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Research Paper

Differential immunochemical characterization of monoclonal antibody directed against the beta-casein and its proteolysis products by the plasmin

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Abstract

Milk is extremely complex both by its multiphasic physical nature and by the diversity of its molecular constituents whether they are its protein particularly. Caseins (CN), represents of the major protein fraction of this dairy undergoes preferential degradation from proteolysis by plasmin activities in Beta-casein (β-CN) with entrainment of gamma casein (γ-CN) and proteose-peptones. In this work aims to study the β-CN antigenicity and its degradation products in order to develop a milk quality indicator based on the determination of the degree of casein proteolysis. A approach consists in producing and characterizing monoclonal antibodies making it possible to differentiate β-CN from its fragments degraded by plasmin. Immunization and fusion in mice with whole caseins followed by pre-selection of hybrids or clones by use of the indirect ELISA technique and finally determination of affinity constants (Ka) of monoclonal antibody. Interaction between the antibody and the target protein that can be easily monitored by immunoassays such as ELISA. Most of the supernatants studied react better with the degradation products than with the native protein, with each antigen and selected these that recognize strongly the three antigens and those recognizing preferentially either β-CN, either β-5P(f1-105/7), either β-CN(f106-209), (f108-209). Finally, it emerges from these results that the SNC46 and 80 have the best discriminating character. They react exclusively with the C-terminal products of β-CN in immunoblotting as well as by calculation of affinity constants. For the C-terminal fragments against β-CN 1, the SNC25 and 35 also exhibit pronounced reactivity, a weak reactivity observed against the N-terminal fragments of β-CN, in ELISA and in Immunoblot. Monoclonal antibodies will make an important contribution to the study of functional proteins.

Keywords: Milk, Beta-Caseins, Anticorp, monoclonal, ELISA.

Introduction

Milk is a complex food by its physical nature and its structural diversity by its nutritive chemical composition. Proteins, fats, carbohydrates and minerals[1]. Caseins (CN), represents 80% of the proteins in bovine milk, are the major protein fraction of this dairy fluid[2,3]. Caseins (CN) is the main protein component of cow's milk (approximately 23 mg mL^{-1})[4,5]. Four types of caseins are mainly present in bovine milk: α S1, α S2, β and κ. These rheomorphic proteins adapt their structure to changes in environmental conditions[6] and assemble to form colloidal aggregates, in the form of casein micelles. Due to their great technological importance, the colloidal properties of milk by casein micelles play a major role in the structural and functional properties of milk protein concentrates. Industry pasteurization and sterilization are the various heat treatments applied to milk in can also have denaturing effects on milk proteins but not directly on CNs. The proteolysis is a major contributor to changes in the texture and flavor of milk[7] and the quality of the products derived from it[8]. One of the

major proteins in milk is the Beta-casein (β-CN), undergoes preferential degradation with entrainment of gamma casein (γ-CN) and proteose-peptones, the proportions of which are linked to proteolytic activities. B-casein contains about 9% α helical structure and about 25% β structure[9]. The micelle also consists of a few peptide fragments (γ caseins) resulting from the proteolysis of β-CN by plasmin and minerals (7% of the dry weight) including 90% of calcium phosphate[10,11]. The protein-peptone fraction in milk has been defined as the proteins that remain in solution after heat treatment at 95°C and acidification to pH 4.7 [12].

Two fractions PP3 appertaining to proteoses peptones (also known as lactophorin) and PP5 contain phosphorus, it represents β-CN (f1–105) resulting from the proteolysis of plasmin[13]. Hydrolysis causes a loss of interaction between the antibody and the target protein that can be easily monitored by immunoassays such as ELISA. In this approach were consisted to follow proteolysis events occurring As a result of their loose function and structural of peptides caseins (CNs) production of monoclona antibodies targeting several epitopes in B-casein particularly. In this work aims to study the β-CN antigenicity and its degradation products in order to develop a milk quality indicator based on the determination of the degree of casein proteolysis. A first approach consists in producing and characterizing monoclonal antibodies making it possible to differentiate β-CN from its fragments degraded by plasmin.

Materials and Methods

Biochemical reactive preparation

Whole casein was extracted from a pool of fresh raw milk (Holstein breed) by three cycles of precipitation at pH 4.6, washing with distilled water and solubilization at pH 7 according to[14] Nitschmann and Lehmann (1947). Mixture of C-terminal peptides of β-CN (β-CN-1P(f29-209), (f106-209) and (f108-209)) was prepared from plasmin treated β-CN, by precipitation at pH 4.6 according to the method of [15] Wilson and al., (1989). The Skimmed milk heated up to 95°C for 30 min was used to prepare a mixture of N-terminal peptides of β-CN (β-CN-4P(f1-28), -5P(f1-105/7) and –1P(f29-105/7) according to Aschaffenburg R, (1946) [16]. Purification of β-CN (f1-105/7)) was realized by hydrophobic interaction FPLC on TSK-Phenyl-5PW (Pharmacia) following the method of Paquet et al. (1988)[17]. Purified antigens (Ag) was dialyzed against distilled water then lyophilized. The polyarylamide gel electrophoresis in 4,5 mol/l urea 0,38 mol/l Tris HCl buffer (pH 8,9), and sodium dodecyl sulphate-polyacrylamide gel (0,1% SDS, 0,38 mol/l tris HCl buffer (pH 8,9)[19] were performed for all antigens preparations. The molecular weight calibration kit (Pharmacia) was used to estimate the molecular weight of colored antigen bands in PAGE-SDS, Amino-acids composition was determined[20].

Immunization and fusion

Eight-week-old BALB/c mice by Jico (IFA CREDO) was used. Two shares A and B nine mouse each were immunized intraperitoneally and respectively with whole and hydrolyzed casein. For the two forms of used casein (240 µg), the solution was emulsified with an equal volume of complete Freund adjuvant (ACF) to the first injection (day J1) and incomplete (AIF) during reminders (day J19 and J33). To the last day (J47) antigens were administered without adjuvant. Nowinsky and al.,[21] Three days after, they having been sacrificed by cervical dislocation. The selection of hybrids has undertaken in minimum middle of EAGLE containing sodium pyruvate (1mM), glutamine (2mM), Penicillin antibiotics (100 U.I/ml), Streptomycin (100 µg/ml), azaserine (10-5 M) as inhibitor, hypoxanthine (5.10-5 M) as source of bases precursor puriques, and 10% of calf fœtus serum (SVF), inactive by the heat. The threes week-old-BALB/c mice thymocytes were used as nutrient cells (106 cells/ml of middle). When hybrids are well developed, the supernatant was replaced by a middle without azaserine, then, by a middle without hypoxanthine (complete middle).

Pre-selection of hybrids

The supernatants of most precocious clones (10 to 12 days after the fusion) were preselected by the use of indirect ELISA technique with two simple sites. To preserve its conformation the antigen was taken in "sandwich" between two antibody coming from different species. The first is a rabbit antibody anti bovine β-CN adsorbed on the solid phase[23]. It takes the β-CN, where will come to fix the anti β-CN monoclonal immunoglobulin (Ig) of mouse, possibly present in preparations to test on microtiter plates (96-well, Nunc) (adaptation of the method described by Mahana et al. (1998)[24].

Selection of hybrids

All supernatants whose optic density (DO) was the double of that negative witnesses (supernatants of wells where no hybrid is appeared) are then amplified in culture during 48 hours and submitted to the

semiautomatic analysis on the β-CN and its proteolysis fragments. Selected hybrid cells are then preserved in nitrogen liquidates (2.10^6 cells 0,7 ml^{-1} of SVF containing 5% of dimethylsulfoxyde) and cloned by limit dilution. Under clones are controlled for their activity and their specificity.

Immunoreactivity of β-CN, β-CN (f1-105/7) and β-CN (f106/8-209)

Obtained clones are tested by ELISA against the β-CN, mother protein and against its fragments of degradation, in occurrence fraction containing β-CN-5P(f1-105/7) and that containing fragments β-CN (f106-209), (f108-209). Reactivity of different antigens with supernatants of hybridomas culture (monoclonal antibody) was also undertaken by Western blotting after transfer on nitrocellulose filter (Schleicher and Schuell, Dassel, Germany)[25]. The insoluble substrate used for revelation is bromochloro indolyl /nitro-blue of tetrazolium (BCIP/NBT) (Sigma). Three types of narrow bands was prepared. On those of type A were transferred the protein mother and its fragments N- and C-terminals. On those of type B has been transferred only the fraction containing γ_2 -, γ_3-CN while those of the type C they contain only γ_2-, γ_3-CN and γ-CN-5P(f1-105/7).

Determination of affinity constants (Ka) of monoclonal antibody (AcM) of culture supernatants

The quantification of free antigen was made by ELISA whose principle is described above. The free antigen is isolated from that entering in the formation of antigen-antibody complex by precipitation of these last by polyethylene-glycol 6000 (PEG 6000). Total concentrations in antibody contained in each supernatant culture (SNC) have been determined spectrophotometrically at 280 nm against witness supernatant without antibodies by using as extinction coefficient Ɛ=1,43 (A280 1mg ml^{-1} of immunoglobulin solution).

Results and Discussions

Fusion for the first cellular hybrids

Results of the two fusion concerning the splenocytes coming, for one, from the immune mouse by the whole β-CN and, for the other, from the one immunized by hydrolyzed β-CN, are presented in the table 1. The first cellular hybrids appear at the end of ten days after the fusion and the first tests have been able to be undertaken in the course of the fifteen next days. Thus on 126 viable clones coming from the mouse SH, 18 were positive and only 6 specific clones being able to reply to needs of a differential dosage were retained. Similarly, on 67 clones coming from the mouse SE, 29 was positive and only 7 have shown an interesting activity.

Table 1: Cloning efficiency of clones

Fusion	Total number of wells	Clones having pushed	Frequency	***Cloning efficiency
*Fusion H-β-CN	480	126	26,25%	>85%
**Fusionβ-CN	408	67	16,40%	>90%

*Fusion H- β-CN: Clones coming from splenocytes of immune animals by the hydrolyzed β-CN
**Fusion β-CN: Clones coming from splenocytes of immune animals by the whole β-CN
***Loi de poisson[27]

Evaluation by ELISA of discriminatory power of monoclonal antibodies selected clones

The clones showing interesting activity against the three tested antigens are retained. We have counted clones reacting the most strongly, in absolute values, with each antigen and selected these that recognize strongly the three antigens and those recognizing preferentially either β-CN, either β-5P(f1-105/7), either β-CN(f106-209), (f108-209). Figure 1 shows the main clones retained.

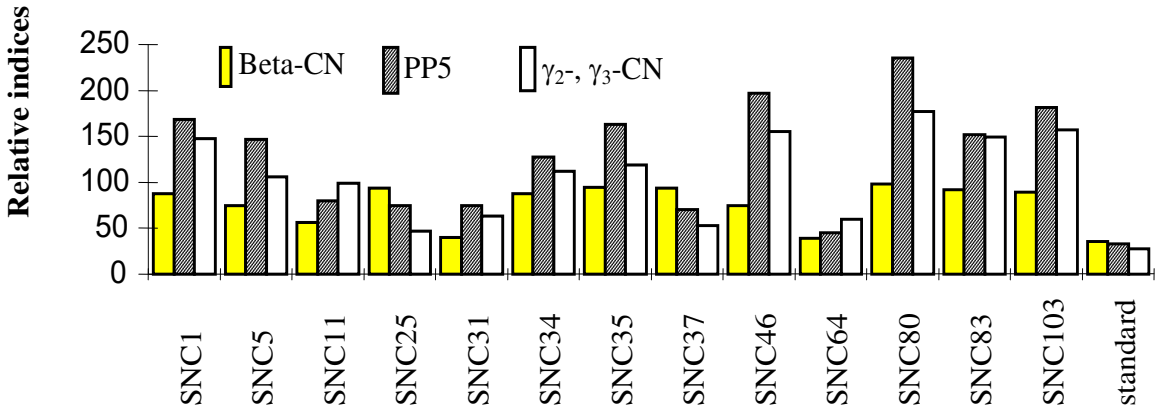

Figure 1: culture supernatants reactivity retained in ELISA, against three antigens types. (Results are converted in relative indices by taking as reference 100, for each antigen, the mouse serum of the fusion. The 100% corresponds thus to a value in equal optical density to 2,5 noticed for this witness. The value of the negative witness equals to a DO of 0,9).

Hybridomas culture supernatants (SNC) reactivity in immunoblot

The study in immunoblotting of culture supernatants reactivity will allow to determine the possible contamination of degradation products them some by others. Such contamination could have explained reactions crossed between fragments C- and N-terminals met in ELISA. 11 of 13 supernatants of tested culture against antigens of narrow bands of type A don't show reactivity. Alone SNC25 and SNC35 give interpretable results. The SNC35 reacts both with the β-CN and the γ_2-, γ_3-CN, while the SNC25 reacts only with the β-CN (figure 2 (A)). For the narrow bands of type B all the supernatants react with fragments of β-CN proteolysis (figure 2 (B)).

However, SNC31, 94, 80 and 103 have to be concentrated by centrifugation on centrisart tube for 15 min at 3000 rotates/min to reach colorings that remain weak intensity (figure 2 (C)). On narrow bands of type C Alone SNC25 and 35give a positive response. The SNC35 reacts with the three antigens, giving net bands, while the SNC25 it recognizes only protein fractions containing γ_2-, γ_3-CN and β-5P(f1-105/7) (figure 2 (D)). At this effect, narrow bands of type A, B and C have been prepared. On those of type A have been transferred the β-CN, β-5P(f1-105) and β-CN(f106/8-209). The narrow bands of type B and C contain respectively the β-CN, PP3, β-5P(f1-105) and β-CN(f106/8-209).

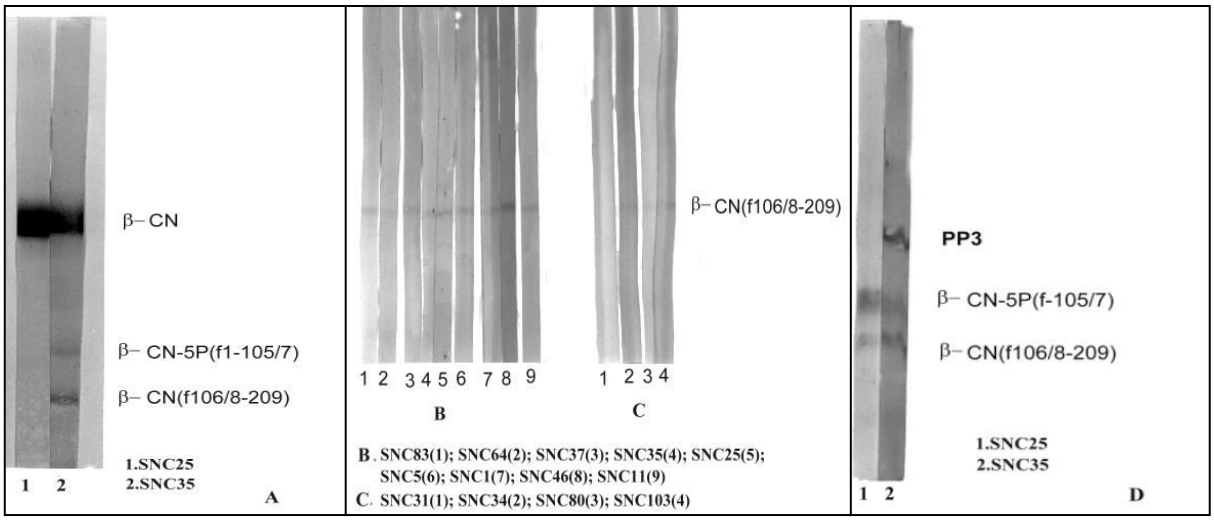

Figure 2: different culture supernatants activity against β-CN and its fragments of degradation. Revelation of activity by apparition of degradation products of BCIP/NBT

Determination of monoclonal antibodies affinity constant (Ka) of culture supernatants
Concentrations of SNC 25, 35, 46 and 80 are respectively 21,1, 19,7, 10,6 and 33,8 µg/mL. These concentrations as well as those in linked and total antigen allow establishing rights whose slopes translate the affinity of each culture supernatant for the three studied antigens (Table 2).

Table 2: Affinity constants of the different studied antibodies ($M^{-1}.L$)

Culture supernatants	Tested antigens	Affinity Constants
25	β-CN	$0,09.\ 10^7$
	β-CN(f106/8-209)	$1,2.\ 10^7$
	β-CN-5P(f1-105/7)	/
35	β-CN	$0,27.\ 10^7$
	β-CN(f106/8-209)	$0,58.\ 10^8$
	β-CN-5P(f1-105/7)	$0,93.\ 10^7$
46	β-CN	/
	β-CN(f106/8-209)	$0,17.\ 10^8$
	β-CN-5P(f1-105/7)	/
80	β-CN	/
	β-CN(f106/8-209)	$0,27.\ 10^7$
	β-CN-5P(f1-105/7)	/

All monoclonal antibodies obtained against these peptides react however with the mother protein, but with a variable and lesser degree from one clone to the other. The monoclonal antibodies directed against the fraction containing the part N-terminal of the β-CN recognize the (or the) even(s) epitope (s) easily accessible(s) on the whole protein. Monoclonal antibodies recognize less easily the part C-terminal containing γ_2- and γ_3-CN, withdrawn inside the protein because of its raised hydrophobic character. In our case, the crossed recognition of this region and the whole protein can explain only by strong segmental movements that would animate this protein in conditions of temperature and used solvent (28, 29, 30). At the light of the cloning efficiency of obtained antibody (>85%), crossed reactions noticed by ELISA between fragments of degradation of the β-CN cannot be attributable to the antibody heterogeneity (31), but to the contamination between antigens if so is that these last have no sequential epitope in common.

At this effect, the majority of SNC tested in immunoblotting whether 11 on 13, recognize exclusively γ_2- and γ_3-CN. However, these fragments C-terminals react only when they are isolated and deposited in concentration raised on the nitrocellulose narrow bands (narrow bands of type A). The same supernatant don't react with this antigen, if this one is transferred in the presence of β-CN or PP5. This can due to differences of supernatants affinity against these compounds. To this effect, the supernatant 25 react positively with γ_2- and γ_3-CN isolated, but, in the presence both of β-CN and γ_2-, γ_3-CN, it recognizes only the whole casein.

Drawn results of the determination of affinities of the different monoclonal antibodies for the three studied antigens do not corroborate totally these obtained in immunoblotting. This shows that antigens can behave differently when they was fixed on a solid support and when they was in phase liquidates. Determination of Ka shows eventually that all supernatants of retained culture, and therefore monoclonal antibodies that they contain, possess good affinities for fragments C-terminals of the β-CN. The SNC25 presents a good affinity against γ_2-, γ_3-CN superior that against the mother protein. The SNC35 has also a good affinity for fragments C-terminals, but not for the β-CN and the PP5 for which the results expression through Scatchard method is somehow significant.

Conclusion

Most of studied supernatants react better with products of degradation that with the native protein. It emerges finally from these results that SNC46 and 80 have the best character discriminating. They react exclusively with products C-terminals of the β-CN in immunoblotting as well as by calculation of affinity constants. SNC25 and 35 present equally a pronounced reactivity against fragments C-terminals of the β-CN, but have in more a weak crossed reactivity against fragments N-terminals of the β-CN, in ELISA and Immunoblotting. However, the determination of affinity constants does not

confirm this crossed reactivity with the composed N-terminals. Indeed, no sensitive variation of these free antigens at the equilibrium is disclosed in the middle of reaction by ELISA. Finally the discriminatory characters of selected monoclonal antibodies make them the candidates reactive for a differential dosage of degradation products in presence of their mother protein. The utilization of such monoclonal antibodies for the recognition of the epitopes localized on fragments γ_2-, γ_3-CN will allow to construct efficient and specific dosage systems even if they use imperfectly purified antigens. The quantification of these degradation products in presence of their mother protein by ELISA becomes thus achievable thanks to monoclonal antibodies that we have characterized, offered a precise control tool of the quality of milk. In addition these monoclonal antibody will bring an important contribution to the functional protein study.

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