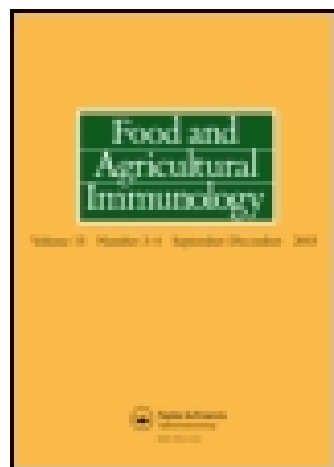




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Study of β -casein Denaturation by Microparticle-enhanced Nephelometric Immunoassay

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Rabbit anti-native bovine β -casein antiserum reacted with native β -casein and fragments $f(1-105/7)$ and $f(106-209)$ formed during β -casein proteolysis by plasmin. Agglutination of β -casein-coated microparticles by anti-native β -casein antiserum was weakly inhibited by β -casein $f(1-105/7)$ and β -casein $f(106-209)$ (0.04 and 1.4%, respectively, compared with native β -casein). Immunoreactivity of these β -casein peptides in microparticle-enhanced nephelometric immunoassay was more preserved in the whole β -casein than in its isolated fragments. The protein concentration producing 50% inhibition of the β -casein-coated microparticle agglutination with anti-native β -casein antiserum increased during β -casein denaturation. A microparticle-enhanced nephelometric immunoassay, quantifying changes of this inhibiting protein concentration, permitted detection of alteration of the immunoreactivity of β -casein during its plasmin proteolysis and heat denaturation, providing an adequate test for the integrity of the whole molecule.

Keywords: β -casein, β -casein denaturation, β -casein peptides, immunonephelometry, particle immunoassay, plasmin activity

INTRODUCTION

Beta-casein (β -CN) determination has been performed recently in bovine milk by a microparticle-enhanced nephelometric immunoassay (El Bari *et al.*, 1991). Hydrophilic monodisperse microspheres (MS) coated with β -CN (MS- β -CN) were agglutinated by anti- β -CN antiserum (As). Free β -CN inhibited 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 MS- β -CN conjugate clusters. During this work, slight cross-reactivity between anti- β -CN As and fragments

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of denatured β -CN, co-existing with native β -CN in bovine milk (Eigel *et al.*, 1979), were observed. Proteolysis of β -CN in bovine milk by alkaline proteinase or plasmin (Humbert & Alais, 1979) produces three main 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).

Studies on the antigenic structure of bovine β -CN and the antigenic reactivity of various fragments of β -CN (1–139, 106–209, 1–93 and peptides derived from fragments 1–93 and 94–209) had been previously reported (Otani *et al.*, 1984a, b, c, d, 1987), using immunological methods such as immunodiffusion, immunoelectrophoresis and the precipitin test. We report here a quantitative study, performed by microparticle-enhanced nephelometric immunoassay, of the immunoreactivity of anti-native β -CN As against two fragments of β -CN naturally produced by plasmin in bovine milk, β -CN f(1–105/7) and β -CN f(106–209), compared with its immunoreactivity against native β -CN. A possible application of such an immunonephelometric assay was proposed, to detect the alteration of β -CN during its proteolysis by plasmin and its treatment by heating.

MATERIALS AND METHODS

Chemicals

Acrolein, 2-hydroxyethylmethacrylate, methacrylic acid, polyoxyethylene ether Triton® X-100 and the disodium salt of ethylenediaminetetraacetic acid (EDTA- Na_2) were obtained from Merck (Darmstadt, Germany). *N,N'*-methylene diacrylamide was a product from Eastman Kodak Co. (Rochester, NY, USA). Other chemical reagents were of analytical grade and 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 and 30 g/l of polyethylene glycol (PEG) 6000 (milk nephelometry buffer).

Preparation of β -CN, β -CN Fragments and Anti- β -CN Antiserum

Beta-CN was extracted from a pool of fresh, raw milks according to the method of Aschaffenburg (1963) modified by Garnier *et al.* (1964). Skimmed milk heated up to 95°C for 30 min was used to prepare a mixture of N-terminal fragments of β -CN (β -CN f[1–28], f[1–105, 1–107] and f[29–105, 29–107]) according to Aschaffenburg (1946). β -CN f(1–105/7) was purified by gel filtration (Andrews, 1978) on Ultrogel ACA 54 (IBF, Villeneuve la Garenne, France) and hydrophobic interaction chromatography on Phenyl-Sepharose CL4B (Pharmacia, Uppsala, Sweden) according to Pâquet *et al.* (1988).

A mixture of C-terminal peptides, β -CN f(29–209), f(106–209) and f(108–209), was obtained following the method of Reimerdes and Herlitz (1979) from milk stored for 14 days at 30°C. Beta-CN f(106–209) was purified from this mixture by ion-exchange chromatography on diethylaminoethyl-cellulose (DE 52 Whatman, Maidstone, UK) according to Mikani (1971).

Proteolysis (37°C, from 0 to 75 min) of β -CN by plasmin (EC 3.4.21.7, Sigma Chemical Co., St Louis, MO, USA) was performed by mixing in 1 ml of 0.05 M-borate buffer (pH 8.4), 10 mg of heat-treated β -CN (80°C, 10 min) with 0.28 mg of plasmin (10 units/mg according to the first British standard 78/646).

Heat denaturation of β -CN was carried out by heating β -CN in sealed tubes (7.5 mg ml^{-1} in 0.05 M-phosphate buffer, pH 6.7) to temperatures from 70 to 150°C for 10 min. Heat-processed β -CN was then rapidly cooled before nephelometric study.

Anti- β -CN antisera were obtained by immunizing rabbits with intra-dermal 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, Germany) for the first injection and

Freund's Incomplete Adjuvant for the booster injections which followed (after 13, 36, 65 and 240 days). Antisera were collected 7 days after each booster injection. Reactivity and specificity of antisera were assessed as previously reported (El Bari *et al.*, 1991).

Characterization and Immunoreactivity of β -CN, β -CN f(1-105/7) and β -CN f(106-209)
Polyacrylamide gel electrophoresis in 4.5 M-urea, 0.38 M-Tris-HCl buffer, pH 8.9 (Ng-Kwai-Hang & Kroeker, 1984) and sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (0.1% SDS, 0.38 M-Tris-HCl buffer, pH 8.9) (Laemmli & Favre, 1973; Hillier, 1976; Basch *et al.*, 1985) were performed for all protein preparations with equipment from Hoefer Scientific Instruments (San Francisco, CA, USA). A molecular weight calibration kit (Pharmacia) was used to estimate the molecular weight of protein-staining bands in SDS-polyacrylamide gel electrophoresis.

The amino acid composition of purified β -CN, β -CN f(1-105/7) and β -CN f(106-209) was determined according to Moore and Stein (1963), after hydrolysis with 6 N-HCl at 110°C for 24 h. Amino acid analysis was performed using a Beckman 7300 autoanalyser (Brea, CA, USA).

The reactivities of β -CN, β -CN f(1-105/7), β -CN f(106-209) and plasmin-treated β -CN with anti- β -CN antisera were assayed by Western blotting (Towbin *et al.*, 1979) on cellulose nitrate transfer membrane (Schleicher & Schuell, Dassel, Germany). The goat anti-rabbit immunoglobulin peroxidase conjugate used as labelled antibody was a Diagnostics Pasteur (Marnes, France) product.

TABLE 1. Amino acid composition^a of prepared proteins

Amino acid	Prepared proteins			Theoretical ^b		
	β -CN	β -CN f(1-105/7)	β -CN f(106-209)	β -CN	β -CN f(1-105)	β -CN f(106-209)
Asx	9.4	6.4	3.3	9	6	3
Thr	8.6	4.9	6.6	9	5	4
Ser	13.1	8.6	6.3	16	9	7
Glx	36.0	23.1	13.9	39	24	15
Pro	34.4	13.7	21.6	35	14	21
Gly	5.2	3.2	2.7	5	3	2
Ala	5.2	3.6	2.4	5	3	2
Val	19.2	8.5	8.7	19	9	10
Met	5.3	1.6	2.9	6	2	4
Ile	10.1	6.3	2.6	10	7	3
Leu	22.8	8.1	12.3	22	8	14
Tyr	3.6	0.9	3.1	4	1	3
Phe	8.8	3.7	4.6	9	4	5
Trp	nd ^c	nd	nd	1	0	1
Lys	11.2	7.6	3.8	11	7	4
His	5.4	1.6	3.6	5	1	4
Arg	4.4	2.3	2.2	4	2	2

^aResidue of amino acid per mole protein.

^bAccording to the amino acid sequence of β -CN A² (Ribadeau-Dumas *et al.*, 1972).

^cNot determined.

Immunonephelometric Study of β -CN, β -CN f(1-105/7), β -CN f(106-209), Plasmin-treated β -CN and Heat-processed β -CN

Immunonephelometric studies were carried out by microparticle-enhanced nephelo-

1 2 3 4 -

FIG. 1. Western blotting of prepared proteins (1) β -CN; (2) β -CN f(1-105/7); (3) β -CN f(106-209); (4) plasmin treated β -CN. Reaction of anti- β -CN As after SDS-polyacrylamide gel electrophoresis in pH 8.9 buffer.

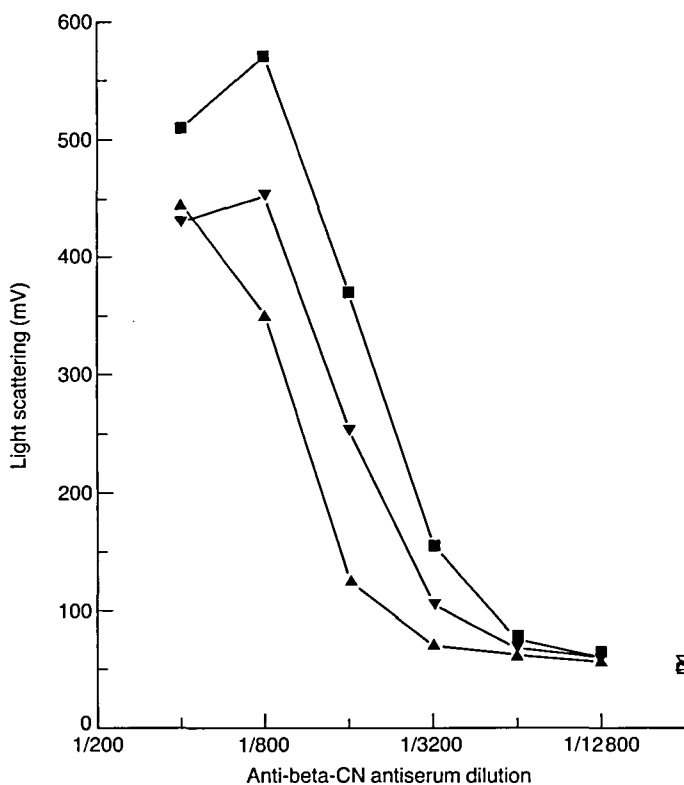


FIG. 2. Intensity of light scattered, as a function of anti- β -CN As dilution, during immunological agglutination of conjugates (30 mg l^{-1}). MS- β -CN (■); MS- β -CN f(1-105/7) (▼); MS- β -CN f(106-209) (▲); intensity of light scattered by the conjugates alone (□, ▽, △); reaction time, 2 h.

TABLE 2. Immunonephelometric study of β -CN and β -CN fragments

	Conjugate		
	MS- β -CN	MS- β -CN f(1-105/7)	MS- β -CN f(106-209)
Last agglutinating anti- β -CN As dilution*	1/3200-1/6400	1/3200	1/1600-1/3200
Maximum inhibition observed (%) with			
β -CN	98	97	98
β -CN f(1-105/7)	77	99	57
β -CN f(106-209)	81	53	98
Concentration (mole l ⁻¹) producing 50% inhibition			
β -CN	8.3 10 ⁻⁷	4.1 10 ⁻⁴	1.1 10 ⁻⁴
β -CN f(1-105/7)	2.3 10 ⁻³	7.4 10 ⁻⁴	1.2 10 ⁻¹
β -CN f(106-209)	5.9 10 ⁻⁵	4.1 10 ⁻²	2.1 10 ⁻³
Cross-reactivity (%)			
β -CN	100	180	1910
β -CN f(1-105/7)	0.04	100	1.7
β -CN f(106-209)	1.4	1.8	100

*Expressed as the last As dilution increasing twofold the light scattered by the conjugate alone.

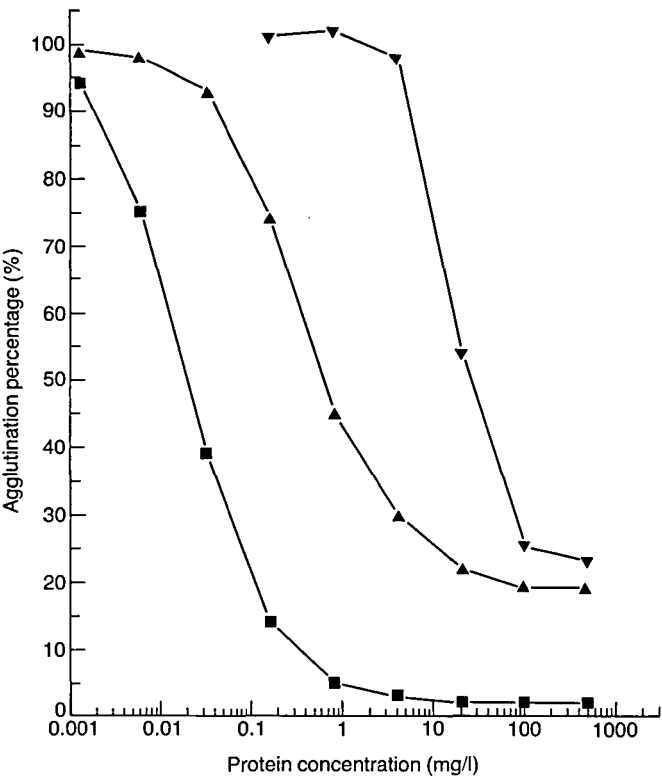


FIG. 3. Variation of agglutination percentage with free protein concentration during inhibition of MS- β -CN (30 mg l⁻¹) agglutination. β -CN (■); β -CN f(1-105/7) (▼); β -CN f(106-209) (▲); anti- β -CN As dilution, 1/1000; reaction time, 2 h.

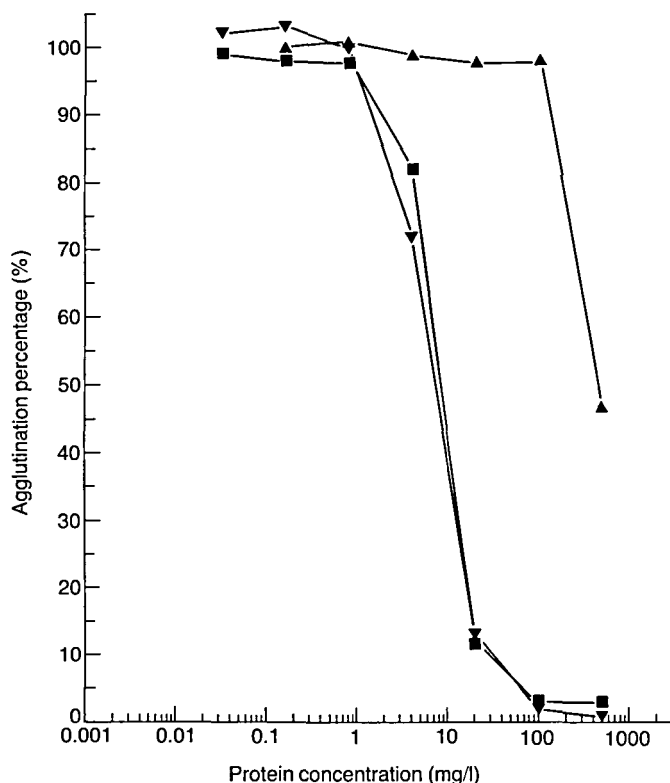


FIG. 4. Variation of agglutination percentage with free protein concentration during inhibition of MS- β -CN f(1-105/7) (30 mg l^{-1}) agglutination. β -CN (■); β -CN f(1-105/7) (▼); β -CN f(106-209) (▲); anti- β -CN As dilution, 1/1000; reaction time, 2 h.

metric immunoassay (Montagne *et al.*, 1991). Hydrophilic and polyfunctional MS (mean dry diameter = 220 nm, SD = 7 nm, $n = 49$) were produced by copolymerization (γ -irradiation, ^{60}Co , 60 krad/cm², 3 h) of acrylic monomers (2-hydroxyethyl methacrylate 59.71 g l^{-1} , acrolein 56.40 g l^{-1} , methacrylic acid 2.40 g l^{-1} , N,N' -methylene diacrylamide 1.51 g l^{-1}) in the presence of SDS (0.91 g l^{-1}). Beta-CN, β -CN f(1-105/7) and β -CN f(106-209) were separately bound on these MS as previously described by El Bari *et al.* (1991), to obtain MS- β -CN, MS- β -CN f(1-105/7) and MS- β -CN f(106-209) conjugates, respectively. These covalent conjugates were stored at 4°C in 0.05 M-borate buffer, pH 8, 0.03 M- NaN_3 .

Immunoagglutination of each conjugate (30 mg l^{-1}) with serial dilutions (1/400–1/12800) of anti- β -CN As was performed in milk nephelometry buffer for 2 h at room temperature. Inhibition of these agglutinations (a conjugate concentration of 30 mg l^{-1} and 1000-fold diluted anti- β -CN As) was assayed with serial concentrations of β -CN, β -CN f(1-105/7), β -CN f(106-209) (0.0013 – 2500 mg l^{-1}), plasmin-treated β -CN and heat-processed β -CN (0.0032 – 7 mg l^{-1}). The protein concentrations inducing 50% of inhibition were calculated from each spline smoothed inhibition curve. Quantification of all agglutinations and agglutination inhibitions was carried out by nephelometric measurements of the light scattered by conjugate clusters formed during each reaction using the Diagnostics Pasteur nephelometer (Nephelia N 600) previously described (El Bari *et al.*, 1991).

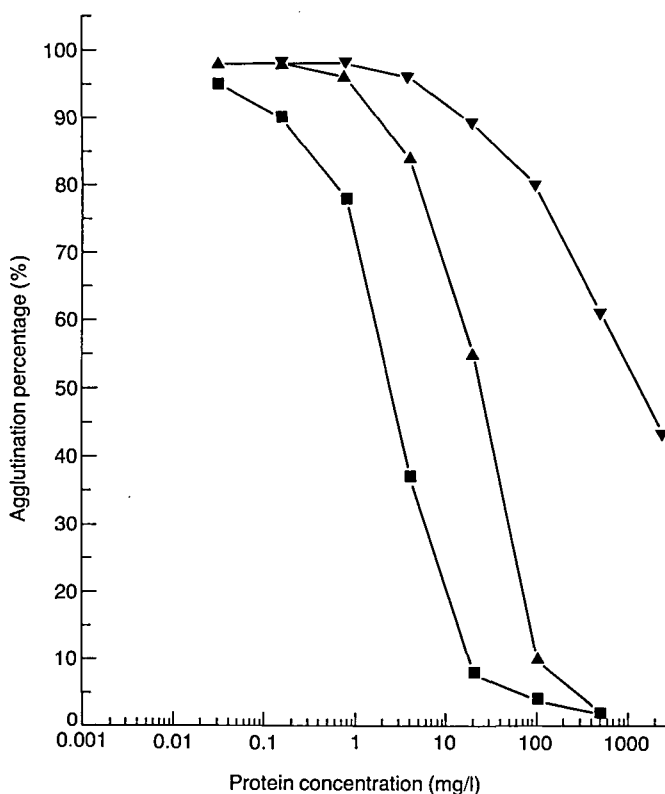


FIG. 5. Variation of agglutination percentage with free protein concentration during inhibition of MS- β -CN f(106-209) (30 mg l^{-1}) agglutination. β -CN (■); β -CN f(1-105/7) (▼); β -CN f(106-209) (▲); anti- β -CN As dilution, 1/1000; reaction time, 2 h.

RESULTS

Characterization of the Protein Preparations

Table 1 gives the amino acid composition of the protein preparations compared with the composition of β -CN, β -CN f(1-105) and β -CN f(106-209) calculated according to the amino acid sequence of β -CN (Ribadeau-Dumas *et al.*, 1972). Results confirmed that the purified proteins correspond to β -CN, β -CN f(1-105/7) and β -CN f(106-209).

Electrophoretic patterns of preparations on polyacrylamide-urea and SDS-polyacrylamide gels showed purity of β -CN, β -CN f(1-105/7) and β -CN f(106-209) reaching 90% and estimated molecular weights of 26 000, 17 000 and 13 000 Da, respectively.

Western blotting (Figure 1) indicated that anti-native β -CN antisera reacted against whole β -CN, β -CN f(1-105/7), β -CN f(106-209) and fragments produced during the proteolysis of β -CN by plasmin (75 min).

Immunonephelometric Study of β -CN, β -CN f(1-105/7) and β -CN f(106-209)

As can be seen in Figure 2, MS- β -CN, MS- β -CN f(1-105/7) and MS- β -CN f(106-209) conjugates were agglutinated by anti- β -CN antisera. However, the levels of light-scattering amplification obtained and the last agglutinating dilutions of antisera (Table

2) varied with each conjugate. They were maximum for MS- β -CN, lower for MS- β -CN f(1-105/7) and minimal for MS- β -CN f(106-209) conjugate.

Agglutination of MS- β -CN conjugate with anti- β -CN antisera was completely inhibited (Figure 3) by free purified β -CN but only inhibited in part by free β -CN f(1-105/7) and β -CN f(106-209). The results shown in Table 2 indicate that the level of cross-reactivity of these free fragments in the inhibition of MS- β -CN agglutination was low, reaching 0.04 and 1.4%, respectively.

Inhibitions of MS- β -CN f(1-105/7) agglutination are presented in Figure 4. The same w/v concentrations of purified β -CN and β -CN f(1-105/7) similarly inhibited the system but a molecular concentration of β -CN slightly lower than that of β -CN f(1-105/7) was necessary to produce 50% inhibition, while the fragment 106-209 had a small effect, with a cross-reactivity of 1.8% (Table 2).

Figure 5 shows the inhibitions obtained for MS- β -CN f(106-209) conjugate agglutination. Although free β -CN f(106-209) and β -CN completely inhibited the agglutination, a molecular concentration of β -CN f(106-209) 19 times higher than that of whole

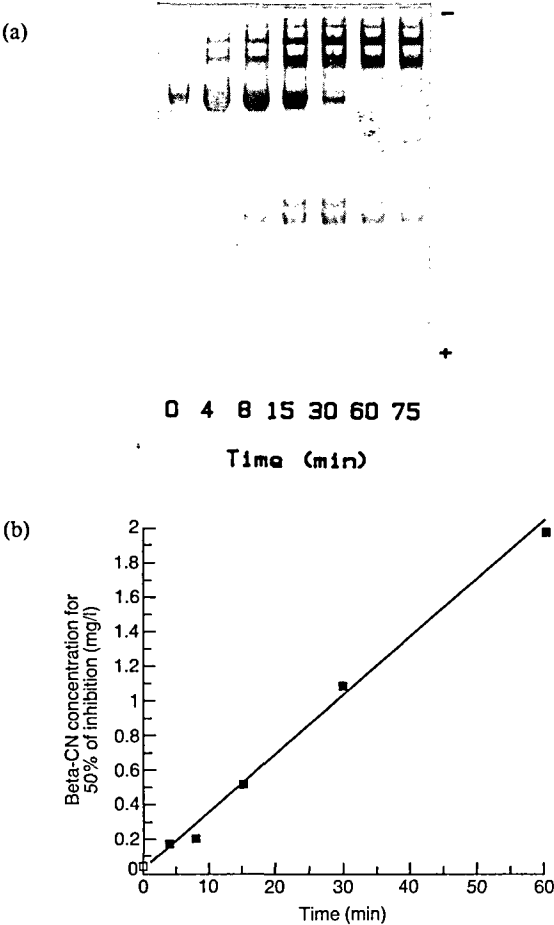


FIG. 6. Influence of proteolysis by plasmin on β -CN. (a) Electrophoretic patterns of plasmin-treated β -CN on polyacrylamide gel in pH 8.9 urea buffer. (b) Concentration of plasmin-treated β -CN inducing 50% inhibition of the agglutination of MS- β -CN (30 mg l⁻¹) with anti- β -CN As (1000-fold diluted) as a function of the proteolysis time (■). Native β -CN (□).

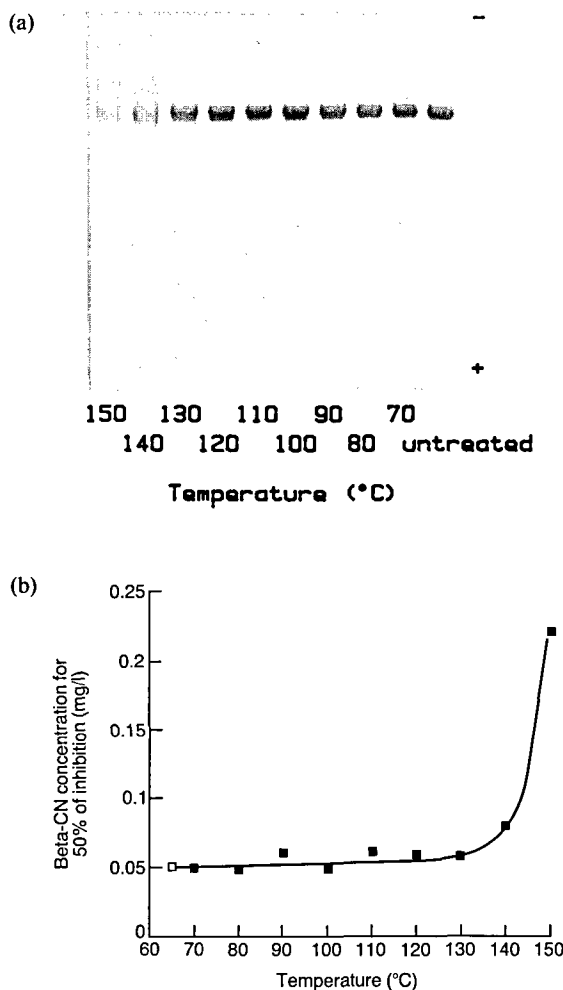


FIG. 7. Influence of heating on β -CN. (a) Electrophoretic patterns of heat-processed β -CN on polyacrylamide gel in pH 8.9 urea buffer. (b) Concentration of heat-processed β -CN inducing 50% inhibition of the agglutination of MS- β -CN (30 mg l^{-1}) with anti- β -CN As (1000-fold diluted) as a function of the temperature (■). Native β -CN (□).

β -CN was necessary to produce 50% inhibition (Table 2). The cross-reactivity of β -CN f(1-105/7) observed in this agglutination reached 1.7%.

Immunonephelometric Study of Plasmin Treatment and Heat Denaturation of β -CN

The early appearance of fragments of β -CN in polyacrylamide-urea gel electrophoresis (Eigel, 1977) showed rapid proteolysis of β -CN by plasmin (Figure 6A). The influence of β -CN proteolysis on the immunoreactivity of the protein was detected by microparticle-enhanced nephelometric immunoassay as early as the fourth minute of the reaction (Figure 6B) by the increase of the protein concentration inducing 50% inhibition of the MS- β -CN conjugate agglutination with anti-native β -CN antisera. This protein concentration gradually increased from 0 to 60 min of proteolysis to reach 40 times the initial concentration.

The effect of heating was found in the same way by the abrupt increase of the

protein concentration inducing 50% inhibition of MS- β -CN conjugate agglutination, observed for temperatures of treatment higher than 140°C (Figure 7B). The appearance of several bands in polyacrylamide-urea gel electrophoresis of heat-treated β -CN (Figure 7A) confirmed the alteration of the molecule at these temperatures.

DISCUSSION

Polyclonal antibodies of rabbit anti-native bovine β -CN antisera recognized whole native β -CN and fragments of the β -CN molecule such as 1-105/7 and 106-209. This result is in agreement with the conclusions of previous studies of the antigenic reactivity of β -CN fragments (Otani *et al.* 1984a, b,c,d, 1987) showing that antigenic sites exist in the N-terminal and the C-terminal regions of β -CN. However, the predominance of the N-terminal region in the repartition of these antigenic sites, observed by these authors using immunodiffusion, immunoelectrophoresis and the precipitin test, was not established by our work carried out by microparticle immunonephelometry. Beta-CN f(1-105/7) and β -CN f(106-209) produced only slight cross-reactivity (0.04 and 1.4% compared with native β -CN) during the inhibition of MS- β -CN conjugate agglutination obtained with anti-native β -CN antisera. This confirmed the applicability of the microparticle-enhanced nephelometric immunoassay of β -CN in bovine milk previously described (El Bari *et al.*, 1991), using MS- β -CN conjugate and anti-native β -CN antisera.

Immunonephelometric studies of MS- β -CN f(1-105/7) and MS- β -CN f(106-209) conjugates show that their agglutinations with anti- β -CN antisera were similarly inhibited at lower molecular concentrations of whole molecule than that observed for its fragments. The immunoreactivity of the whole β -CN molecule, as tested by inhibition of microparticle agglutination, was thus greater than that observed for β -CN peptides, especially for peptide 106-109. The decrease of the antibodies reactivity for isolated β -CN peptides might be due to a loss of epitopes in peptides occurring during the process of fragmentation of β -CN, at the region of fragmentation. This might also be due to possible conformational changes in isolated fragments, involving alteration of the steric fit between antigenic determinants of isolated peptides and the complementary antibody sites. In this last case, thermodynamic changes might be induced in the chemical bonds involved in antigen-antibody interactions, which caused the decrease of antibody affinity (Montagne, 1990), while the unchanged amino acid sequence still preserved the recognition of antigenic determinants by antibodies. However, contrary to the results obtained for β -CN f(106-209), the better preserved ability of β -CN f(1-105/7) to inhibit efficiently agglutination of MS- β -CN f(1-105/7) conjugate showed that epitopes were little modified (if at all) in this fragment compared with the native molecule.

The recognition of fragments of plasmin-treated β -CN by anti-native β -CN antibodies, the possible loss of epitopes in these fragments and the slight affinity of antibodies for those, compared with their affinity for the whole molecule, induced the increase of the concentration of proteolyzed protein inhibiting the MS- β -CN conjugate immunoagglutination. Nephelometric measurement of the protein concentration producing 50% inhibition of MS- β -CN agglutination thus permitted detection of β -CN proteolysis. In the same way, the alteration of β -CN during heating could be detected and the effective temperature could be determined by immunonephelometry. However, the changes of β -CN reactivity observed in this case could be induced by a fragmentation of the molecule or the consequence of an aggregation of β -CN molecules during the heating process, as already reported for other antigens (Humbert *et al.*, 1991; Mahana *et al.*, 1991). Previously described as an immunological method of quantification of β -CN in bovine milk, microparticle-enhanced nephelometric immunoassay thus also appears usable to detect alteration of the immunoreactivity of this

protein during its plasmin proteolysis and heat denaturation and provides an adequate test for integrity of the whole molecule.

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