



Epitope characterization of a supramolecular protein assembly with a collection of monoclonal antibodies: The case of casein micelle

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ABSTRACT

In milk, κ -, β -, α _{s1}- and α _{s2}-casein (CN) are associated into a supramolecular assembly, the micelle. In this work, CN micelles contained in fresh skim milk were used to produce over 100 monoclonal antibodies. The specificity of these probes was determined using libraries of synthetic peptides and peptides fractionated from tryptic hydrolysis of purified CNs. Although κ -CN and α _{s2}-CN are minor proteins in the micelle (ratio 1:1:4:4 for κ , α _{s2}, α _{s1}, β) a proportionally high number of clones were produced towards these two proteins (32 for each), compared to 9 and 29 for α _{s1}-CN and β -CN, respectively. Most of the β -CN and κ -CN epitopes were identified, while about 50% of α _{s1}-CN and α _{s2}-CN antibodies were suspected to react to conformational linear or discontinuous epitopes, since no peptide binding could be identified. Antibody binding to the phosphoserine rich regions of the three calcium sensitive CNs was weak or non-existing, suggesting them to be hidden in the micelle structure together with α _{s1}-CN. The C-terminal glycomacropeptide of κ -CN and the C-terminal moiety of β -CN were well exposed generating the majority of the antibodies specific for these two proteins. The two major antigenic sites of α _{s2} were α _{s2}-CN (f96–114) and (f16–35). Cross-reaction between α _{s2}-CN specific antibodies with α _{s1}-CN illustrated the tangled structure between the two proteins. Immuno-dominant epitopes identified in the present study totally differ from those known for the purified caseins suggesting they were specific for the micelle supramolecular structure.

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1. Introduction

Milk is an important source of protein of the diet both as liquid milk and in a variety of processed forms. The most abundant proteins in cow's milk are the caseins (CNs). Four major CNs can be found in milk, namely α _{s1}-, α _{s2}-, β - and κ -CN in the ratios of 37%, 10%, 37% and 10%, respectively.

The primary structure of the four CNs is known (Farrell et al., 2004) and their secondary structures have been determined by a variety of experimental techniques using circular dichroism (CD), optical rotary dispersion (ORD) and Fourier Transform infrared spectroscopy (FTIR) (Sawyer and Holt, 1993). Less is known about their tertiary structures, since CNs cannot be crystallized and analyzed using standard X-ray crystallography.

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Instead, three-dimensional models refined via energy minimization were constructed for β -CN (Kumosinski et al., 1993a), κ -CN (Kumosinski et al., 1993b) and α _{s1}-CN (Kumosinski et al., 1991). These predicted structures were built from sequence-based secondary structure prediction algorithms in conjunction with global secondary structure results obtained from spectroscopy experiments. All vibrational spectroscopy data are however based on data from globular proteins, thus limiting their value for prediction of less ordered proteins like CNs. At the present time CNs are considered as natively unfolded proteins (Syme et al., 2002).

In milk, individual CNs are associated into large micellar structures present as a colloidal suspension. The micelle organisation and the structure of the individual CNs play a major role in the ability of milk to be transformed into dairy products.

Even though several theoretical models for the organisation of the CN micelle have been proposed, the exact organisation is still unknown. A submicellar model with a hairy layer, an internal sub-structure resembling a mineralized, entangled, or cross-linked web

of chains of CNs and a dual-binding (polycondensation-type) mechanism for gel assembly have been proposed (see review of Horne, 2006). Common features of most proposed models are a more or less dense surface layer of κ -CN surrounding the Ca-sensitive CNs with colloidal calcium phosphate (CCP) as a crucial component for micelle stability. There is however still a controversy on how the individual CNs are organised in the micelle and established models were challenged by recent work, in which shapes and structures were studied directly using high resolution scanning electronic microscopy (FE-SEM) (Dalglish et al., 2004), small-angle X-ray scattering and ultrasmall angle X-ray scattering combined (Marchin et al., 2007) or not (Pignon et al., 2004) with cryo transmission electronic microscopy. The authors proved the CN micelle surface to be more complex than a sphere surrounded by a hairy layer, consisting of tubules protruding to the exterior spaced by large gaps (Dalglish et al., 2004). CNs appear to be organised as an entangled network of protein chains which protuberate at the surface and form an extremely diffuse boundary with the surrounding medium (Marchin et al., 2007).

Although most of the physico-chemical techniques traditionally used to study protein structure are extremely powerful they cannot be applied directly to a complex food matrix like milk. An attractive alternative is to use immunochemical techniques, which due to their sensitivity and specificity are well suited to detect a single protein in a complex environment. Recently, immunochemical techniques were successfully used to distinguish between the forms of native and denatured α -lactalbumin (α -La) in milk. Heat treatment such as ultra high temperature (UHT) or sterilization made certain epitopes disappear as the intensity of the heat treatment increased, whereas new epitopes appeared gradually (Jeanson et al., 1999; Dupont et al., 2004). Similarly, modifications of the β -CN structure were followed on sequence level during the transformation of milk into cheese using antibodies specifically directed against the plasmin sensitive sites in the β -CN sequence (Senocq et al., 2002a; Dupont et al., 2003).

All the CN specific monoclonal antibodies reported in the literature were raised by immunising mice with purified individual CNs or synthetic peptides and antibodies specific for the whole CN micelle have not yet been described. The objective of the current study was thus to produce a panel of monoclonal antibodies mainly specific towards the four CNs as structured within the CN micelle, and to determine if the assembly of CNs into a supramolecular structure changes the nature of the CN immuno-dominant epitopes.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated, chemicals were from commercial origin (Sigma–Aldrich, France).

2.2. Milk

Bovine whole raw milk freshly collected was kindly provided by a local producer (Rolet, France) and kept at 37 °C until further treatment. Milk was then defatted by centrifugation at 1800 $\times$ g and 25 °C during 15 min. The top fat layer was removed and skimmed milk was used for mice immunisation (see below). Total protein content was determined by infrared spectroscopy (IDF, 1996).

2.3. Peptide synthesis

In total, 43 peptides of 18–20 amino acids length covering the entire sequences of the four major CNs were chemically synthe-

sized with a minimum purity of 75% by Epytop (Nîmes, France). The peptide sequences are reported in Table 1. Serine residues were substituted for the phosphorylated serine residues. Peptides were modified by C-terminal amidation, and in some cases by addition of a cysteine residue at N-terminus as indicated in Table 1.

2.4. Casein purification

β -CN was isolated from raw milk according to Senocq et al. (2001). α S₁-, α S₂- and κ -CN were isolated as previously described (Collin et al., 1991). κ -CN was then obtained in a non-glycosylated form as previously described (Vreeman et al., 1986).

2.5. Tryptic hydrolysis of casein

Five mg of β -CN variant H (Senocq et al., 2002b), κ A-CN, α S₁-1B-CN and 1.8 mg α S₂-A-CN, respectively dissolved at 10 mg/ml in 75 mM ammonium acetate pH 8.0, were hydrolysed by TPCK-treated trypsin (Sigma, >10,000 BAEE units/ml) at an enzyme to substrate ratio of 1/500 (w/w) at 37 °C for 15 h. The reaction was stopped by heating the samples to 100 °C for 10 min and sample mixtures were stored at –20 °C. Prior to chromatographic separation the hydrolysates were diluted with an equal volume of 0.1 M Tris pH 8 containing 8 M urea. κ -CN and α S₂-CN sample mixtures were incubated for 1 h at 37 °C with 2 μ l 0.5 M dithiothreitol (DTT) to break the cysteine disulfide bridges, followed by centrifugation at 1800 $\times$ g. All CN hydrolysates were filtered through a 0.45 μ m PVDF Millex filter (Millipore, France).

2.6. Reversed phase-high performance liquid chromatography (RP-HPLC–MS) fractionation

CN hydrolysates were fractionated on a C18 column (4.6 mm $\times$ 150 mm, 5 μ m, Grace VYDAC, 218TP5415) at a flow rate of 0.8 ml/min and 40 °C using an Agilent HP1100 chromatographic system (Agilent Technology, France). The mobile phase consisted of deionised water with 0.106% TFA (solution A) and acetonitrile 80% in water with 0.1% TFA (solution B). After column equilibration with 5% of solvent B, a gradient elution was performed as follows: 0–3 min, 5%; 3–60 min, 5–60%; 60–62 min, 60–90%; 62–64 min, 90%; 64–70 min, 90–5%; 70–75 min, 5%. Peaks were detected both by UV-absorbance (214 nm) and on line ESI-MS as described by Molle and Leonil (2005). Resolved peaks from two to three 100 μ l injections of the respective hydrolysed CN were manually collected and freeze dried.

2.7. Production of monoclonal antibodies

Immunisation with defatted raw milk was carried out by the foot pad route essentially according to Jeanson et al. (1999). Briefly, defatted milk was diluted 37.5 times in physiological water in order to obtain an 800 μ g protein/ml solution. This solution was then emulsified 1:1 (v/v) in Freund adjuvant (complete for the first immunisation, incomplete for the booster injection). Fifty microliters (i.e. 20 μ g of proteins) of the emulsion were injected per foot pad. Popliteal lymph nodes from two immunised mice were pooled. Lymphocytes were fused in a ratio of 5:1 to Sp2/O-Ag14 myeloma using filter fusion according to Buttin et al. (1978). Polyethylene glycol (PEG 1000, Merck Chemicals Ltd., UK) was allowed to permeate through monolayers of mixed cells adsorbed on membrane filters. Culture supernatants of resulting hybridoma were collected during the log growth phase and screened by ELISA for Ig binding to dry milk and bovine protein fractions. Secreting hybridomas were subcloned by limiting dilution.

Table 1Synthetic peptides used for mapping of monoclonal antibodies directed towards β -CN, κ -, α ₁-CN and α ₂-CN.

Peptide	Amino acid sequence	Peptide	Amino acid sequence
β -CN		κ -CN	
1–19	RELEELNVPGEIVESLSSS	1–16	QEQNQEPIRCEKDER
20–39	CEESITRINKKIEKFQSEEQ ^a	17–35	FFSDKIAYIPIQYVLSRY
42–56	CEDELQDKIHFAETQ ^a	36–54	PSYGLNYYQKQPVALLNNQ
57–75	SLVYPFGPIPNSLPQNIP	55–73	FLPYPPYAKPAVRSPAQI
76–93	PLTQTPTVVPPFLQPEVM	74–92	LQWQVLSNTVPAKSCQAQP
94–113	CGVSKVKEAMAPKHKEMPFA ^a	98–115	HPHPLHSFMAIPKKNQD
114–132	YPVEPTESQSLTLTDVEN	112–130	KNQDKTEIPTINTIASGEP
133–150	LHLPLPLLSQSWMHQPHQP	131–150	TSTPTTEAVESTVATLEDSP
151–166	LPPTVMFPPQSVLSLS	150–169	PEVIESPPEINTVQVTSTAV
167–178	CQSKVLPPVQKAV ^a		
184–202	CDMPIEAFLLYQEPVLGPVR ^a		
193–209	CYQEPVLGPVRGPFPIIV ^a		
α ₁ -CN		α ₂ -CN	
1–19	RPKHPIKHQGLPQEVNLNEN	1–15	KNTMEHVSSEESII
19–37	NLLRFFVAPFPEVFGKEKV	16–35	SQETYSQKEKNMAINPSKENL
38–55	NELSKDIGSESTEDQAME	36–55	CSTFCKEVVRNANEEYSIG
56–74	DIKQMEAESISSSEIVPN	56–75	SSSEESAEVATEEVKITVDD
75–92	SVEQKHQKEDVPSEYRL	76–95	KHYQKALNEINQFYQKFPQY
93–111	GYLEQLRLKKYKVPQLEI	96–114	LQYLYQGPIVLPNPWDQVKR
112–128	VPNSAEERLHSMKEGIH	115–132	NAVPIPTPLNREQLSTSE
129–148	AQQKEPMIGVNQELAYFYPE	133–151	ENSKKTVDMESTEVTFTKK
149–166	LFRQFYQLDAYPSGAWYY	152–170	TKLTEEEKNRLNFKKISQR
167–184	VPLGTQYTDAPSFSIDIPN	171–190	YQKFALPQYLYKTVYQHOKAM
185–199	PIGSENSEKTTMPLW	190–207	KPWIQPKTKVIVVRYL

^a Peptides with addition of a cystein residue at N-terminus.

2.8. Characterization of monoclonal antibodies

2.8.1. Characterization by indirect ELISA

This method was used to screen the hybrid clones for binding to bovine milk, specificity towards bovine protein fractions (β -CN, κ -CN, α ₁-CN, α ₂-CN, BSA, α -La, β -lactoglobulin (β -Lg) and lactoferrin (Lf)) and to map the antigenicity with (1) synthetic peptides mimicking the full sequence of the four CNs and (2) tryptic peptides from hydrolysis of the four CNs. For the screening assay bovine protein fractions were dissolved in 0.1 M carbonate buffer, pH 9.6 (CB) and coated in parallel at 0.5 μ g/ml. For antigenicity mapping, synthetic peptides were initially dissolved at 1 mg/ml in distilled water and coated at 0.5 μ g/ml in CB. Clones which did not react with synthetic peptides were further tested against tryptic peptides. Positive results with synthetic peptides were confirmed with tryptic peptides when overlapping peptides were available. The lyophilized tryptic peptides were initially dissolved in water to a concentration of 0.1 mg/ml and further diluted at 0.5 μ g/ml in CB before being coated. In addition a coating with 5–20 times higher concentration of tryptic peptides was performed to screen for weak interaction. The ELISA was performed according to Senocq et al. (2001).

2.8.2. Interaction between κ -CN (f113–169) specific antibodies and glycosylated or non-glycosylated κ -CN by Biacore

Binding and dissociation of glycosylated and non-glycosylated κ -CN with κ -CN (f113–169) specific antibodies were investigated using a Biacore 3000 instrument (General Electric Europe GmbH, France) in order to determine if glycosylations were involved in the antigen–antibody interaction. Sensor chip CM5 (carboxy-methylated dextran), HBS-EP buffer (10 mM HEPES, 3 mM EDTA, 0.15 M sodium chloride, 0.005% P20, pH 7.4), Rabbit Anti Mouse Immunoglobulins (RAM, α -Mouse Immunoglobulins), 10 mM sodium acetate pH 4.5 and amine coupling kit [(0.05 M NHS (N-hydroxy-succinimide), 0.2 M EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride)) and 1 M ethanol amine–hydrochloric acid, pH 8.5] were obtained from General Electric.

RAM were immobilized covalently on a CM5 sensor chip by amine coupling as described previously (Jonsson et al., 1991). The final immobilization level was 11,000 RU, corresponding to 11 ng/mm². RAM was immobilized on flow cell 2, while flow cell 1 being used as a reference cell.

A continuous flow was maintained at 20 μ l/min. 40 μ l of supernatant of hybrid clones, recognizing κ -CN (f113–169), was injected over the RAM surface at a dilution of 1/5 in HBS-EP buffer, followed by a 20 μ l injection of either glycosylated or non-glycosylated κ -CN at a concentration of 10 μ g/ml. Regeneration of the chip surface was achieved with 10 mM glycine–HCl at pH 1.7 and 10 mM NaOH injected in series (20 μ l of each).

2.8.3. Isotyping

Determination of Ig sub-classes was carried out on 45 hybridomas supernatants producing α ₁-, α ₂-, β - or κ -CN specific antibodies using commercial kits (Interchim, France).

2.9. In silico predictions of disorder and order of κ [113–169] and β [114–169]

The on-line “Predictor of Natural Disordered Regions” (PONDR) program was assessed in term of its ability to predict order and disorder in κ -CN (f113–169) and β -CN (f114–169) primary amino acid sequences. This program was accessed through the server: www.pondr.com/.

3. Results

3.1. Characterization of tryptic peptides

A summary of the tryptic peptides obtained in this study is shown in Table 2. For β -CN and α ₁-CN basically the whole sequences were covered with tryptic peptides complementary to the synthetic peptides, including all phosphoserine residues. For α ₂-CN all phospho-groups in the N-terminal half of the protein (P8, P9, P10, P16, P56, P57, P58 and P61) were covered by α ₂-CN

Table 2Tryptic peptides obtained from β -, κ -, α _{s1}- and α _{s2}-CN.

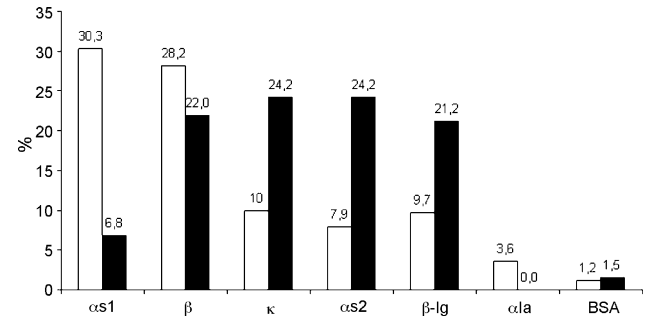
Peptide	Measured molecular mass	Amino acid sequence ^a
β-CN		
1–25	3123.23	RELEELNVPG EIVEU ^U UUE ESITR
33–48	2062.19	FQEEQQQTEDELQDK
49–97 ^b	5360.47	
114–169	6363.88	YPVEPFTESQSLTLTDVENL HLPLPLLSQWMHQPHQ PLPPTVMFPQSVLSLSQSK V LPVPQK AVPY PQR
170–176	779.8	
177–183	829.9	
184–202+	2186.99+	DMPQAFLLYQEPVLGPVR
69–97	3096.2	+SLPQNIPPLTQTPVVVPP FLQPEVMGVSK QAFLLYQEPVLGPVR
188–202	1729.04	
κ-CN		
1–16	2013.58	BEQNQEPIR CEKDER
25–34	1251.44	YIPQY VLSR
69–86	1979.84	SP AQILQWQVLS DTPVAK
87–97	1193.24	SCQA QPTTMR
98–112	1736.54	HPH PHLSFMAIPP KK
113–169	6023.14	NQDKTEIP TINTIASGEP TSTPTTEAVE STVATLEDUPEVIESPPEIN TVQVTSTAV
α_{s1}		
4–7	493.3	HPIK
8–22+	1760.09	HQGLPQEVLENENLLR + NPIGSE
194–199	+747.7	
23–34+	1384.19+	FFVAPFPEVFGK+
152–193	4718.0	QFYQLDAYPSGAWYYVP LGTQYTDAPSFSDIPNPIGSENSEK EKNELSK DIGUEUTEDQAMEDIK QM EAEU ^U UUEE IVPNUVEQKH IQKEDVPSEY YLGYLEQLRLK YKVPQLEIVPNUAEER
104–119	1952.54	
125–132	909.7	EGIHAQ QK
133–151	2316.44	EPMIGVNQ ELAYFYPELFR
α_{s2}		
1–24+	3133.28+	KNTMEHV ^U UUEESIUQET ^Y KQEK+
2–24	3005.33	NTMEHV ^U UUEESIUQET ^Y KQEK
25–32	873.44	NMAINPSK
25–41	1900.34	NMAINPSKENLCSTFCK
46–70	3008.78	NANEEYSIG ^U UUEEUA ^U AEVATEEVK
71–76	689.81	ITVDDK
92–114	2867.03	FPQYLQYLYQGPIVLPWDQVQR
138–149	1466.24	TVDMETEVFTK
153–158	747.70	LTEEEK
174–181	979.49	FALPQYLK
182–188	902.99	TVYQHQQK
189–197	1097.99	AMKPWQPK

^a Phosphorylated serine residues indicated by U.^b Sequence β (49–97) collected from the α _{s2}-hydrolysate which contained β -casein due to contamination.

(f1–24) and α _{s2}-CN (f46–70), respectively. In the C-terminal half α _{s2}-CN (f138–149) contained P143 while no peptide with P129 and P131 was obtained. A majority of the peptides obtained from α _{s2}-CN were very short resulting from the high content of lysine residues of this protein compared to the other CNs (24, 14, 11 and 9 lysine residues in α _{s2}-, α _{s1}-, β - and κ -CN, respectively), therefore providing more potential tryptic cleavages. For κ -CN the sequence κ -CN (f113–169) contained the phospho-group P149, but there was no glycosylation.

3.2. Production of hybridoma

Immunisation of two mice with skimmed milk yielded 132 hybridomas secreting antibodies specific to milk proteins. Among

**Fig. 1.** Comparison of the percentage of antibodies specific to each protein (■) and their respective concentration in milk in g/l (□).

those, 2 were directed against BSA, 28 towards β -Lg and 102 were CN specific. No α -La or Lf antibody specific secreting hybridoma was obtained although α -La is present in milk at relatively high concentrations (1 mg/ml) and is known to be immunogenic. Towards β -, κ - and α _{s2}-CN, 29, 32 and 32 antibodies, respectively were produced, while only 9 antibodies directed towards α _{s1}-CN were obtained. The difference in immunogenicity between the different milk proteins is highlighted in Fig. 1, as the percentage of antibodies specific to each protein in relation to their theoretical respective concentration in milk.

The result of an immunisation with skimmed milk would be expected to reflect the abundance and immunogenicity of the respective CN in combination with the actual exposure of individual epitopes to the immune system of the host animal. The two most abundant milk proteins are α _{s1}-CN (30.3%) and β -CN (28.2%). 22% of the clones bound to β -CN while only 6.8% were directed towards α _{s1}-CN. As for κ -CN (10% in milk) and α _{s2}-CN (7.9% in milk), the number of milk protein clones was proportionally higher (24.2% for each). Since all the lymph nodes of the mice were pooled within one fusion experiment and not treated independently, a statistical analysis of these data cannot be performed in order to demonstrate that the number of α _{s1}-CN specific clones is significantly lower than expected. However, if we consider that α _{s2}-CN and κ -CN concentrations in milk are 3 times lower than that of α _{s1}-CN and that 32 specific clones were obtained towards these proteins, we could have expected to generate around 100 α _{s1}-CN specific clones which is much more than the 9 clones obtained.

A few antibodies were found to interact with more than one of the CNs during initial screening with ELISA. This cross-reaction was confirmed after subcloning for five clones, all having α _{s2}-CN as their main antigen and all cross-reacting with α _{s1}-CN to about 55%, 18%, 16%, 15% and 12%, respectively. Binding to specific epitopes is further discussed in the following section.

The isotype determination of the 45 α _{s1}-, α _{s2}-, β - or κ -CN specific antibodies gave 19 IgG1, 1 IgG2a, 5 IgG2b, 1 IgG3, 3 IgM, 14 showing IgG1 and G3 cross-reactions and 2 showing reactivity towards all the anti-isotype conjugates. These results show that even if the immunisation protocol of the mice is short (fusion 17 days after the primary injection) it does not lead to a high proportion of IgM isotypes.

3.3. Mapping of casein antigenicity with ELISA using synthetic and tryptic peptides

Mapping with the synthetic peptides (Table 1) coated on ELISA plates revealed the binding sites on CN for 41% of the produced monoclonal antibodies. Complementary screening with tryptic peptides increased the number of antibodies with identified binding to 72%. Binding to synthetic peptides were confirmed using tryptic peptides whenever overlapping sequences were avail-

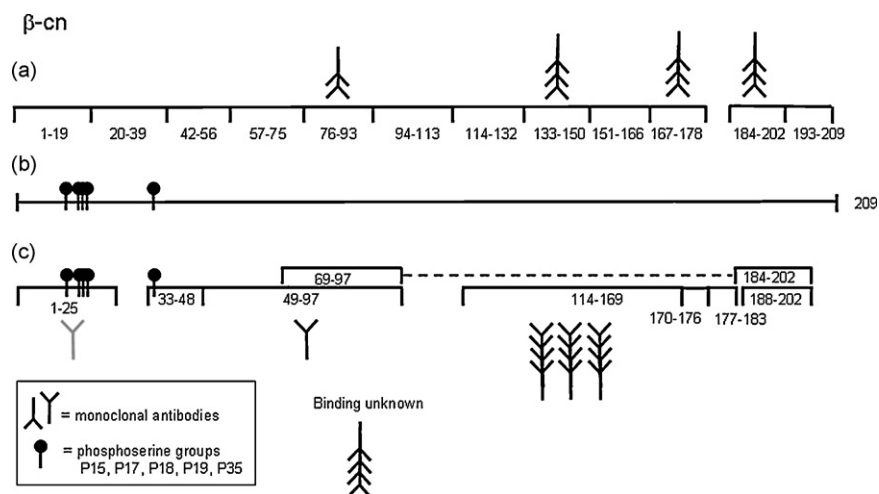


Fig. 2. Specificity of anti- β -casein monoclonal antibodies reacting towards (a) synthetic peptides and (c) tryptic peptides only (no recognition of synthetic peptides observed). Under (b) location of the phosphoserine groups of β -casein are indicated. Antibodies showing weak binding to the casein are represented in grey.

able. For β -CN, specific binding for 25 of 29 (86%) clones were identified, while the corresponding figures for κ -, α _{S1}- and α _{S2}-CN were 28 of 32 (88%), 4 of 9 (44%) and 16 of 32 (50%), respectively.

3.3.1. β -Casein

Figs. 2–5 show the mapping of β -, α _{S1}-, α _{S2}- and κ -CN specific antibodies and indicate the number of antibodies binding to the synthetic fragments (a), the positions of phosphoserine groups and/or glycosylated residues (b) and the antibodies binding to tryptic peptides only (c). For β -CN, most of the antibodies were directed against the C-terminal part of the molecule. Interaction with sequence 4P(f1–25) was only identified for one clone and the binding was weak, since a second assay with a 20-fold higher concentration of the peptide solution was required for detection in ELISA.

3.3.2. α _{S1}-Casein

Binding of the nine clones directed primarily towards α _{S1}-CN is shown in Fig. 3(a–c). ELISA reactivity of α _{S1}-CN specific antibodies towards synthetic and tryptic peptides was generally weaker than that of the β -CN specific antibodies towards their corresponding peptides. Only one of the nine α _{S1}-CN specific antibodies bound to a synthetic peptide, namely α _{S1}-CN (f93–111). Additional binding to tryptic peptides occurred in the centre part of the molecule from about residue 104–151. Five of the α _{S1}-CN specific antibodies were not classified and none of the antibodies tested showed binding to the phosphoserine rich region 46–75.

3.3.3. α _{S2}-Casein

For α _{S2}-CN most of the antibodies were directed towards α _{S2}-CN (f16–35) or α _{S2}-CN (f96–114) as shown in Fig. 4(a–c). Binding to tryptic peptides confirmed the results from the synthetic peptides,

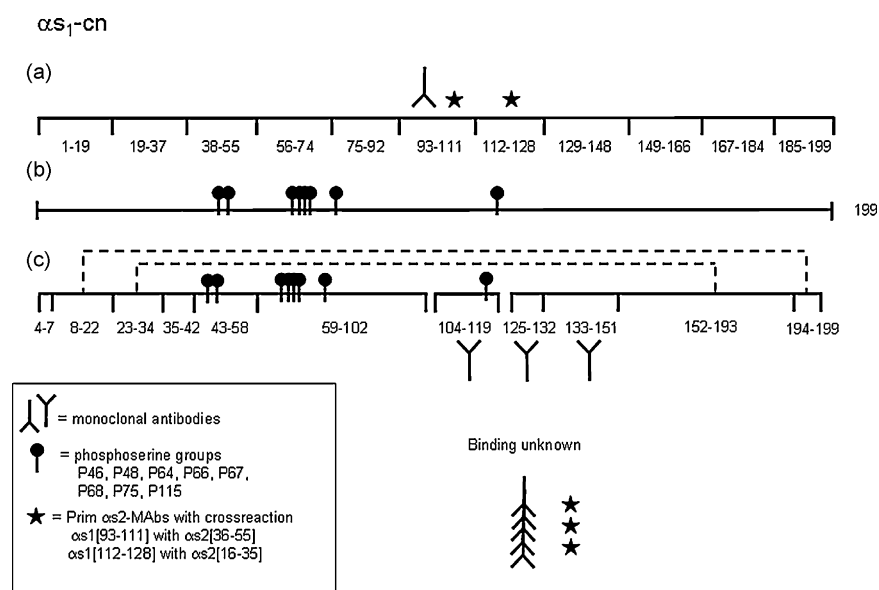


Fig. 3. Specificity of anti- α _{S1}-casein monoclonal antibodies reacting towards (a) synthetic peptides and (c) tryptic peptides only (no recognition of synthetic peptides observed). Under (b) location of phosphoserine groups of α _{S1}-casein are indicated. Antibodies primarily directed towards α _{S2}-CN cross-reacting with α _{S1}-CN, are indicated with a star.

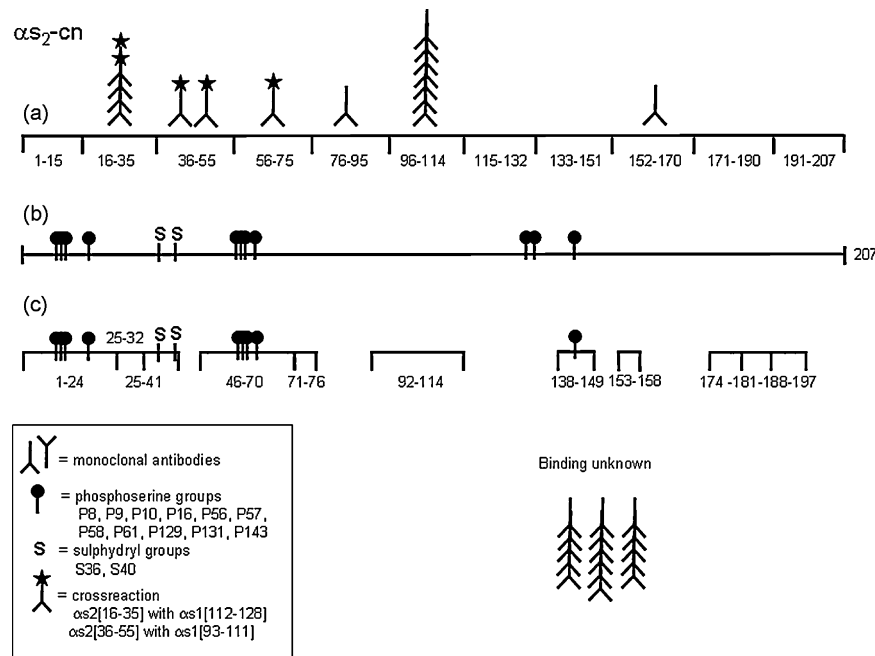


Fig. 4. Specificity of anti- α_2 -casein monoclonal antibodies reacting towards (a) synthetic peptides and (c) tryptic peptides only (no recognition of synthetic peptides observed). Under (b) location of phosphoserine groups of α_2 -CN are indicated. Antibodies cross-reacting with α_1 -CN are indicated with a star.

but no additional epitopes were identified. The four antibodies directed towards α_2 -CN (f16–35) bound to the tryptic fragments α_2 -CN (f1–24)4P and α_2 -CN (f25–32) to a similar extent but not to α_2 -CN (f36–55), suggesting that the epitope would be centred on the 24–25 peptide bond. In contrast, the two antibodies directed towards α_2 -CN (f36–55) bound α_2 -CN (f15–35) to about 30%, indicating a slightly overlapping epitope around amino acid 36. The low number of characterized antibodies could be due to the small length of the tryptic fragments directed toward the C-

terminal half (about 75–207) of the molecule and their subsequent lack of reactivity in ELISA. The 3D structure of the area with two cystein residues in position 36 and 40 engaged in interchain disulphide linkage forming α_2 -dimers with parallel or antiparallel orientation (Rasmussen et al., 1992), may also contribute to a low epitope recognition using linear α_2 -peptides. In addition, it could be noted that no antibodies bound exclusively to α_2 -CN (f1–24) containing phosphoserine groups at P8, P9, P10, P16 or to α_2 -CN (f138–149) containing one phosphoserine-group at P143.

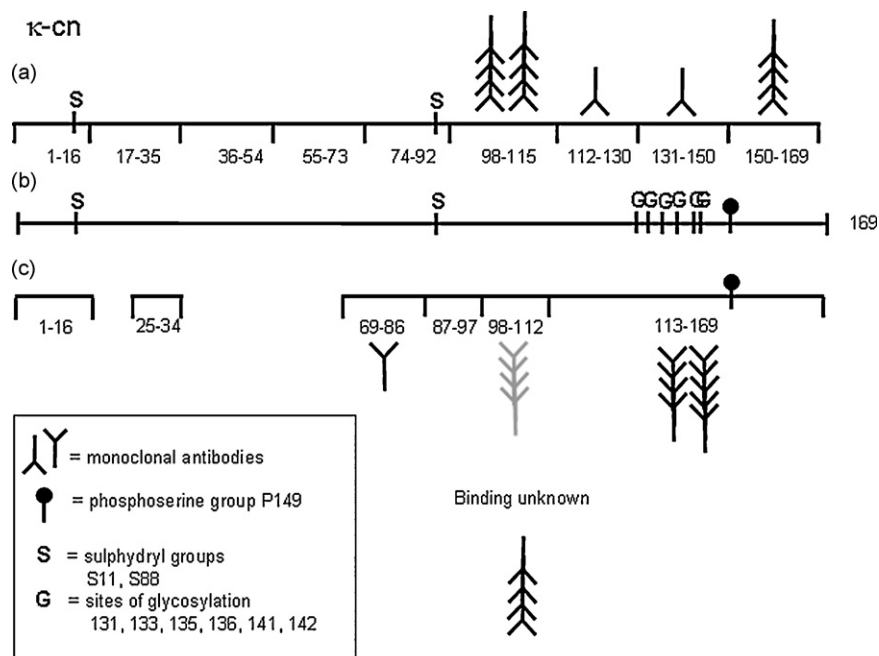


Fig. 5. Specificity of anti- κ -casein monoclonal antibodies reacting towards (a) synthetic peptides and (c) tryptic peptides only (no recognition of synthetic peptides observed). Under (b) location of sulphhydryl groups (S) region of glycosylation (G) and phosphoserine groups of κ -casein are indicated. Antibodies showing weak binding to the casein are represented in grey.

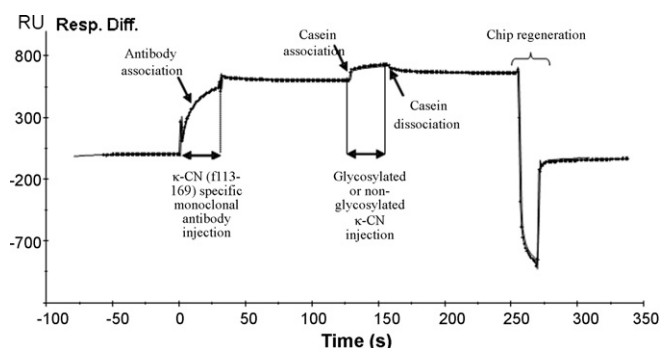


Fig. 6. Type of sensorgrams obtained when κ -CN (f113–169)-specific monoclonal antibodies were captured on a RAM Biacore surface and glycosylated (---) or non-glycosylated (·····) κ -CN were injected afterwards.

3.3.4. Cross-reaction between α ₁- and α ₂-casein

Five antibodies primarily directed towards α ₂ bound to specific motifs of α ₁ even after subcloning. Cross-reacting antibodies are indicated with stars in Figs. 3 and 4. Cross-reaction could either be due to common motifs in the amino acid sequence, or to an actual liaison between the proteins in the micelle structure. Cross-reacting antibodies were all directed to motifs in the N-terminal half of α ₂ between amino acids 16 and 75 as seen in Fig. 4. One antibody directed towards α ₂-CN (f36–55) is particularly interesting due to its strong cross-reaction with α ₁-CN (f93–111) (about 55%). A second antibody reacted with both α ₂-CN (f16–35) and α ₁-CN (f112–128) (cross-reaction of about 18%). The three remaining antibodies reacting with α ₂-CN (f16–35), α ₂-CN (f36–55) and α ₂-CN (f56–75) bound to unknown epitopes of α ₁-CN.

3.3.5. κ -Casein

Fig. 5(a–c) shows that all κ -CN specific antibodies were directed towards the C-terminal half of the protein (the glycomacropeptide) except one that reacted towards the κ -CN (f69–86) synthetic peptide. Nine antibodies, which did not interact with any of the synthetic peptides, bound strongly to the long C-terminal tryptic peptide κ -CN (f113–169). About 50% of κ -CN is in a glycosylated form in position around (f131–142) (Molle and Leonil, 2005). Since none of the peptides used in this study were glycosylated, the nine antibodies specific for κ -CN (f113–169) were tested for their ability to interact with both intact glycosylated and non-glycosylated κ -CN. A similar binding of these antibodies to both forms was observed (Fig. 6), suggesting that the sugars are not involved in the antigen–antibody interaction. Only four antibodies interacted with the hydrophilic N-terminal κ -CN (f150–169), while eight bound to peptide κ -CN (f98–115), in the more hydrophobic area close to the chymosin sensitive site at Phe₁₀₅–Met₁₀₆. Four additional antibodies, which did not bind to any synthetic peptides during initial screening, interacted weakly with the same area when a 10 times higher concentration of tryptic peptide κ -CN (f98–112) was used.

4. Discussion

Individual protein structures need to be visible to the immune system of the host animal to generate antibodies. They also need to be immunogenic. The question is whether the antibodies produced in the present study reflect the structure of a more or less intact CN micelle or simply show a difference in immunogenicity between different epitopes. How come that so few antibodies were generated towards α ₁-CN, and so many towards κ -CN and α ₂-CN, counter to what one would expect from the ratios of the respective protein in the micelle (ratio 1:1:3:3 for κ , α ₂, α ₁, β)?

Do the antibody specificities reflect the structural organisation of protein monomers into a supramolecular assembly?

The generation of an immune response involves the collaboration of several different cell types. Thus for the production of an antibody both B and T lymphocytes specific for the particular antigen are required, each cell expressing a surface receptor capable of recognizing a distinct region within the molecule. However, whereas the B-cell epitope is generally present in the native antigen, T-cells only recognize the antigen when it is associated with the major histocompatibility complex (MHC) glycoproteins, a restriction which dictates that the antigen must be processed and presented by an appropriate antigen presenting cell (Unanue, 1984). Antigen processing by the host has been the topic of several studies in the 1970s and 1980s. Most of the results were obtained on globular proteins, like myoglobin (Berzofsky et al., 1987) and it is difficult to be sure that a supramolecular structure like the CN micelle would behave exactly in the same way. It is well accepted that antigen processing will dramatically modify the structure of the antigen that will be degraded by proteases, releasing hidden epitopes buried inside the structure. However, motifs at the surface of an antigen (protein, virus, etc.) will remain intact and able to stimulate the immune system thus eliciting an immune response; this principle has been the basis of most of the vaccine productions. Indeed, it was suggested that most of the antibodies made by conventional immunisation with globular proteins are directed against antigenic determinants found in the native molecule and not represented in the denatured protein or in fragments of it. The B-cell clones are mostly selected by protein antigens that appeared not to have suffered extensive conformational changes (Benjamin, 1991). Since hybridoma are the results of a fusion between B-lymphocytes and myeloma cells, it is therefore not surprising to generate antibodies directed against intact native part of a structure like the CN micelle.

If parts of the CN micelles are kept intact during immunisation, the sites of interaction of the produced antibodies would truly reflect the epitopes that are exposed at the micelle surface. The fact that a high number of clones directed towards the hydrophilic C-terminal half of κ -CN was generated, is consistent with the general conception that this protein is mainly located at the micelle surface (Horne, 2006).

Our results would suggest that α ₁ and all the phosphoserine clusters of the three calcium sensitive CNs (α ₁-, α ₂- and β -CN) are deeply buried in the micelle structure, since very few, if any antibodies were obtained against these domains. Instead hydrophobic regions like β -CN (f114–169) and α ₂-CN (f96–114) would be more exposed. This is consistent with the Holt model as illustrated by Horne (2006), in which the interior of the micelle is described as a chain of linked nanoclusters composed by the phosphoserine regions and CCP surrounded by dangling hydrophobic regions. It is possible that these “dangling” hydrophobic regions would be more accessible to the immune system than the nanoclusters.

One way to get a clearer answer to our question is to compare the specificity of the antibodies produced using the CN micelle as an immunogen to those produced when purified CNs were used for immunisation. Immunisation of mice and rabbits with pure β -CN have shown that the protein has multiple immuno-dominant epitopes distributed throughout the whole sequence (Otani et al., 1984, 1987; Mizumachi et al., 1999; Senocq et al., 2001). The phosphoserine rich fragment β -CN (f1–25), was recognized as an antigenic region both in rabbit (Otani et al., 1987) and in mice (Senocq et al., 2001). In the latter study, a third of the monoclonal antibodies produced were directed towards the N-terminal extremity of β -CN which is totally opposite to what is observed in the present study. This would support the conclusion that the phosphoserine clusters are involved in micellar nanocluster formation,

and consequently not exposed to the immune system of the mice when milk is used for immunisation.

The analysis of κ -CN epitopes also shows differences when comparing CN micelle and purified protein as immunogen. Two monoclonal antibodies produced in mice were suggested to have epitope centred around κ -CN (f92–94) and κ -CN (f134–136), respectively (Kuzmanoff et al., 1990). This would suggest this part of the protein to be more immunogenic. However, in the present study, only 1 out of the 32 anti- κ -CN antibodies bound to the N-terminal half of this CN, supporting the idea of an intact micelle. Another factor could be the possibility of cross-linking by disulphide bridges in this area (at Cys11 and Cys88) as bovine κ -CN has been shown to exist as homomultimers (Rasmussen et al., 1999).

A similar analysis can hardly be conducted for α ₂-CN since only one single monoclonal antibody has been reported so far with no identification of the epitope recognized (Leung et al., 1991). By studying the kinetics of peptide release from tryptic hydrolysis of purified bovine α ₂-CN, the C-terminal extremity (153–207) was identified as the most accessible region, prior to the N-terminal half of the protein (1–91/113), while the medium part (92/115–150) appeared to be more protected (Tauzin et al., 2003). In our study on the other hand, the main part of the identified clones were directed towards supposable medium to highly protected regions, namely α ₂-CN (f16–35) and (f96–114), both situated in hydrophobic sections of the protein (Swaigood, 2003). Again, differences in the organisation of the protein may be suspected. In milk, α ₂-CN and α ₁-CN appear to have a tangled structure, which is indicated by the difficulties to separate and isolate them. The cross-reactions identified in the current study between α ₂-antibodies directed towards α ₂-CN (f16–35) with α ₁-CN (f112–128), and α ₂-CN (f36–55) with α ₁-CN (f93–111) may indicate possible positions of interactions between these two proteins.

Finally, the case of α ₁-CN could also indicate an influence of the micelle structure on the specificity of the antibodies obtained. Indeed, in our study only 6 antibodies recognized this protein although it was the most abundant in the mixture used for immunisation. Is this result due to a low immunogenic potential of this protein or is it the result of the hidden position of this protein in the micelle structure? Information about relative immunogenicity of the different CNs is difficult to find. In our laboratory we have recently obtained 192 anti- α ₁-CN secreting hybridomas during immunisation with purified α ₁-CN showing that this protein still has important immunogenic properties (unpublished data). It was also reported that three strains of mice responded highly to α ₁-CN (Ametani et al., 1988). These data would therefore support the hypothesis that the limited number of α ₁-CN specific antibodies obtained in the present study was mainly due to the location of α ₁-CN buried inside the CN micelle structure.

The present study also showed that several antibodies were able to bind a peptide obtained by tryptic hydrolysis of the native protein whereas they did not recognize any of the synthetic peptides covering the whole CN sequence. Indeed, 9 antibodies reacted only with tryptic peptides κ -CN (f113–169) and not with synthetic peptides κ -CN (f112–130), κ -CN (f131–150) and κ -CN (f150–169). Similarly, 12 antibodies recognized only tryptic peptide β -CN (f114–169) and not synthetic peptides β -CN (f114–132), β -CN (f133–150), β -CN (f151–166) and β -CN (f167–178). This difference in reactivity for κ -CN (f113–169) cannot be the results in differences in glycosylation since the produced antibodies reacted similarly with both glycosylated and non-glycosylated κ -CN. However, the structural analysis of this fragment using the model previously published by Kumosinski et al. (1993b) shows the presence of an α -helix between the residues 136 and 146 that could be absent in the shorter peptides synthesized. On the other hand, β -CN (f114–169) does not contain any particular structure according to the model

of Kumosinski et al. (1993a). Areas κ -CN (f125–156) and β -CN (f153–177) that are encrypted within peptides κ -CN (f113–169) and β -CN (f114–169), respectively showed the highest PONDR scores (>0.8) demonstrating that these regions were the most disordered in κ -CN and β -CN (data not shown). This result tends to indicate that peptides κ -CN (f113–169) and β -CN (f114–169) both contain sequences that showed drastic modifications of their conformations that could explain the differences observed with more structured shorter synthetic peptides.

Finally, cross-reactivity has been observed for two antibodies that reacted with both α ₁-CN (f112–128) and α ₂-CN (f16–35) and α ₁-CN (f93–111) and α ₂-CN (f36–55), respectively. For each antibody, sequence comparison between the two fragments gave low percentages of identity (14.3% for α ₁-CN (f112–128)/ α ₂-CN (f16–35), 4.8% for α ₁-CN (f93–111)/ α ₂-CN (f36–55)). These quite low levels of identity can hardly explain the observed cross-reaction. Indeed, even if it has been shown by molecular modelling and energy calculations that as few as three to five residues in the so-called “contact epitope” contributed to the binding energy of the interaction (Novotny et al., 1989), X-ray crystallography also showed that 15–22 amino acid residues were identified as being “in contact” with the antibody paratope (Pellequer et al., 1991). Therefore, another possible hypothesis in order to explain the cross-reactivity would be the formation of structural motif caused by the proximity of α ₁- and α ₂-CN in the micelle structure.

In conclusion, the present study suggests that the specificity of monoclonal antibodies raised against a protein assembly reflect its structural organisation. This approach could constitute an interesting way of characterizing protein assembly structures in complex environments.

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