Monoclonal Antibodies against Bovine β-Casein: Production and Epitope Characterization

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Production and epitopic characterisation of monoclonal antibodies against bovine β-casein

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SUMMARY. Twenty one murine monoclonal antibodies (MAbs) were produced against bovine β-casein (β-CN). Eight epitopic groups of MAbs recognising six discrete determinants (4-8, 14-24, 33-48, 49-91, 177-183, 184-209) were defined from ELISA reactivity with peptides and related homologues from 3 species (ovine, human and equine). Thirteen distinct epitopes were discriminated by cross-examination of the MAbs reactivity in BIAcore. Determinants 14-24, 49-91 and 184-209 were found to encompass at lest 4, 2 and 4 epitopes respectively. No MAb was bovine specific, but one at least discriminated ovine/caprine casein from bovine. Terminal sequences 1-28 and 177-209 included ~80% of the discriminated epitopes, and the most conserved ones. This panel of MAbs can provide specific probes for β-CN proteolysis fragments.

The caseins make up the major protein fraction of bovine milk (~80%). They represent the products of 4 linked genes, the caseins α_{S1}, a_{S2}, β and κ. Bovine casein β (β-CN) accounts for ~40% of the casein content. Its primary structure is known from chemical and gene sequencing (Swaisgood, 1992). It consists of 209 residues (~24000 Da), with phosphorylated serine residues clustered at N-termini (5 mole P/mole). It occurs in milk as a mixture of several variants (Ng-Kwai-Hang & Grosclaude, 1992). Eight variants (A^1, A^2, A^3, B, C, D, E, F) differing by 2-5 single-point substitutions have been fully characterised, and variants A^1, A^2, and B account for the large majority of Western cattle heterogeneity. None was found to arise from post-translational modification, although substitutions in variants C and D cause defect of phosphorylation. Homologue β-casein was found in milks of all species. Its sequence in human, sheep, rat, mouse, rabbit and other species indicates a rapid divergence arising from point mutations, without segmental rearrangements (Stewart *et al*., 1987). Bovine β-CN differs from the other closely related caseins by (i) a higher rate of hydrophobic residues, (ii) a low phosphorylation rate and (iii) a high proline content (17%). Its secondary structure reflects these characteristics (Swaisgood, 1992). Experimental and theoretical data agree on a low rate of periodic structure (30%), especially α-helix (10%), but many proline-induced β-turns (34%)(Kumosinski *et al.*, 1993). Owing to the impossibility to crystallise the molecule, secondary and tertiary structure (Kumosinski *et al.*, 1993) could be only predicted. All data suggest an organisation opposite to that of typical globular proteins: conserved domains with strictly defined structure occur at both termini, and are linked by an open and flexible intermediate region.

The products of β-CN cleavage in milk reflect both its structure and the specificity of the native enzymes. The main native protease, plasmin (EC 3.4.21.7), cleaves apart the hydrophilic and heat resistant domain β-CN (f1-28), the intermediate region β-CN (f29-105/-107) and the hydrophobic C-termini β-CN (f106/108-209). These major fragments occur in large amount in fresh milk (typically 10-20% of β-CN in weight) (Andrews, 1983; Andrews & Alichanidis, 1983). Plasmin is associated with casein micelles, and is incorporated in dairy foods, *e.g.* cheeses, where it contributes significantly to the induced proteolysis. The contribution of plasmin to β-CN proteolysis is thought to significantly increase the release of peptides and hydrophobic amino acids in the sapid fraction of cheeses. The objective monitoring of plasmin action could contribute to modelling desirable pathways of flavour development. Biochemical fractionation of peptides is complex, and is not specific for β-CN (Ferranti *et al.*, 1997b). In contrast, immunochemical detection has a great potential for descriptive analysis of proteolysis of specific casein fractions (Addeo *et al.*, 1995). Provided that specific probes are produced, the degradation of β-CN segments can be monitored and quantified (Pizzano *et al.*, 2000). Specific antibodies are currently raised against peptides (Pizzano *et al*., 1999), although the reactivity of anti-peptide antibodies with the intact parent protein is ambiguous. Moreover, these reagents come in limited supply, and may not be reproduced. In contrast, hybridoma technology (Köhler & Milstein, 1975) allows the continuous production of monospecific antibodies, which corresponding epitopes might be investigated in detail (Kobayashi *et al.*, 1991). β-CN presents many β-turn, and a high interspecies divergence which should contribute to its

immunogenicity. Four previous studies reported however only four specific MAbs (Nagaune *et al.*, 1988; Kuzmanoff *et al.*, 1991; Oudshorn *et al.*, 1994; Anguita *et al.*, 1995)., and thus supported the current opinion that caseins are poorly immunogenic. Since the MAbs raised to a protein usually vary in specificity and reactivity, a large collection is preferable to select probes with pre-requisite characteristics. The objective of this study was to produce a large panel of characterised MAbs against β-CN, as probes for its plasmin-induced fragments. Twenty-one murine MAbs were compared for isotypes, ELISA reactivity with proteins and peptides, and reactivity with β-CN in BIAcore. Thirteen non-redundant MAbs bound six discrete determinants distributed along β-CN sequence, with a marked bias in favour of the termini.

MATERIALS AND METHODS

Reagents

Unless otherwise stated, chemicals of analytical grade were purchased from Prolabo (Merck eurolab, D-64293 Darmstadt, Germany). BIAcore 3000 was used with registered chemicals (BIAcore AB, S-75450 Uppsala, Sweden).

Inactive plasminogen was isolated from fresh bovine blood according to the procedure of Deutsch & Mertz (1970), and frozen at -20°C. Immediately prior to use, aliquots (70 g/l) were treated for 10 min at 37°C with urokinase (EC 3.4.21.73) (Choay, Sanofi Winthrop) at an enzyme:substrate ratio of 100 UI/mg, and thus activated to plasmin (EC 3.4.21.7).

β-casein isolation

Individual milk was obtained from the genetically typed () herd of the Coopérative agricole Graindorge (14140 Livarot, France). β-CN from a cow typed as homozygous for β-CN A^2 was analysed in electrospray ionisation mass-spectroscopy (ESI-MS) according to Léonil *et al.* (1995). It was found in agreement with the variant reported by Jimenez-Flores *et al.* (1987), and its primary sequence (A^2 substituted for leucine at position 93) was used.

Milk fat was removed after 15 min centrifugation (1800 g, 4°C). Whole casein was obtained from defatted milk by isoelectric precipitation at pH 4·6 according to the procedure of Wei & Whitney (1984).

Crude β-CN was obtained in gram amounts by the batch-wise ion exchange procedure of Leaver & Law (1992), dialysed against water, and lyophilised. The purity determined on urea-polyacrylamide gel electrophoresis (PAGE) (Andrews, 1983) was ~ 90%. Crude β-CN was purified to homogeneity by the fast protein liquid chromatography (FPLC) ion exchange procedure of Andrews *et al.* (1985), scaled to fit a HR16/10 column (Pharmacia Biotech. AB, S-75450 Uppsala, Sweden). Briefly, 600 mg of crude β-CN were loaded onto 20 ml of Q Sepharose FF (Pharmacia), and eluted at 1 ml/min with a linear gradient of NaCl over 0·09-0·32 M in 25 mM-Tris-HCl–4·5 M-urea–0·08 mM-DTT buffer, pH 7·0. Fractions automatically collected over 5 min were checked in urea-PAGE for homogeneity, pooled, dialysed against water, and lyophilised.

Other antigens

Individual mare's milk was obtained from the farm of the Lycée Agricole (21140 Semur-en-Auxois, France). Mare's whole casein was prepared like bovine's. β–CN from cow buffalo's milk (*Bubalus bubalis*) was a gift from D. Lebars (INRA, 78352 Jouy-en-Josas, France). Whole casein from cow's milk was purchased from Merck, that from ewe, goat, and human milk from Sigma (Sigma-Aldrich, 38297 St.Quentin-Fallavier, France), and bovine serum albumin (BSA) from Pierce (Interchim, 03103 Montluçon, France). Other bovine proteins were isolated from bovine bulk milk according to the following chromatographic procedures: α-Lactalbumin (α-La) and β-Lactoglobulin (β-Lg) according to

Jeanson *et al.* (1999), casein fractions α_S and κ according to Collin *et al.* (1991), immunoglobulins G (IgG) according to Levieux (1974).

Mapping of β-casein antigenicity

β–casein hydrolysis. Plasmin was used to perform limited cleavage of β-CN, specific for the bonds lys-X and arg-X. Aliquots of purified β-CN (10 g/l) in 75 mM-ammonium acetate were adjusted with NaCl at 0, 2·5 and 5 g/l, and at each pH 5·5, 6·0 and 6·5, then plasmin was added in a weight ratio of 1:100, and the solution was incubated at 37°C for 0·5 and 8 hours. The reaction was stopped by boiling for 10 min at 100°C. Prior to chromatographic separation, hydrolysates were diluted with an equal volume of trifluoroacetic acid (TFA) 1·05 ml/l 8 M-urea buffer and filtered through 0·45 µm.

Reverse phase-high performance liquid chromatography (RP-HPLC) fractionation. β-CN hydrolysates were fractionated on a C_{18} Zorbax column (4·6x150 mm, 300 Å, 3·5 µm, Agilent Technologies, Interchim), as described by Beuvier *et al.* (1997). Briefly, 5 nmoles of hydrolysed β-CN were loaded on the column and eluted at 0·8 ml/min by a linear gradient of acetonitrile over ~2-22 % volume, with TFA at 1ml/l as a modifier. Peptides were detected by u.v. absorbance (214 nm), and identified by on-line ESI-MS analysis as described by Léonil *et al.* (1995). Resolved homogenous peaks were collected from further injections of 20 nmoles of hydrolysed β-CN and vacuum-dried at 30°C. Peptides were designated according to the recommended nomenclature (Eigel *et al.*,1984).

Production of monoclonal antibodies

Monoclonal antibodies were produced essentially as described by Jeanson *et al.* (1999). Immunisation with 20 µg of purified β-CN was carried out by the footpad route (Mirza *et al*., 1987). For each experiment, popliteal lymph nods from two immunised mice were pooled. Lymphocytes were fused in a ratio of 5:1 to Sp2/O-Ag-14 myeloma according to two procedures. Essentially, in mass fusion (Köhler & Milstein, 1975) polyethylene glycol (PEG 1000, Merck) (450g/l water) was drop-wise added onto pellets of mixed cells, whereas in filter fusion (Buttin *et al.*, 1978), it was allowed to permeate through monolayers of mixed cells adsorbed on membrane filters. Supernatant media of log phase hybridoma culture were screened in enzyme linked immunosorbent assay (ELISA) for Ig binding to β-CN.

ELISA

The reactivity of the Ig secreted in culture supernatant was assayed in the antigen coated on plate (ACP) mode.

For screening assay, β-CN was coated at 0·5 mg/l 0·1 M-carbonate buffer, pH 9·6 (CB). For cross-reactivity assays, bovine protein fractions (β-CN, BSA, α-La, β-Lg, α_S-CN, κ-CN, IgG) (0·5 mg/l CB) or whole caseins (cow, cow-buffalo, human, ewe, goat and mare) (1 mg/l CB) were coated simultaneously. For antigenicity mapping, peptides initially

dissolved in 1 ml 0·05 M-phosphate buffer–0·15 M-NaCl, pH 7·2 (PBS), were coated at the optimised dilution that produced similar detection signal as β-CN (0·5 mg/l CB). Aliquots of 100 µl antigens were incubated, for two hours at 37°C, in wells of a flat-bottomed 96-well microtitration plate (Nunc Maxisorp, Polylabo 67023 Strasbourg, France). The remaining binding sites were blocked by incubating 250 µl gelatine (Merck) (10 g/l PBS-T buffer, i.e. Tween 20 (Merck) 0·55 g/l PBS) for one hour at 37°C in the wells. Culture supernatants (100 µl) were incubated in the wells, for one hour at 37°C, at proper dilution in PBS with a final concentration of 0·55 g/l Tween 20. They were diluted ½ for screening. For the following assays, dilution was optimised whenever possible as to produce a ~2·0 units absorbance increase in screening conditions. Bound Ig were detected by incubating 100 µl of goat anti mouse immunoglobulin alkaline phosphatase conjugate (0·33 ml/l PBS-T) for one hour at 37°C in the wells (Jackson Immunoresearch, Interchim). Wells were rinsed between each incubation step for 15 s with four changes of 250 µl PBS-T. Following the last rinsing, 100 µl *p*-nitrophenyl phosphate (1 g/l 1 M-diethanolamine-HCl–1 mM-$MgCl_2$–0·1 mM-Zinc acetate) (Sigma) were incubated in the wells. After one hour, the absorbance at 405 nm was read against a blank, and corrected for background signal in the absence of antigen. Threshold for alien casein recognition was set at 4% of bovine signal. All assays, except screening, were performed in duplicate

Isotyping. Light and heavy Ig chains were specifically detected in screening conditions using secondary antibodies picked from the Kit 37502 from Pierce (Interchim).

Biosensor assay using BIAcore 3000

IgG MAbs were assayed for reactivity with β-CN using BIAcore 3000 (Fägerstam *et al.*, 1992). Kinetics measurement were performed from single runs. Additive assays were performed without labelling antibodies using on-line detection (Daiss & Scalice, 1994).

Capture assay. The carboxy-methylated dextran surface (CM5) was conditioned for immobilisation of IgG MAbs as follows: 35 µl of a 0·05 M-N-hydroxy-succinimide–0·2 M-N-ethyl-N'-(3-dimethyl-aminopropyl) solution, then 35 µl of rabbit anti-mouse Fc γ (30 mg/l 10 mM-sodium acetate buffer, pH 4·8), and 20 µl of 1 M-ethanolamine hydrochloride, pH 8·5 solution were injected in sequence at 5 µl/min. Undiluted supernatants were injected at 10 µl/min for 3 min. Unoccupied binding sites of the matrix were blocked with IgG1, IgG2 and IgG3 from mixed mice ascitic fluids non-reactive with β-CN. Purified β-CN (10 mg/l HBS buffer i.e. polysorbate 20 50 µl/l 10 mM-HEPES–3 mM-EDTA–0·15 M-NaCl, pH 7·4) was injected at 10 µl/min for 3 min. The total mass immobilised on the sensor surface was recorded on-line throughout all steps as a proportional increase of the surface plasmonic resonance. For comparison of β-CN capture, resonance was read at 3 min after injection of IgG and β-CN. The mass ratios of bound antigen and antibody were corrected to molar ratio using the following Mw values: 146 kDa for IgG1 and G2, 170 kDa for IgG3 and 24 kDa for β-CN.

Pair-wise sandwich assay. MAbs were pair-wise assayed within the reactivity groups established for tryptic mapping (Table 2), with single MAb IV pooled with group V. Primary MAb was assayed for capture of β-CN, then secondary MAb was injected for binding to captured casein in the same conditions.

Sequence analysis

Primary sequence of β-CN from ewe, goat, human, cow-buffalo, cow, and zebra (*Equus grevyi*) (respectively n° P11839, P33048, P05814, Q9T5I0, P02666, and Q28401 in SwissProt-trEMBL) were compared with free software Multalin 5.4.1 (INRA, 31326 Toulouse, France) set on default parameters. Sequence for bovine β-CN was corrected according to Jimenez-Flores *et al.* (1987). Percentage homology was calculated as aligned residues matching bovine's. Scales for hydrophilicity (Hopp & Woods), solvent accessibility (Boger *et al.*) and antigenicity (Parker *et al*.) were calculated from primary structure using free release ANTHEPROT V4.9 (IBCP-CNRS UPR 412, 69367 Lyon, France) with default parameters and five residues window.

RESULTS

Production of hybridoma

Table 1 features the outcome of three independent fusion experiments carried each with a pair of mice. 21 hybridoma secreting anti β-CN Ig were produced. All carried κ light chains, whereas several heavy chain classes (G1, G2a, G3, M and A) were found. One experiment (filter protocol) yielded IgG MAbs of sub-classes exclusively 2a and 3, whereas other experiments yielded a marked majority of sub-classes 1.

In ACP-ELISA, low cross-reactivity was detected with β-Lg, IgG, α_S- and κ-CN, but not with α-La and BSA. MAbs displaying the highest levels of cross-reactivity were found directed at β-CN C-terminus (group V). They frequently recognised multiple test antigens, which suggested non-specific binding rather than casual sequence homology. Systematic cross-reaction with β-Lg originated apparently from contamination of the test antigen solution with β-CN.

Mapping of β–casein antigenicity

The MAbs were assigned to five groups (I to V) according to their reactivity with 5 fragments of β-CN in ACP-ELISA (Table 2). HPLC fractions containing peptides shorter than 25 residues were not detected. The epitopes recognised by MAbs from groups I, III and V were unambiguously present in peptides β-CN (f1-25), β-CN (f49-97), and β-CN (f184-209) respectively. MAbs designated as II and IV did not recognised these peptides, but recognised respectively β-CN (f33-97) and β-CN (f177-209). Tentative determinants appeared as 33-48 and 177-184 respectively. However, it is possible that the residues 49-52 and 184-187 had been too involved in their epitopes.

Reactivity with alien sequence variants

In ACP-ELISA, no MAb discriminated the casein from goat and sheep, nor that from cow and cow-buffalo. Each MAb detected casein from at least one non-bovine specie. They were discriminated into eight classes by their specificity towards ovine, equine and human casein (Fig. 1). The number of MAbs cross-reacting with ovine (20), human (7), and with both (6) decreased with decreasing sequence homology (respectively 91%, 53% and 51%). MAb I-1 reacted only faintly with equine and human casein.

Epitope delineation

The sequences of cow, buffalo, ewe and human β-CN were compared for the substituted residues within the plasmin-cleaved peptide antigens. The sequence for equine β-CN has not been reported, but a partial sequence for Zebra is available, which is likely very close. Four determinants, 4-8, 14-24, 177-183 and 184-190 could be delineated without ambiguity, and probable sites of binding were refined for other MAbs (Fig. 2). Residues critical to the epitope of MAb IV were precisely identified at position 180-181. Similarly to MAb IV, MAb V-1 also recognised horse sequence, and was thus attributed an adjacent epitope, i.e. 184-190. MAb I-2 was assigned to the sequence 4-8, common to ruminants. MAbs I-4,6 and MAbs I-3 and I-7 were attributed to the conserved domain 14-24, common to human and

ruminants, and partially shared by equine. MAbs I-3 and I-7 recognised however an epitope not common to equine casein, and thus included at least one unidentified mutated residue.

BIAcore epitope mapping

In BIAcore assay, capture of β-CN by any MAb from group I, II or III prevented totally the further binding of all MAbs from the same group. Within the remaining MAbs IV and V, assayed pair-wise, non-interfering binding to β-CN was observed for MAb IV, MAb V-2 and a set composed of MAbs V-1, V-4 and V-5, which mutually interfered (Table 3).

Capture of β-CN by immobilised IgG monoclonal antibodies

The MAbs assayed in biosensor were rated for their ability to capture β-CN in solution (Table 4). MAb I-1 achieved 91% of the optimal monovalent binding. In contrast, MAbs III-1 and III-3 did not bind β-CN, whereas they did in the ACP-ELISA screening. These MAbs were apparently directed against β-CN epitopes specifically exposed upon conformation changes subsequent to coating. The contrasted capture ability discriminated markedly sub-groups of MAbs within the groups I to V.

DISCUSSION

Previous constructions of hybridoma against β-CN relied on intra-peritoneal immunisation, and each highlighted apparently a single dominant epitope, though clear data often lack. MAb 1C3 recognises β-CN (f193-202) (Nagaune *et al.*, 1988), whereas three MAbs CAS-OT# recognise redundantly β-CN (f105-193) (Oudshorn *et al.*, 1994). In addition, MAb AH4 recognises an unidentified bovine-specific epitope (Anguita *et al.*, 1995), and five MAbs named 58-# recognise an identical unknown epitope (Kuzmanoff *et al.*, 1991). According to Mirza *et al.* (1987), footpad route increases the positive clone rate (100% *vs* 8%), the specificity spectrum, and particularly emphasises conserved epitopes. Using this protocol, we achieved only a low positive clone rate, possibly owing to low intrinsic immunogenicity of β-CN, but we elicited an unprecedented panel of specificity. A single IgA was obtained. IgA may be obtained in mice specifically upon oral immunisation route and/or collection of gut associated lymphocytes, and seldom result from other protocols. Oral immunisation probably occurred, either previously to the primary injection, *via* the diet, or simultaneously, by casual ingestion of the immunogenic mixture. Dietary β-CN digests enhances class-switch towards IgA in mice, possibly *via* cytokine(s) stimulation (Otani *et al.*, 2000).

All MAbs recognised β-CN fragments, which indicated they recognised continuous epitopes. Typically 10% of MAbs raised to globular proteins recognise peptides (Pellequer *et al.*, 1991), which was observed for bovine β-Lg (~36 kDa) (Venien *et al.*, 1997). The striking difference for β-CN (100% *vs* 10%) accounts probably for the high proportion of aperiodic structure (70% *vs* 45%), and the lack of stable tertiary structure in β-CN. Owing to the generalised cross-reactivity with peptides, all the continuous epitopes could be circumscribed.

Seven antigenic areas were defined in ELISA assays, including three individual epitopes, tentatively delineated as 4-8, 178-183 and 185-190, and four larger determinants, defined as 14-24, 33-48, 49-94 and 184-209. The

determinant 14-24 was also found to contain two epitopes at least, one common to all assayed species, and the other differing in equine sequence. The eight corresponding groups of MAbs were further split into 13 epitopic classes according to (i) the additivity assays and (ii) the contrasted rates of β-CN capture, in BIAcore assays. The determinants 14-24 and 49-94 were found to contain at least respectively 4 and 2 distinct but probably overlapping epitopes. The determinant 184-209 contained three distinct epitopes probably overlapping and one non-overlapping one (V-2).

The fusion process immortalises only a restricted population of the collected lymphocytes that constitute the individual immune response, typically 10^{-5}-10^{-6}. Moreover, Otani *et al.* (1987) have demonstrated that individual responses of rabbits immunised with β-CN varied dramatically. The specificity panel we achieved sums the responses of six mice and thus features a cross section of β-CN immunogenic determinants. Otani *et al.* have investigated β-CN immunogenicity by the analysis of individual rabbit polyclonal antibodies raised either to bovine β-CN (1984; 1987) ot to β-CN digest (1990). They have identified discrete antigenic determinants at the sequences 1-25, 26-60, 61-93, 94-102, 103-109, 110-144 and 157-185. In addition, they have found the peptide β-CN (f186-209) immunogenic. Our experiments failed to detect determinants in the sequence 94-144. This discrepancy may/might be originated in the different host we used (mouse *vs* rabbit). The comparison of the sequence homology for the three species at 94-144 does/does not support this hypothesis. Alternatively, a bias should be considered in our collection. The screening step relied exclusively on ACP-ELISA with coated β-CN, and we may have failed to detect some antibodies specific for a native form. As much as 7% of specific MAbs non-reactive in ACP ELISA with β-Lg have been reported by Venien *et al*. (1997). β-CN is dramatically less organised than β-Lg, as was illustrated by the lower rate of ELISA specific MAbs we obtained (10% *vs* 25%). It is thus likely that a lesser rate, if any, of such MAbs would be elicited against β-CN.

The determinant 14-24, recognised by MAbs I-3,7, coincides with the phosphorylated cluster. This sequence has been found a potent determinant common to α_S- and β- casein in immunised mice, and in allergic humans, and its reactivity has been shown to depend upon proper state of phosphorylation (Bernard *et al.*, 2000). No cross-reactivity with α_S-CN was observed for MAbs I-3,7. The determinant 3-12 has not been found dependant upon phosphorylation (Pizzano *et al*., 2000). It would be interesting to investigate the reactivity of MAbs I-1,7 with dephosporylated species, since phosphatases are acting in cheese (Ferranti *et al*., 1997a).

β-CN terminal sequences 1-25 and 184-209 represented each ~12% of the sequence in length. They carried each 5 out of the 13 distinct determinants (~38%) we identified, and particularly carried all the epitopes conserved in two or more non-bovine species. Stewart *et al.* (1987) reported that the N-terminal domain 1-50 was highly conserved in most species (including cow, human, rat, ewe), in particular at the phosphorylated cluster, but that the C-terminus was highly variable. It is now a widespread affirmation that protein termini are highly immunogenic. This opinion arises partly from the confusion of immunogenicity for the reactivity towards antipeptide antibodies. This characteristic implies a structural similarity with the peptide, and has thus been correlated with flexibility and/or surface exposure in globular

proteins (Pellequer *et al.*, 1991). These parameters are favourable in the N-terminus, and all three immunogenicity scales based on hydrophilicity, solvent accessibility, and statistical propensity predicted the determinant 14-24. On the contrary, these parameters are not favourable in the C-terminus, and all three scales failed to predict the epitope 193-209. The scales were generally not efficient for predicting β-CN determinants: the determinants 14-24 and 33-49 were predicted by all three scales, the epitope 177-184 only by solvent accessibility and statistical propensity, and the determinant 4-8 only by hydrophilicity scale. Conversely, all three predicted a consensual epitope in the area 100-110, that we did not observed, whereas Otani *et al.* did (1987).

The produced panel of specificity enables the specific detection of fragments of β-CN released by plasmin, i.e. β-CN (f1-28), β-CN (f29-105/-107), and β-CN (f106/108-209). The immunodetection of these peptides should help in objective assessment of plasmin contribution to proteolysis in cheese. In particular, phosphopeptides may be potential indexes for cheese ageing certification. Pizzano *et al*. (2000) reported that the determinant 3-12 was rapidly degraded in cheese, and thus useless as a marker. In contrast, the peptide β-CN (f16-22) accumulates in hard-type cheeses, and is therefore a good candidate as an ageing index (Ferranti *et al.*, 1997a). Five MAbs from group I are directed at four distinct epitopes within 14-24, and may enable its immunometrical detection in the pH 4·6 soluble fraction of cheeses. In other respects, considerable efforts have been devoted to the production of bovine specific reagents suitable to detect minute adulteration of ovine or caprine milk in cheeses made thereof (Dupont ????). Antibodies directed at caseins include only one MAb, raised against an unidentified epitope of β-CN (Anguita *et al*., 1995). MAb IV might prove useful in this issue, since its epitope is a good marker for γ-caseins that accumulate upon degradation of β-CN in cheese, and might therefore be detectable in extensively ripened cheeses (Pizzano *et al*., 1999).

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FIGURE LEGENDS

Fig. 1. Distribution of 21 anti bovine β-casein MAbs according to their reactivity with ovine, human and equine whole casein in ACP-ELISA. Group size when >1 is given in brackets.

Fig. 2. Tentative epitopes of specific anti bovine β-casein MAbs from 7 reactivity classes as determined from their reactivity with tryptic fragments, buffalo, ovine and human sequence variants in ACP-ELISA.

1 **Table 1**. *Outcome of three independent fusion experiments of activated murine lymphocytes*

	fusion	growing /lymphocytes	positive /hybrids	isotype					
				G1	G2a	G3	M	A	κ
1	mass	$1{\cdot}1.10^{-6}$	6·0%	4				1	5
2	filter	$1{\cdot}9\ .10^{-6}$	6·6%		5	1	1		7
3	filter	$3{\cdot}3\ .10^{-6}$	5·9%	9					9

2

3

Table 2. *Mapping of bovine β–casein antigenicity in ACP-ELISA towards 21 specific MAbs*

molecular mass (Da)		peptide	reactive MAb group §					nb
measured†	theoretical‡		I	II	III	IV	V	
3122·73±0·1	3123·0	β-CN -4P(f1-25)	+					7
7344.96.±1.0	7346.2	β-CN -1P(f33-97)		+	+			8
5302·17±0·6	5301·2	β-CN (f49-97)			+			5
3722·6 ± 0·1	3722·5	β-CN (f177-209)				+		6
2910·9 ± 0·9	2910·5	β-CN (f184-209)				+	+	5

† average molecular mass ± SD calculated for multiple multiprotonated ions in RP-HPLC analysis coupled to ESI-MS detection of hydrolysed β-CN

‡ average molecular mass calculated according to the sequence of probable match (Jimenez-Flores *et al.,* 1987)

§ classification of reactive MAbs in ACP-ELISA

Table 3. *Interfering (greyed) or non-interfering pair-wise binding to bovine β-casein of specific MAbs with close epitopes. Binding is expressed as a molar % of maximal capture of the secondary MAb.*

MAb	captured				
immobilised	IV	V-2	V-1	V-4	V-5
IV	-				
V-2	*nd*	-		*nd*	
V-1	+	+	-		
V-4	+	+	-	-	
V-5	+	+	-	-	-

nd : non determined

Table 4. *Capture of β–casein (molar % of Ig) by immobilised specific IgG MAbs in biosensor assay*

MAb	% Ig	MAb	% Ig	MAb	% Ig	MAb	% Ig
I-1	91	II-2	17	III-4	31	V-1	78
I-7	39	II-3	11	III-2	21	V-5	41
I-2	35			III-5	21	V-2	31
I-4	28			III-1	0	V-4	6
I-5	23			III-3	0		
I-6	19						
I-3	9			IV	12		

MAb	% Ig	MAb	% Ig	MAb	% Ig
I-1	91	II-2	17	IV	12
I-7	39	II-3	11	V-1	78
I-2	35	III-4	31	V-5	41
I-4	28	III-2	21	V-2	31
I-5	23	III-5	21	V-4	6
I-6	19	III-1	0		
I-3	9	III-3	0		

FIGURES