

Topography of the Casein Micelle Surface by Surface Plasmon Resonance (SPR) Using a Selection of Specific Monoclonal Antibodies

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S Supporting Information

ABSTRACT: Several theoretical models of the casein micelle structure have been proposed in the past, but the exact organization of the four individual caseins (α_{s1} , α_{s2} , β , and κ) within this supramolecular structure remains unknown. The present study aims at determining the topography of the casein micelle surface by following the interaction between 44 monoclonal antibodies specific for different epitopes of α_{s1} , α_{s2} , β , and κ -casein and the casein micelle in real time and no labeling using a surface plasmon resonance (SPR)-based biosensor. Although the four individual caseins were found to be accessible for antibody binding, data confirmed that the C-terminal extremity of κ -casein was highly accessible and located at the periphery of the structure. When casein micelles were submitted to proteolysis, the C-terminal extremity of κ -casein was rapidly hydrolyzed. Disintegration of the micellar structure resulted in an increased access for antibodies to hydrophobic areas of α_{s1} - and α_{s2} -casein.

KEYWORDS: casein micelle, antibody, interaction, SPR, biosensor

INTRODUCTION

Identification of protein–protein interactions is currently driven by studies on the building mechanisms of supramolecular assemblies in different fields such as biology or nanotechnology. Current techniques for studying protein–protein interactions such as radioisotope or fluorescence labeling are often laborious and time-consuming. In addition, fluorescence methods can be associated with experimental artifacts such as quenching and high background. Immunosensors have gained growing attention because of their low detection limit and their ability to analyze heterogeneous and complex samples. Surface plasmon resonance (SPR) enables the study of interactions between molecules with no labeling.¹ SPR is a surface-based biosensing technique that has been extensively used to study macromolecular interactions such as antigen–antibody binding extended to cellular organisms such as viruses.² SPR has been applied to milk proteins for quantifying caseins in milk,^{3,4} following casein–casein^{5,6} or casein–polysaccharides interactions.⁶ Our aim is to examine the potential of SPR-based immunosensors used as probes for exploring the surface of a supramolecular assembly, the casein micelle.

The self-assembly of casein into a higher ordered structure called casein micelle in milk is well documented. Casein micelles are particles of colloidal size that can be described as a supramolecular assembly, a system consisting of multiple molecular entities, proteins and minerals, held together and organized by means of noncovalent intermolecular binding interactions.⁷ In bovine milk, casein micelles are constituted of four different

phosphoproteins, α_{s1} , α_{s2} , β , and κ -caseins, in the approximate molar ratio 4:1:3.5:1.5 as well as minerals, essentially calcium and phosphate, called colloidal calcium phosphate (CCP). Casein micelles are highly porous structures hydrated with about 4 g of water/g of casein. The casein micelle is known to be a more or less spherical particle with a size distribution varying from 20 to 600 nm in diameter, with a median size between 100 and 200 nm.⁸ Currently, the structure of the casein micelles has not been unequivocally elucidated. Many models have been proposed and can be regrouped into two kinds of models: the submicelle model⁹ and the nanocluster model.^{10–13} In the first model, the casein micelles are composed of smaller proteinaceous subunits, the submicelles, linked together via the CCP. In the second model, the subunits are nanoclusters of CCP randomly distributed in a chain web of caseins, for which the homogeneity is controversial.¹³ For all of these models, the structures of the surface and of the interior of the casein micelle are still unknown and remain matters of debate due to their importance in the functional properties of casein micelles.¹⁴ Most models agree that molecular chains of the C-terminal end of κ -casein protrude from the micelle surface, forming the highly hydrophilic hairy layer responsible for the stability of the micelles. The nanocluster model¹⁰ emphasizes the role of

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calcium phosphate, but information on the surface is scarce. The dual-binding model¹¹ takes hydrophobic interactions and calcium phosphate bridging into account, and as a consequence κ -casein is relegated on the surface. However, the casein micelle surface seems to be more complex than a simple sphere surrounded by a fairly smooth hairy layer.¹⁵ Recently, new evidence about the micellar surface has been presented by using high-resolution microscopy techniques.¹² The surface is seen by high resolution in scanning microscopy (FE-SEM) as constituted of tubules, postulated to be mainly constituted of κ -casein, protruding to the exterior of the micelle separated by large gaps.¹⁵ This model was reinforced recently by the description of the casein micelle as an interlocked lattice structure formed from linear and branched caseins held through calcium phosphate nanocluster sites.¹³

The nature of caseins on the external structure has been investigated by electron microscopy coupled with lectin-labeled gold markers,¹⁶ gold labeled caseins,¹⁷ and labeled specific antibodies.¹⁸ However, the results were dependent on the method of labeling and/or the preparation techniques so that no unequivocal conclusions on the nature and location of the different casein components in the micelle were drawn from these studies. From a study on the casein micelle composition as a function of its size,¹⁹ the outer layer of the micelles would be composed of nearly equivalent amounts of α_s ($\alpha_{s1} + \alpha_{s2}$) and κ -caseins and a small amount of β -casein. In the same way using artificial casein micelles, it was suggested that α_{s1} - and α_{s2} -caseins are present with κ -casein at the surface of the casein micelles.²⁰ However, there are no experimental data to confirm these results on native casein micelles. Taken together, these results raise again the question about the nature of the caseins present at the surface. Is there a preferential location of one of the three other caseins (α_{s1} -, α_{s2} -, and β -casein), or are they all present at the surface? Do they expose a preferential area of their sequence in interaction with the serum phase?

To address these issues, a panel of 44 different monoclonal antibodies directed toward specific epitopes of α_{s1} -, α_{s2} -, β -, and κ -caseins²¹ was used in the present study to evaluate the accessibility to casein-specific antibodies at the surface of the casein micelle. Interactions between antibodies and casein micelle were studied by SPR to establish the nature of the casein sequence accessible on the surface and consequently to gain more insight on its surface.

MATERIALS AND METHODS

Chemicals. Unless otherwise stated, chemicals were from commercial sources (Sigma-Aldrich, Saint-Quentin Fallavier, France). α_{s1} -, α_{s2} -, β -, and κ -caseins were purified as previously described.⁴

Casein Micelle Separation. Whole raw milk was obtained from the experimental farm of INRA (Méjusseau, Le Rheu, France). The fat was removed by centrifugation at 3000g for 20 min at 25 °C. Sodium azide (0.02%, w/v) was added to prevent microbial growth. Casein micelles were separated by ultracentrifugation at 70000g for 1 h at 25 °C in a Sorvall ultracentrifuge (Thermo Fisher Scientific, Courtaboeuf, France) using a T865 8 × 28 mL fixed-angle rotor (Thermo Fisher Scientific). The casein micelles were then dispersed in imidazole buffer (20 mM imidazole, 50 mM NaCl, 5 mM CaCl₂, pH 6.7) to reach with a good accuracy the original concentration of caseins (about 26 mg/mL of caseins). Imidazole buffer was used because its pH and ionic strength are close to those of whey, therefore keeping the micelle structure in its

Table 1. Mean Diameter of Casein Micelle Fractions Obtained with Different Centrifugation Conditions

fraction	centrifugation conditions	micelle mean diameter ^a (nm)
F1	10000g, 15 min	183.0 ± 6.9
F2	15000g, 15 min	166.6 ± 4.6
F3	30000g, 15 min	137.9 ± 3.3
F4	45000g, 15 min	124.1 ± 5.6
F4 + 20 mM EDTA	45000g, 15 min	ND ^b
F5	60000g, 15 min	95.4 ± 2.3
F6	70000g, 60 min	112.4 ± 2.7

^a Means of five measurements. ^b ND = non detectable.

native form. Dissolution was achieved by stirring the mixture for 2 h at 37 °C.

Six casein micelle fractions (F1–F6) were obtained as previously described²² using differential centrifugation according to the scheme in Table 1. Fraction 4, considered to be most representative of the medium casein micelles with a narrow size distribution,²² was used for the topography of the casein micelle surface on Biacore. Fractions 1, 4, and 6 were used for determining the impact of the particle size on the micelle topography.

To demonstrate that the signal obtained with casein micelles with SPR was related to the micelle structure, the same experiment was repeated, but the micellar structure was disrupted by the addition of a calcium-chelating agent that solubilized the individual caseins. To reach this goal, the same micelle fractions were resuspended after centrifugation in 20 mM imidazole, 50 mM NaCl, 5 mM CaCl₂, and 20 mM EDTA, pH 6.7. Therefore, the micelle sample and the micelle + EDTA one had exactly the same protein composition. The only difference was that caseins were associated in the micelle in the first one, whereas they were soluble in the second.

Micelle Size. The average diameter of casein micelles was measured at 20 °C by photon correlation spectroscopy on a Malvern Zetasizer 3000 (Malvern Instr., Orsay, France), using a He–Ne laser light ($\lambda = 633$ nm) and a scattering angle of 90° as described previously.²²

Monoclonal Antibodies. Forty-four mouse monoclonal antibodies were chosen for their specificity among the INRA collection, to cover as much as possible the whole sequences of α_{s1} -, α_{s2} -, β -, or κ -casein.

Topography of the Casein Micelle Surface on Biacore.

Surface Preparation. All of the binding experiments were made on a Biacore 3000 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Rabbit anti-mouse antibodies (RAM, GE healthcare) were immobilized covalently on a CM5 sensor chip (GE Healthcare), by amine coupling, as described previously.²³ The immobilization level was 11000 resonance units (RU), corresponding to 11 ng/mm² RAM. RAM were immobilized on flow channel (Fc) 2. Flow channel 1 (Fc1) was used as reference cell. Fc1 was activated by the mixture of EDC + NHS and was saturated with 1 M ethanolamine in the same conditions as described for Fc2. Although casein micelle and EDTA-dissolved caseins unspecific binding on the reference flow cell was low (<3.2% of the specific binding signal), reference subtraction was systematically used to process raw data.

Optimization of Monoclonal Antibody Dilution. Twenty microliters of serial dilutions (1:2, 1:5, 1:10, 1:50, and 1:100 in HBS-EP) of monoclonal antibody culture supernatants was injected on the RAM surface to capture comparable amounts of Ig for each monoclonal antibody.

Determination of the Monoclonal Antibodies Binding Capacity toward Their Respective Antigens. Binding capacity is traditionally used

Table 2. Characteristics of the Monoclonal Antibodies Used in the SPR Measurements

sequence ^a	casein	immunogen	epitope location	binding capacity (%)
α_{s1} (f1–18)A	α_{s1}	peptide	N-ter	46.9
α_{s1} (f1–18)B	α_{s1}	protein	N-ter	32.4
α_{s1} (f1–18)C	α_{s1}	protein	N-ter	37.8
α_{s1} (f19–37)	α_{s1}	protein	close to N-ter	85.4
α_{s1} (f75–92)	α_{s1}	protein	interior	33.5
α_{s1} (f93–111)	α_{s1}	milk	interior	40.0
α_{s1} (f104–119)	α_{s1}	milk	interior	44.7
α_{s1} (f125–135)	α_{s1}	milk	interior	14.2
α_{s1} (f129–148)	α_{s1}	protein	interior	22.8
α_1 (f133–151)	α_{s1}	milk	interior	46.9
α_{s1} (f185–199)	α_{s1}	peptide	C-ter	71.4
α_{s2} (f16–35)A	α_{s2}	milk	close to N-ter	80.8
α_{s2} (f16–35)B	α_{s2}	milk	close to N-ter	76.9
α_{s2} (f36–55)	α_{s2}	milk	interior	76.7
α_{s2} (f56–75)	α_{s2}	milk	interior	147.3
α_{s2} (f76–95)	α_{s2}	milk	interior	52.7
α_{s2} (f96–114)A	α_{s2}	milk	interior	88.9
α_{s2} (f96–114)B	α_{s2}	milk	interior	80.8
α_{s2} (f96–114)C	α_{s2}	milk	interior	126.7
α_{s2} (f152–170)	α_{s2}	milk	interior	82.0
α_{s2} (f190–207)	α_{s2}	milk	C-ter	88.3
β (f1–25)	β	protein	N-ter	86.0
β (f42–56)A	β	protein	interior	54.1
β (f42–56)B	β	protein	interior	51.1
β (f49–97)	β	milk	interior	45.5
β (f76–93)	β	protein	interior	39.1
β (f114–169)	β	milk	interior	90.9
β (f133–150)A	β	milk	interior	123.8
β (f133–150)B	β	milk	interior	48.6
β (f133–150)C	β	milk	interior	52.3
β (f167–178)	β	milk	interior	33.2
β (f184–202)	β	protein	close to C-ter	93.8
β (f193–209)	β	protein	C-ter	33.8
κ (f1–16)	κ	peptide	N-ter	19.8
κ (f69–86)	κ	milk	interior	72.3
κ (f98–115)A	κ	milk	interior	111.2
κ (f98–115)B	κ	milk	interior	99.9
κ (f98–115)C	κ	milk	interior	52.0
κ (f112–130)	κ	milk	interior	80.8
κ (f113–169)	κ	milk	interior	96.3
κ (f131–150)	κ	milk	interior	99.1
κ (f150–169)A	κ	milk	C-ter	144.1
κ (f150–169)B	κ	milk	C-ter	100.0
κ (f150–169)C	κ	milk	C-ter	111.9

^aSequence of the casein specifically recognized by each monoclonal antibody. Letters A, B, and C correspond to different antibodies recognizing the same epitope.

on Biacore experiment to characterize the ability of an antibody to bind an antigen. It corresponds to the percentage of casein bound compared

to the maximum binding capacity ($R_{\max}$), where

$$R_{\max} = (\text{analyte } M_w / \text{ligand } M_w) \times \text{ligand response} \times \text{valence}$$

If we consider that the stoichiometry of the interaction between a 24 kDa antigen and a 144 kDa antibody is 1:1 (which is usually the case with IgG-type antibodies) and that 600 RU of monoclonal antibodies was captured on the RAM surface, then the $R_{\max} = (24/144) \times 600 = 100$ RU.

If an 80 RU response is observed, then the binding capacity is 80%.

When binding capacities of >100% are observed, either the stoichiometry of the reaction is not 1:1 (which can be the case with IgM antibodies, for instance, or when antigens are oligomerized) or the observed binding is limited by the transport of analyte from bulk solution to the surface, so-called mass transport.

The binding capacity of each monoclonal antibody toward its respective antigen was evaluated on Biacore by injecting 20 μ L of each culture supernatant followed by 10 μ L of a 10 μ g/mL solution of its corresponding antigen (α_{s1} , α_{s2} , β , or κ -casein) in HBS-EP. Binding capacity, as shown in the right column of Table 2, was defined as the percentage of casein bound compared to the theoretical maximum binding.²⁴ This value was taken into account in the final expression of the results, making allowance for the difference between high response due to high-affinity antibodies and high response due to high exposure of epitopes in the micelle structure.

Topography of the Casein Micelle Surface on Biacore. (a) *Optimization of the Assay.* Different solutions were tested to perform casein micelle/monoclonal antibody interaction. Milk permeate (MP) bound significantly to the immobilized antibodies, decreasing the signal obtained when micelles were injected on the sensor surface, probably because MP contained casein peptides able to bind antibodies, decreasing the number of available binding sites for the casein micelles. Simulated milk ultrafiltrate buffer (SMUF)²⁵ generated insoluble material that affected the optical signal. Therefore, imidazole buffer was used as running buffer for the interaction.

Four different flow rates (5, 10, 20, and 30 μ L/min) were compared by injecting casein micelles on four different antibodies specific for one of the four caseins. A 20 μ L/min flow rate was selected for the experiment and maintained over the sensor surface. In a similar manner, a range of casein micelle concentrations was injected onto these four antibodies to select the dilution to apply to the micelle sample. Finally, a casein concentration of 1 μ g/mL was used for the whole experiment; using an identical concentration of caseins for all of the antibodies allowed their binding signals to be compared.

(b) *Description of the Assay.* Forty microliters of a specific monoclonal antibody was injected, followed by 20 μ L of micelle fraction F4 diluted at 1 μ g/mL in imidazole buffer. Finally, a sensor chip was regenerated with a 20 μ L injection of 100 mM glycine-HCl, pH 1.7, followed by 20 μ L of 10 mM NaOH. The level of binding of each monoclonal antibody onto the RAM surface corresponded to the increase in RUs after monoclonal antibody injection (RU1). Binding of the casein micelle on each monoclonal antibody corresponded to the increase in RUs after micelle injection (RU2). Dissociation between the casein micelle and each monoclonal antibody was estimated by calculating the decrease in RUs due to the release of the casein micelle by the monoclonal antibody during the 70 s following micelle injection (RU3). Analyses were performed in duplicate.

(c) *Results Calculation.* Binding of micelles or EDTA-dissolved caseins was normalized between antibody injections by calculating the binding per 1000 RU of antibody captured using the equation $(RU2 \times 1000)/RU1$. Then, these values were divided by the casein binding capacity to take antibody casein binding capacity into account. Micelle dissociation was established by calculating the percentage of micelles that dissociated from the antibodies according to the formula $[(RU2 - RU3)/RU2] \times 100$.

Dissociation of Micelle Structure and Impact on Biacore Signal. The program generated on Biacore for the topography of the casein micelle surface was applied again on fraction F4 dissolved in 20 mM imidazole, 50 mM NaCl, 5 mM CaCl₂, and 20 mM EDTA, pH 6.7, to disrupt the micelle structure. Report points and calculations were identical to those established on the native casein micelle.

Impact of Particle Size on Micelle Topography. Micelle fractions F1, F4, and F6 were injected on captured monoclonal antibodies α_{s1} -casein (f93–111), α_{s2} -casein (f96–114), κ -casein (f150–169), and β -casein (f1–25). Analyses were performed in duplicate.

Evolution of Casein Micelle Topography during Proteolysis. Topography of the micelle was studied by SPR using monoclonal antibodies during UHT milk incubation with proteolytic *Pseudomonas fluorescens* using strain CNRZ 795 as previously described.²⁶ A 500 mL flask of UHT milk was inoculated with strain CNRZ 795 at 4.5×10^5 cfu/mL and incubated for 5 days at 37 °C. This resulted in the destabilization of milk. Then, the milk sample was centrifuged at 50000g during 1 h at 20 °C. The supernatant was collected, stored at –20 °C, and considered to be a source of *P. fluorescens* protease as previously described.²⁶ Ten milliliter UHT milk samples were inoculated with 10 μ L of the protease preparation and incubated for 6 weeks at 4 °C to make the proteolytic phenomenon happen slowly, and 1 mL aliquots were collected aseptically after 1 day, 3 weeks, and 6 weeks. Topography of the micelle surface was performed on the milk prior to inoculation (reference) and on the aliquots collected after 1 day, 3 weeks, and 6 weeks as described above. Results were expressed as percentage of increase or decrease of the SPR signal in comparison with the signal of the reference. Analyses were performed in duplicate.

RESULTS AND DISCUSSION

Particle Size. Differences in micelle diameter were observed between fractions F1–F6, the diameter decreasing from F1 to F5 (Table 1). However, the differences observed were not important, and F6 showed a diameter even higher than that of F5. Dissolution of fraction F4 in 20 mM EDTA led to the total disruption of the micelle structure, and no particles were detected. The casein micelle topography experiment was made on fraction F4.

Antibody Binding Capacity. The binding capacity of the antibodies used in this study was calculated from the binding of a 10 μ g/mL solution of pure α_{s1} -, α_{s2} -, β -, or κ -casein in percent of maximum theoretical binding (Table 2). If we assume that casein binding on monoclonal antibodies is monovalent, the binding capacity varied between 14 and 147% for the antibodies used. For β - and α_{s1} -casein antibodies the mean binding capacities were 63% (SD = 29%) and 43% (SD = 20%), respectively, whereas the corresponding values for α_{s2} - and κ -casein were higher, that is, 90% (SD = 27%) and 90% (SD = 33%), respectively. These differences in terms of binding capacity may be partly explained by the way the antibodies were raised. Indeed, most of the antibodies against α_{s2} - and κ -casein used in the present study were raised using raw milk as an immunogen (all α_{s2} -caseins and 10 of 11 κ -caseins), resulting in antibodies with high binding capacities.²¹ In contrast, a large proportion of the β - and α_{s1} -casein-specific antibodies used was either raised toward the purified caseins (6 of 12 β -casein-specific and 5 of 11 α_{s1} -casein-specific antibodies, respectively)²⁴ or toward a synthetic peptide (the N- and C-termini of α_{s1} -casein).⁴ If we compare the antibodies directed toward β - and α_{s1} -caseins, there are actually two groups, one with low binding capacities (33.2–54.1%, $n = 8$, for β and 14.2–46.9%, $n = 9$, for α_{s1}) and one with high binding capacities (86.0–123.8%, $n = 4$, for β and 71.4–85.4%, $n = 2$,

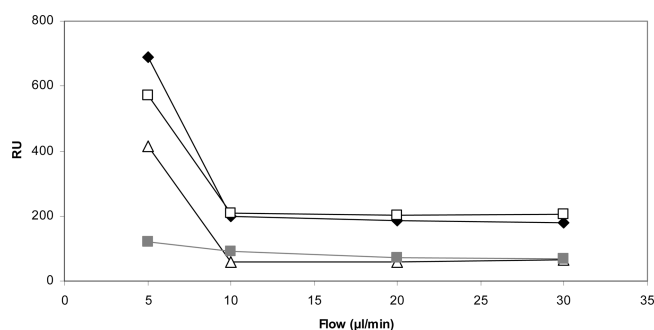


Figure 1. Effect of flow rate (in μ L/min) on the level of interaction between the casein micelle and monoclonal antibodies α_{s1} -casein (f93–111) (◆), α_{s2} -casein (f96–114) (■), β -casein (f76–93) (□), and κ -casein (f69–86) (△).

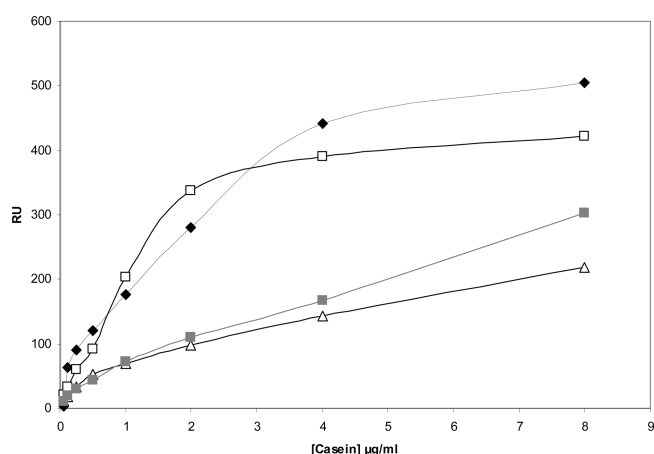


Figure 2. Effect of casein micelle concentration (in μ g/mL) on the level of interaction between the casein micelle and monoclonal antibodies α_{s1} -casein (f93–111) (◆), α_{s2} -casein (f96–114) (■), β -casein (f76–93) (□), and κ -casein (f69–86) (△).

for α_{s1}). Analysis of the six high-binding capacity anti- α_{s1} - and β -casein antibodies shows that immunization with milk gave high binding capacity antibodies directed toward some epitopes in the interior of the protein sequence, whereas immunization with pure caseins gave high binding capacity antibodies directed toward the extremities of β - and α_{s1} -casein. This is probably a sign of difference in antigen exposure during immunization.

Assay Conditions. An increase in the binding level of casein micelle injected onto the sensor surface was observed at very low flow rate and a stabilization of the signal at higher flow rates (Figure 1). At a 5 μ L/min flow rate, it is probable that one micelle binds to a first antibody, then dissociates from it, and rebinds to another antibody because of the low flow rate increasing the observed signal artificially. Therefore, 20 μ L/min was chosen for the experiment. When a range of casein micelle concentration was injected onto the four casein-specific antibodies to select the dilution to apply to the micelle sample, the antibodies gave a linear response when concentrations in casein between 0.5 and 2 μ g/mL were injected (Figure 2). At higher concentrations, a plateau was reached at 2 μ g/mL for antibody β -casein (f76–93) and at 4 μ g/mL for antibody α_{s1} -casein (f93–111). Therefore, a casein concentration of 1 μ g/mL was used for the whole experiment; using an identical concentration of caseins for all of the antibodies allowed their binding signals to be compared.

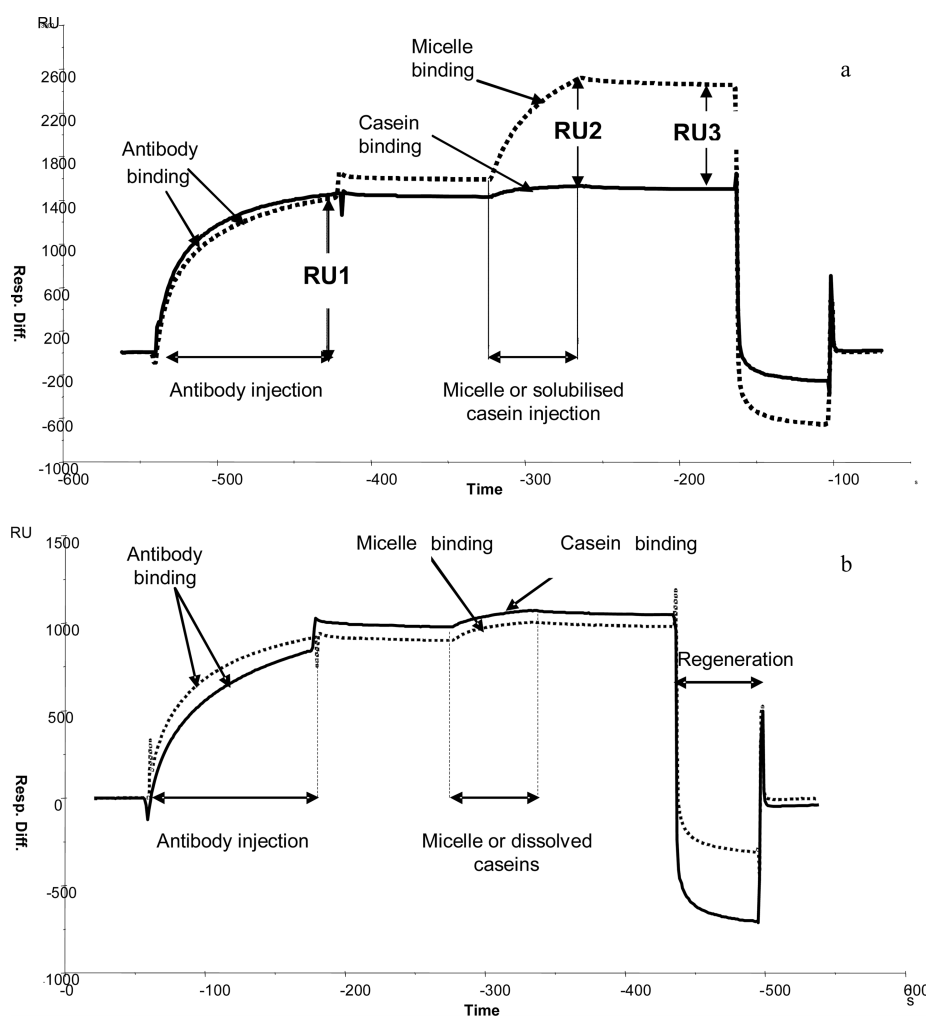


Figure 3. Typical sensorgrams obtained with Biacore by injecting casein micelles (---) or EDTA-solubilized caseins (—) on captured monoclonal antibodies κ -casein (f131–150) (a) and α_{s1} -casein (f93–111) (b). In panel a, RU1, RU2, and RU3 correspond to the level of binding of the monoclonal antibody, of the casein micelle, and of the casein micelle after 70 s of dissociation, respectively.

Micelle Topography. A first analysis of the interactions between the casein micelle and the 44 antibodies was performed. Once these 44 interactions were finished, a replication of the whole experiment was performed using the same samples. Duplicates were similar, as demonstrated by standard deviations ranging from 0.4 to 4.1%, indicating that storage of the casein micelle in imidazole buffer did not affect the interaction with the antibodies throughout the duration of the experiment. Typical sensorgrams obtained with Biacore by injecting casein micelles and EDTA-dissolved caseins on captured monoclonal antibodies are represented in Figure 3, panels a and b, respectively (see also the Supporting Information). Binding of micelles and EDTA-dissolved caseins to α_{s1} -, α_{s2} -, β -, and κ -casein-specific monoclonal antibodies is shown in Figure 4, panels A–D, respectively.

α_{s1} -Casein. Micelle binding to α_{s1} -casein antibodies ranged from 124 to 341 RU, as shown in Figure 4A. The highest micelle bindings were via epitopes α_{s1} -casein (f1–18)A, α_{s1} -casein (f75–92), and α_{s1} -casein (f129–148), followed by the C-terminal α_{s1} -casein (f185–199). These four sequences correspond to peptides in or near the three main hydrophobic areas of the protein, namely (f1–44), (f90–113), and (f132–199). However, because none of the available antibodies were specific for the two hydrophilic sections where phosphorylations occur at

positions 46, 48, 64, 66, 67, 68, and 75, we are not able to conclude on the accessibility of hydrophobic sections compared to hydrophilic ones. Antibodies α_{s1} -casein (f1–18) A, B, and C showed different behaviors toward the casein micelle, antibody A giving significantly higher response than antibodies B and C. Antibody A was raised using the N-terminal synthetic peptide as immunogen, whereas antibodies B and C were produced against the purified proteins. Because the contact zone of an antibody on its antigen consists of three to six amino acids, it is still possible that within the f1–18 area, these three antibodies bind different epitopes.

Addition of EDTA disrupted the micelle structure and led to a general decrease of the binding levels observed or no significant modification of them except for antibody α_{s1} -casein (f129–148), which showed a slight increase in binding from 320 to 406 RU.

α_{s2} -Casein. α_{s2} -Casein is the most hydrophilic casein but seems to be less available to antibody binding than α_{s1} -casein if we look at the primary results with micelle binding ranging from 33 to 130 RU (Figure 4B). Extremely low micelle binding was even observed with antibodies specific to the N-terminal moiety of the protein, antibodies α_{s2} -casein (f16–35A), α_{s2} -casein (f16–35)B, α_{s2} -casein (f36–55), and α_{s2} -casein (f56–75)

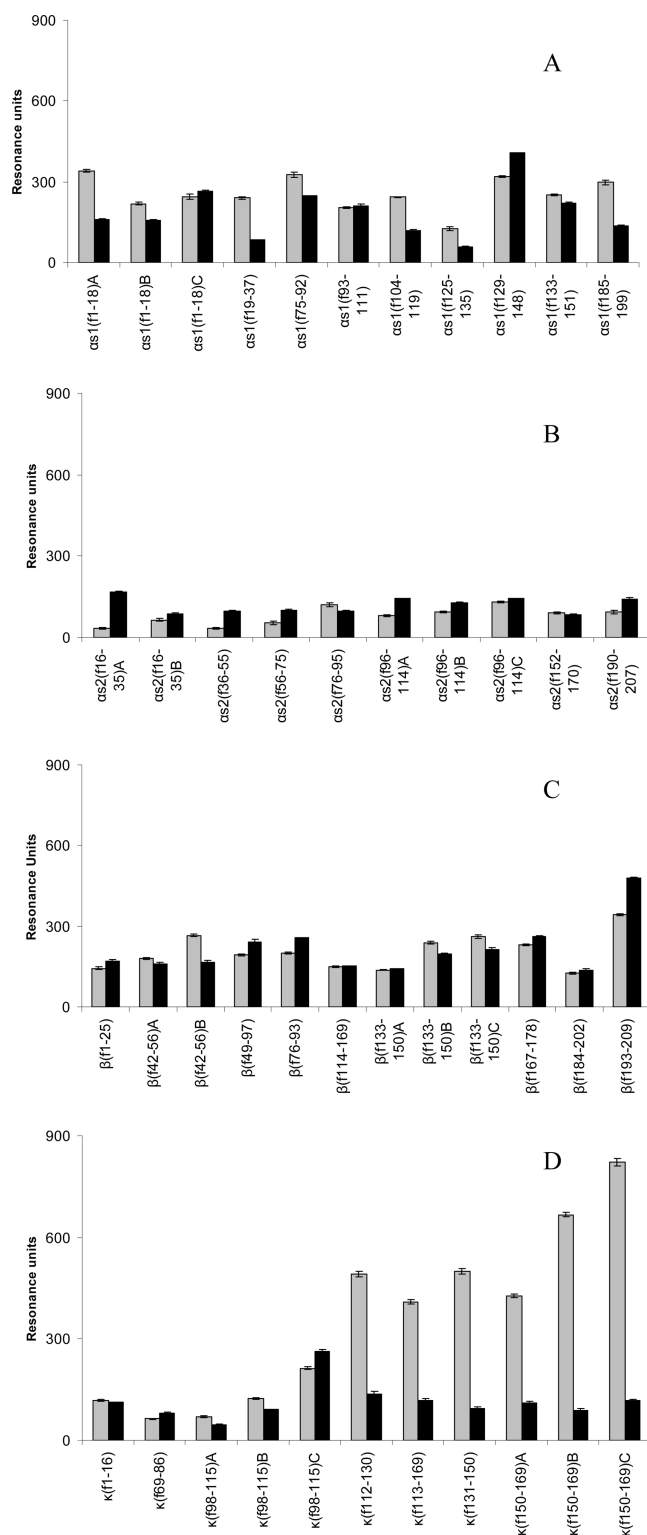


Figure 4. Micelle (■) and EDTA-dissolved caseins (■) binding on α_{s1} - (A), α_{s2} - (B), β - (C), and κ -casein (D) and specific monoclonal antibodies. Values were corrected to take antibody binding capacity into account. The antibodies are named after their site of interaction. The y-axes were kept identical to facilitate comparison of the results obtained for different caseins.

showing binding values of 33, 63, 33, and 55, respectively. In contrast, the mean binding level for the antibodies recognizing

epitopes in the C-terminal part of the protein was 102 RU. In the α_{s2} -casein (f96–114) region, results were identical between antibodies A, B, and C. These results indicate that α_{s2} -casein is less accessible for antibody binding than the other caseins in the micelle and that α_{s2} -casein seems to be oriented in the micelle structure, its N-terminal part being hidden in the core of the micelle and hardly detectable by specific antibodies. When EDTA was added to disrupt the micelle structure, binding levels increased for 8 of the 10 α_{s2} -casein-specific antibodies. When casein micelle is disrupted, the α_{s2} -casein monomers liberated from the structure are free to interact with the specific antibodies.

A higher immunoreactivity of the epitopes corresponding to the hydrophobic area i.e. α_{s2} -casein (f152–170) and α_{s2} -casein (f190–207), than those directed against the hydrophilic area i.e. α_{s2} -casein (f56–75) was observed. The same observation was made for α_{s1} -casein although no antibodies specific for epitopes in the hydrophilic area were in our possession. Both α_{s2} - and α_{s1} -caseins were accessible to antibodies indicating a proportion of these 2 molecules is accessible at the micelle surface. This confirms the hypothesis based on calculation postulating that the outer layer of the micelles would be composed of nearly equivalent amount of α_s (α_{s1} + α_{s2}) and κ -casein and a small amount of β -casein.¹⁹ Both α_{s2} - and α_{s1} -caseins are the most phosphorylated caseins. Their clusters of phosphoserines are supposed to make them involved in the protein shell of nanocluster allowing at the same time the growth of the casein network through attractive interaction between the hydrophobic areas of each casein.²²

β -Casein. Figure 4C shows that, for β -casein, micelle binding ranged from 125 to 342 RU with no tendency for a particular area to show higher values, except for the C-terminal extremity of the protein. Disruption of the micelle structure with EDTA did not change dramatically the level of binding observed, except for the C-terminal extremity of the molecule with an increase in binding from 342 to 479 RU. In contrast, the most hydrophilic part of β -casein, that is, β -casein (f1–25), does not seem to be more exposed than the more hydrophobic parts of the protein. The three β -casein (f133–150) binding antibodies A, B, and C showed slight differences in the binding of micelles as well as of soluble caseins. β -Casein, which is the most hydrophobic of the four caseins, is an amphiphilic molecule with a highly hydrophobic (C-terminal) and a hydrophilic (N-terminal) part. Interestingly, our results showed that the C-terminal section provided more significant responses compared to the N-terminus, which was the less bound by corresponding antibodies. This indicated a preferential exposure of this C-terminal part of β -casein at the surface, whereas the N-terminal part was less exposed. Our result is in agreement with the fact that 40% of β -casein is cross-linked to nanoclusters and consequently has a role in structuring casein micelle.²⁷ Four of the five phosphoserine residues that β -casein contains are located on the N-terminal part (residues 1–25); this section is supposed to interact with calcium phosphate to form nanoclusters and, as a consequence, cannot be well exposed at the surface of casein micelles. Despite the hydrophobic nature, our result showed that the C-terminus of β -casein was accessible for binding with very large molecules such as antibodies immobilized on the sensor surface.

κ -Casein. When native micelles were injected on captured monoclonal antibodies, the highest binding was obtained for the hydrophilic C-terminal half of κ -casein. The closer to the C-terminal, the higher the binding obtained was. The binding in this area was comparatively higher [ranging from 410 RU for

κ -casein (f113–169) to 822 RU for κ -casein (f150–169)C] than that obtained with other caseins. The binding in the N-terminal half of the κ -casein as determined with antibodies κ -casein (f1–16) and κ -casein (f69–86) was lower than the one in the C-terminal half of the protein as shown in Figure 4D. Differences between the three antibodies directed toward κ -casein (f98–115) were seen, with response values ranging from 70 to 213 RU. However, the central zone of κ -casein was the part of the protein that showed the less binding toward the antibodies. Disrupting the casein micelle structure by chelating the calcium with EDTA led to dramatic changes in the signal obtained, especially with C-terminal κ -casein-specific monoclonal antibodies. Indeed, when EDTA was added, signals obtained with these antibodies decreased by factors of 4–7 to reach values even lower than those obtained with the three other caseins. For instance, casein micelle dissolution with EDTA decreased the binding on κ -casein (f150–169) from 822 to 118 RU. These results suggest that κ -casein is well exposed at the periphery of the micelle structure, allowing the capture of a large supramolecular structure like the casein micelle by an antibody. When the micelle structure is disrupted, only dissociated (or nonmicellar) κ -casein will be able to be captured by the antibodies, causing a drastic decrease in the binding signal. The mean value obtained for κ -casein when micelles were disrupted using EDTA was 114 RU and therefore lower than those of α_{s1} -casein (188 RU) and β -casein (215 RU), because κ -casein is approximately 3 times less concentrated in milk than α_{s1} - and β -caseins.

The results obtained in this study showed that capture of high molecular weight particles such as the casein micelle by specific antibodies was possible on a Biacore sensorchip. In the case of C-terminal κ -casein-specific antibodies, the epitope was extremely accessible at the micelle periphery, allowing a strong interaction with the antibodies. This gave birth to a stable complex between the micelle and the antibody and high corresponding binding levels. In contrast, α_{s2} -casein-specific antibodies were unable to bind strongly their corresponding epitopes because they were less accessible at the periphery of the micelle. The complexes formed were labile and the levels of binding lower. When micelles were dissolved using EDTA, monomers of casein bound to the captured antibodies. The difference in molecular weight between a casein micelle (around 1.3×10^6 kDa) and a monomer (19–25 kDa) is so important that we may have expected an even more important decrease of the signal when EDTA was added. It is probable that the binding of big particles such as casein micelles on a sensorchip coated with antibodies is limited because of the small surface available for binding (flow cell surface of 1.2 mm^2 , height of $5 \text{ }\mu\text{m}$) and steric hindrance for interacting with antibodies. Binding of casein monomers, on the other hand, is not limited by steric conditions and availability of binding sites on the carboxymethyl dextran layer.

Our results raise a key question: how does the location of several hydrophobic regions at the casein micelle surface conciliate with its high hydration? It is known that the hydrophobic interactions are driven by the gain in entropy, leading to the expulsion of water molecules. Consequently, the hydrophobic side chains are largely buried within the folded structure and are inaccessible to solvent. If this has been proved for globular proteins, this organization can be different, taking into account that caseins are intrinsically unstructured proteins, but because they are incompletely folded, caseins must inevitably expose to the solvent at least some regions of structure that are usually buried. It can be assumed that the flexibility of caseins must help

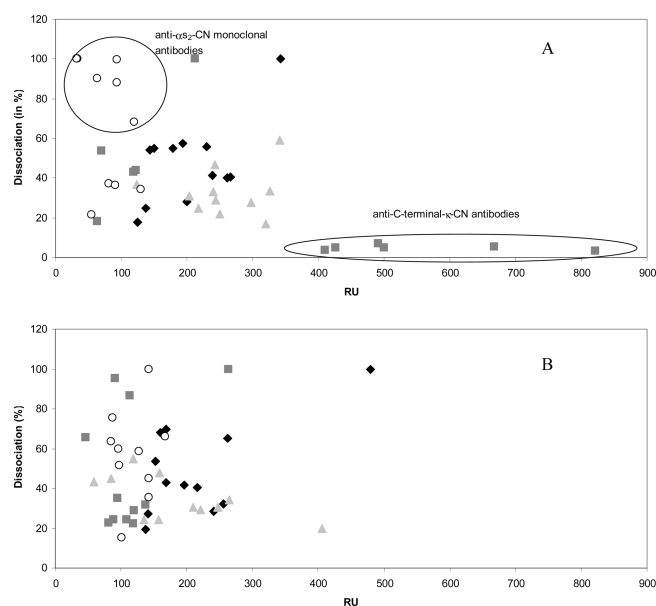


Figure 5. Dissociation of the antigen–antibody complexes versus the level of binding of the antigen on the captured anti- α_{s1} - (▲), α_{s2} - (○), β - (◆), or κ -casein (■) antibodies (in RU). The antigens were either casein micelle (A) or EDTA-dissolved caseins (B).

the polypeptidic chain to distend and organize at the micellar interfaces of the particle. To do so, they need to release some free water molecules, and we hypothesized that these cavities at the surface as well as in the interior of the casein micelles may be formed by release of water molecules. Hence, it can be considered that the surface may have large pockets or cavities, tending to minimize these exposed hydrophobic surfaces which remain, nevertheless, accessible for binding with antibodies. Consequently, models of the casein micelle must be compatible with these large cavities present at the surface. Particular insight has come from the use of high-resolution microscopies that clearly showed a surface with large pore clefts^{15,22} that was recently taken into account in models proposed for the casein micelle.^{12,13} Together, our results raise the question of where the surface of the micelle is to be found as recently asked.¹² They support the fact that the casein micelles must be considered as an open structure because large molecules such as dextrans²⁸ and enzymes²⁹ were shown to be able to diffuse within casein micelles.

Stability of the Antigen–Antibody Complexes. Dissociation of the antigen–antibody complex was plotted against level binding for each antibody used, when micelle (Figure 5A) or EDTA-dissolved caseins (Figure 5B) were injected on the antibodies. In Figure 5A, most of the antibodies specific to α_{s2} -casein were discriminated from the others and characterized by a low stability (high dissociation) of the complex and low binding levels. In contrast, C-terminal κ -casein antibodies were characterized by a very high stability of the complex and high levels of micelle binding. When EDTA-dissolved caseins were injected (Figure 5B), markers were distributed randomly on the graph and no group of antibodies was discriminated from the others. These results demonstrate the important differences existing between α_{s2} - and κ -caseins in the micelle structure. When the C-terminal extremity of κ -casein is well exposed at the periphery of the structure, allowing its capture by the antibody to form a very solid and stable complex, α_{s2} -casein is less accessible at the

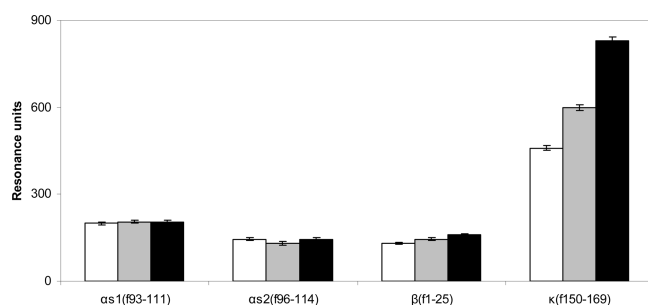


Figure 6. SPR signal (in resonance units) obtained on large (F1) (white bars), medium (F4) (gray bars), and small (F6) (black bars) casein micelles with monoclonal antibodies α_{s1} -casein (f93–111), α_{s2} -casein (f96–114), β -casein (f1–25), and κ -casein (f150–169).

periphery of the structure, allowing the formation of few complexes of low stability.

Micelle Size. Micelle diameters of fractions F1, F4, and F6 were 183, 124.1, and 112.4 nm, respectively. Binding of F1, F4, and F6 micelles to anti-casein antibodies α_{s1} -casein (f93–111), α_{s2} -casein (f96–114), β -casein (f1–25), and κ -casein (f150–169) was tested. The results are presented in Figure 6. For κ -casein, the binding levels to the C-terminal sequence κ -casein (f150–169) were 30 and 81% higher for the medium micelles (F4) and the small ones (F6) compared to that of the large micelles (F1). Similar results were observed for β -casein but with lower increases (11 and 23%), whereas values for the different fractions were similar for α_{s1} - and α_{s2} -caseins. The comparison of the large and small casein micelles, which differ by their composition in κ -casein, showed that the interaction is well correlated to the respective κ -casein concentrations. The small casein micelles, which are richer in κ -casein, gave a higher response than the large casein micelles. From high-resolution microscopy images, modeling describes the casein micelle surface as not smooth with clefts penetrating the micelle.¹⁵ Indeed, the amount of κ -casein in milk is insufficient to cover the micellar surface on its own. κ -Casein would be grouped at the extremity of the tubules rather than like a surrounding continuous brush on the surface of the casein micelles. These local high concentrations of κ -casein at the surface can explain the high intensity of response obtained by SPR here. Taken together, our SPR results on κ -casein are in agreement with the surface location of κ -casein, confirming that this technique is relevant as a surface probe.

Evolution of the Casein Micelle Topography during Proteolysis by Proteases from Psychrotrophic Bacteria. Changes at the micelle surface occurred slowly, and only slight changes were observed after 1 day of incubation (Figure 7A). However, after 3 weeks of incubation, the SPR signal showed a decrease for epitopes located at the C-terminal extremity of κ -casein and at the N-terminal extremity of β -casein to a lesser extent (Figure 7B), showing that these two areas were the most affected by proteolysis. In contrast, signals obtained with α_{s1} - and α_{s2} -caseins and some β -casein epitopes increased during proteolysis, showing an increased accessibility of these areas at the micelle surface. Most of these areas are rather hydrophobic, particularly areas α_{s1} -casein (f93–111), α_{s1} -casein (f129–148), and β -casein (f167–178). Analysis of the sample stored for 6 weeks confirmed the modifications observed after 3 weeks of incubation (Figure 7C). The pronounced difference in SPR response between κ -casein and the three other caseins, α_{s1} -, α_{s2} -, and

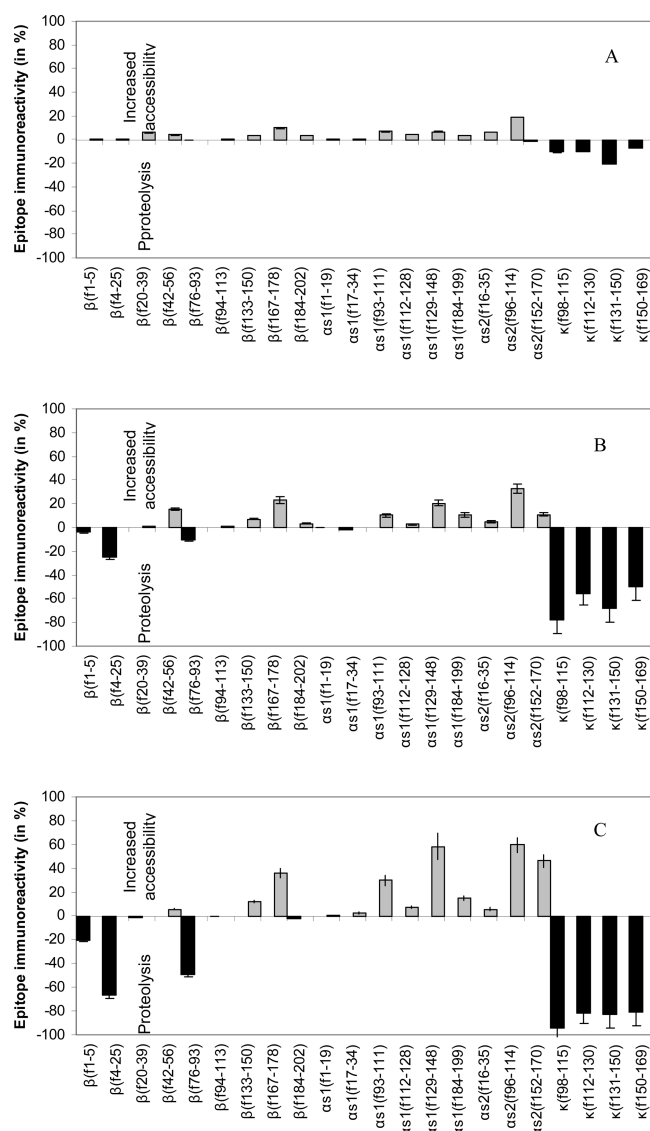


Figure 7. Evolution of the casein micelle surface after 1 day (A), 3 weeks (B), and 6 weeks (C) of proteolysis by *Pseudomonas fluorescens* proteases as determined by SPR using casein-specific monoclonal antibodies. Results are expressed as percentage of increase (gray bars) or decrease (black bars) of the SPR signal as compared with unproteolyzed milk (binding ratio).

β -casein (Figure 4), showed that the κ -casein is clearly the most accessible part of the casein micelle, which is consistent with previous work using ^1H NMR.³⁰ Its C-terminal part gave a considerably higher response than the N-terminal part of κ -casein, suggesting a highly accessible position. The accessibility of the C-terminal extremity of κ -casein was also clearly confirmed when micelles were submitted to proteolysis. Our results show that this area was the most extensively and quickly hydrolyzed by proteases because of its high accessibility to enzymes from psychrotrophic bacteria. Comparatively, the N-terminal κ -casein molecule had a lower accessibility to antibodies. This section, highly hydrophobic and positively charged, could play the role of anchor of κ -casein in the casein micelle core through electrostatic and hydrophobic interactions with other caseins. When the casein micelle was submitted to proteolysis, both α_{s1} - and α_{s2} -caseins appeared to be more reactive toward

their specific monoclonal antibodies. This increased interaction with monoclonal probes can be attributed to the opening of the micelle structure by the protease action, resulting in a higher access for the antibodies to their specific epitopes. However, when micelles were submitted to proteolysis (3 weeks) that induced a partial disruption of the casein micelle structure, the region of β -casein (f4–25) was shown to be extensively hydrolyzed by microbial proteases.

The current understanding of the casein micelle surface indicates the presence of an outer loosely packed structure looking like a “hairy” layer enriched in κ -casein at the surface, whereas the other caseins are more or less present at this surface. In the present work, the combined use of SPR and casein-specific monoclonal antibodies turned out to be an interesting approach for a molecular analysis of the casein sequences accessible at the casein micelle surface. This information may also help to validate theoretical models of casein micelle by providing a deeper understanding of its topography.

On the basis of our results, an open structure model prevails to explain the preferential location of κ -casein as “patches” at the surface and the presence of all caseins at the surface as well as their better accessibility by proteolysis. Besides the location of the C-terminal part of κ -casein at the surface, the finding of hydrophobic regions of these caseins such as sequences β -casein (f193–209), α_{s1} -casein (f185–199), α_{s1} -casein (f75–92), α_{s2} -casein (f96–114), α_{s2} -casein (f76–95), and α_{s2} -casein (f190–207) is of particular significance to promote this open model. The areas hydrophobic at the surface are not compatible with the submicelle model that locates hydrophilic areas at the surface of the submicelles for binding with calcium phosphate to hold together the submicelles.⁹ Taken together, our results will be in accordance with a nonhomogeneous surface with gaps at the surface resulting in the organization of caseins.

■ ASSOCIATED CONTENT

S Supporting Information. Overlay plot of all the whole sensorgrams obtained with Biacore by injecting casein micelles (Figure S1a) or EDTA-solubilized caseins (Figure S1b) on the 44 casein-specific monoclonal antibodies captured by RAM. Focus on the specific response of casein micelles (Figure S2a) or EDTA-treated micelles (Figure S2b, same scale; Figure S2c adjusted scale). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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