\text{-CN}$, $n=13$, $r=0.998$). Percentages of recovery were ranged between 82% and 112% (mean = 100.2% , $\text{SD}=9.04\%$, $n=13$). Fragments of denatured β -CN were not highly recovered in the β -CN assay; 3.9% and 12.1% for mixture of β -CN f(29–209), f(106–209) and f(108–209) and mixture of β -CN f(1–28), f(1–105, 1–107) and f(29–105, 29–107) respectively.

DISCUSSION

Hydrophilic monodispersed MS with predetermined diameter can be produced by copolymerization of acrylic monomers under gamma-irradiation. Purified β -CN is covalently bound to these MS via the formation of imine bonds between aldehyde sites on MS and primary amino groups of the protein. Agglutination of thus obtained MS- β -CN conjugate with an anti- β -CN antiserum is inhibited by purified β -CN and milk β -CN. Measurement of the light scattered by agglutinates of the MS- β -CN conjugate with a specially designed nephelometer allows β -CN quantification in a microparticle-enhanced nephelometric immunoassay.

This β -CN Nephelia is easy to perform (one step, no washing, nor phase separation), fast (30 min), sensitive ($1 \mu\text{g l}^{-1}$ of β -CN significantly detected) and fully automatizable. Its specificity is assessed by the complete inhibition of the MS- β -CN conjugate immunoagglutination with free β -CN, and at the same time by the low interference of other main milk proteins (lower than 0.1% for α_5 -CN, α -LA, and β -LG, about 5% for K-CN probably due to a contamination of the commercial preparation used). Analytical recovery and precision studies confirm the accuracy and the repeatability of the

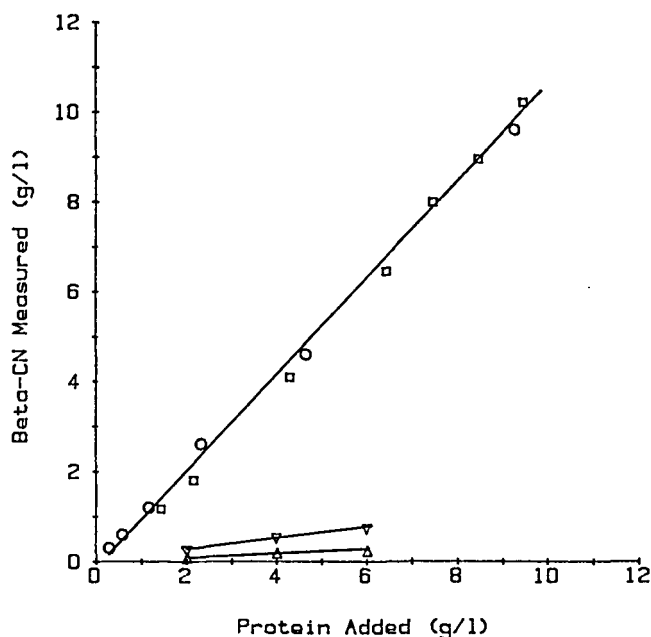


FIG. 4. Analytical recovery of β -CN. Diluted milk (O), spiked milk with purified β -CN (□), spiked milk with mixture of β -CN f(29-209), f(106-209) and f(108-209) (Δ), spiked milk with mixture of β -CN f(1-105, 1-107), f(29-105, 29-107) and f(1-28) (∇).

assay which allows measurement of native β -CN in the presence of its main derivated C- and N-terminal fragments without great interference from them.

The great sensitivity obtained in this assay is not necessary, of course, for milk β -CN quantification. However, it allows a high dilution of milk, and sample blank measurement or milk pretreatment (skimming, extraction) can be avoided. The chosen competitive mode avoids under-evaluation of analyte in the antigen excess condition, as may be seen in all immunoassays based on the classical antigen-antibody precipitation, and gives the possibility of hapten determination (Duheille *et al.*, 1984).

Measurement, by microparticle-enhanced nephelometric immunoassay, of several human serum or plasma proteins has already been reported (Montagne *et al.*, 1991; Cuilliere *et al.*, 1991) for clinical analysis and some of these applications are the basis of diagnostics kits already produced. The β -CN assay here described shows that this method is likewise usable for analysis in the dairy industry. A similar application to other caseins (K-CN, α -CN) and whey proteins (α -LA, β -LG, and Immunoglobulins G) in cow milk and dairy products has also been foreseen and will shortly be published. Microparticle-enhanced nephelometric immunoassay, using a new hydrophilic support specifically conceived for that use and an adapted nephelometer, indeed appears as an alternative method for routine and research determination of a large variety of molecules, which today are measured by more complex methods in dairy and food-processing analysis.

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