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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RUN TITLE: Monoclonal antibodies against bovine β-casein

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2   SUMMARY. Twenty one murine monoclonal antibodies (MAbs) were produced against bovine β-casein (β-CN). Eight

3   epitopic groups of MAbs recognising six discrete determinants (4-8, 14-24, 33-48, 49-91, 177-183, 184-209) were

4   defined from ELISA reactivity with peptides and related homologues from 3 species (ovine, human and equine).

5   Thirteen distinct epitopes were discriminated by cross-examination of the MAbs reactivity in BIAcore. Determinants 14-

6   24, 49-91 and 184-209 were found to encompass at lest 4, 2 and 4 epitopes respectively. No MAb was bovine

7   specific, but one at least discriminated ovine/caprine casein from bovine. Terminal sequences 1-28 and 177-209

8   included ~80% of the discriminated epitopes, and the most conserved ones. This panel of MAbs can provide specific

9   probes for β-CN proteolysis fragments.

10

1   The caseins make up the major protein fraction of bovine milk (~80%). They represent the products of 4 linked

2   genes, the caseins α_{S1}, a_{S2}, β and κ. Bovine casein β (β-CN) accounts for ~40% of the casein content. Its primary

3   structure is known from chemical and gene sequencing (Swaisgood, 1992). It consists of 209 residues (~24000 Da),

4   with phosphorylated serine residues clustered at N-termini (5 mole P/mole). It occurs in milk as a mixture of several

5   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

6   substitutions have been fully characterised, and variants A^1, A^2, and B account for the large majority of Western cattle

7   heterogeneity. None was found to arise from post-translational modification, although substitutions in variants C and D

8   cause defect of phosphorylation. Homologue β-casein was found in milks of all species. Its sequence in human, sheep,

9   rat, mouse, rabbit and other species indicates a rapid divergence arising from point mutations, without segmental

10   rearrangements (Stewart *et al.*, 1987). Bovine β-CN differs from the other closely related caseins by (i) a higher rate of

11   hydrophobic residues, (ii) a low phosphorylation rate and (iii) a high proline content (17%). Its secondary structure

12   reflects these characteristics (Swaisgood, 1992). Experimental and theoretical data agree on a low rate of periodic

13   structure (30%), especially α-helix (10%), but many proline-induced β-turns (34%)(Kumosinski *et al.*, 1993). Owing to

14   the impossibility to crystallise the molecule, secondary and tertiary structure (Kumosinski *et al.*, 1993) could be only

15   predicted. All data suggest an organisation opposite to that of typical globular proteins: conserved domains with strictly

16   defined structure occur at both termini, and are linked by an open and flexible intermediate region.

17   The products of β-CN cleavage in milk reflect both its structure and the specificity of the native enzymes. The main

18   native protease, plasmin (EC 3.4.21.7), cleaves apart the hydrophilic and heat resistant domain β-CN (f1-28), the

19   intermediate region β-CN (f29-105/-107) and the hydrophobic C-termini β-CN (f106/108-209). These major fragments

20   occur in large amount in fresh milk (typically 10-20% of β-CN in weight) (Andrews, 1983; Andrews & Alichanidis, 1983).

21   Plasmin is associated with casein micelles, and is incorporated in dairy foods, *e.g.* cheeses, where it contributes

22   significantly to the induced proteolysis. The contribution of plasmin to β-CN proteolysis is thought to significantly

23   increase the release of peptides and hydrophobic amino acids in the sapid fraction of cheeses. The objective

24   monitoring of plasmin action could contribute to modelling desirable pathways of flavour development. Biochemical

25   fractionation of peptides is complex, and is not specific for β-CN (Ferranti *et al.*, 1997b). In contrast, immunochemical

26   detection has a great potential for descriptive analysis of proteolysis of specific casein fractions (Addeo *et al.*, 1995).

27   Provided that specific probes are produced, the degradation of β-CN segments can be monitored and quantified

28   (Pizzano *et al.*, 2000). Specific antibodies are currently raised against peptides (Pizzano *et al.*, 1999), although the

29   reactivity of anti-peptide antibodies with the intact parent protein is ambiguous. Moreover, these reagents come in

30   limited supply, and may not be reproduced. In contrast, hybridoma technology (Köhler & Milstein, 1975) allows the

31   continuous production of monospecific antibodies, which corresponding epitopes might be investigated in detail

32   (Kobayashi *et al.*, 1991). β-CN presents many β-turn, and a high interspecies divergence which should contribute to its

1   immunogenicity. Four previous studies reported however only four specific MAbs (Nagaune *et al.*, 1988; Kuzmanoff *et*

2   *al.*, 1991; Oudshorn *et al.*, 1994; Anguita *et al.*, 1995)., and thus supported the current opinion that caseins are poorly

3   immunogenic. Since the MAbs raised to a protein usually vary in specificity and reactivity, a large collection is

4   preferable to select probes with pre-requisite characteristics. The objective of this study was to produce a large panel

5   of characterised MAbs against β-CN, as probes for its plasmin-induced fragments. Twenty-one murine MAbs were

6   compared for isotypes, ELISA reactivity with proteins and peptides, and reactivity with β-CN in BIAcore. Thirteen non-

7   redundant MAbs bound six discrete determinants distributed along β-CN sequence, with a marked bias in favour of the

8   termini.

9

1	**MATERIALS AND METHODS**
2	*Reagents*
3	Unless otherwise stated, chemicals of analytical grade were purchased from Prolabo (Merck eurolab, D-64293
4	Darmstadt, Germany). BIAcore 3000 was used with registered chemicals (BIAcore AB, S-75450 Uppsala, Sweden).
5	Inactive plasminogen was isolated from fresh bovine blood according to the procedure of Deutsch & Mertz (1970),
6	and frozen at -20°C. Immediately prior to use, aliquots (70 g/l) were treated for 10 min at 37°C with urokinase (EC
7	3.4.21.73) (Choay, Sanofi Winthrop) at an enzyme:substrate ratio of 100 UI/mg, and thus activated to plasmin (EC
8	3.4.21.7).
9	
10	*β-casein isolation*
11	Individual milk was obtained from the genetically typed () herd of the Coopérative agricole Graindorge (14140
12	Livarot, France). β-CN from a cow typed as homozygous for β-CN A^2 was analysed in electrospray ionisation mass-
13	spectroscopy (ESI-MS) according to Léonil *et al.* (1995). It was found in agreement with the variant reported by
14	Jimenez-Flores *et al.* (1987), and its primary sequence (A^2 substituted for leucine at position 93) was used.
15	Milk fat was removed after 15 min centrifugation (1800 g, 4°C). Whole casein was obtained from defatted milk by
16	isoelectric precipitation at pH 4·6 according to the procedure of Wei & Whitney (1984).
17	Crude β-CN was obtained in gram amounts by the batch-wise ion exchange procedure of Leaver & Law (1992),
18	dialysed against water, and lyophilised. The purity determined on urea-polyacrylamide gel electrophoresis (PAGE)
19	(Andrews, 1983) was ~ 90%. Crude β-CN was purified to homogeneity by the fast protein liquid chromatography
20	(FPLC) ion exchange procedure of Andrews *et al.* (1985), scaled to fit a HR16/10 column (Pharmacia Biotech. AB, S-
21	75450 Uppsala, Sweden). Briefly, 600 mg of crude β-CN were loaded onto 20 ml of Q Sepharose FF (Pharmacia), and
22	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,
23	pH 7·0. Fractions automatically collected over 5 min were checked in urea-PAGE for homogeneity, pooled, dialysed
24	against water, and lyophilised.
25	
26	*Other antigens*
27	Individual mare's milk was obtained from the farm of the Lycée Agricole (21140 Semur-en-Auxois, France). Mare's
28	whole casein was prepared like bovine's. β−CN from cow buffalo's milk (*Bubalus bubalis*) was a gift from D. Lebars
29	(INRA, 78352 Jouy-en-Josas, France). Whole casein from cow's milk was purchased from Merck, that from ewe, goat,
30	and human milk from Sigma (Sigma-Aldrich, 38297 St.Quentin-Fallavier, France), and bovine serum albumin (BSA)
31	from Pierce (Interchim, 03103 Montluçon, France). Other bovine proteins were isolated from bovine bulk milk
32	according to the following chromatographic procedures: α-Lactalbumin (α-La) and β-Lactoglobulin (β-Lg) according to

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

2 to Levieux (1974).

3

4 *Mapping of β-casein antigenicity*

5 *β–casein hydrolysis.* Plasmin was used to perform limited cleavage of β-CN, specific for the bonds lys-X and arg-X.

6 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

7 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

8 and 8 hours. The reaction was stopped by boiling for 10 min at 100°C. Prior to chromatographic separation,

9 hydrolysates were diluted with an equal volume of trifluoroacetic acid (TFA) 1·05 ml/l 8 M-urea buffer and filtered

10 through 0·45 μm.

11 *Reverse phase-high performance liquid chromatography (RP-HPLC) fractionation.* β-CN hydrolysates were

12 fractionated on a C_{18} Zorbax column (4·6x150 mm, 300 Å, 3·5 μm, Agilent Technologies, Interchim), as described by

13 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

14 linear gradient of acetonitrile over ~2-22 % volume, with TFA at 1ml/l as a modifier. Peptides were detected by u.v.

15 absorbance (214 nm), and identified by on-line ESI-MS analysis as described by Léonil *et al.* (1995). Resolved

16 homogenous peaks were collected from further injections of 20 nmoles of hydrolysed β-CN and vacuum-dried at 30°C.

17 Peptides were designated according to the recommended nomenclature (Eigel *et al.*,1984).

18

19 *Production of monoclonal antibodies*

20 Monoclonal antibodies were produced essentially as described by Jeanson *et al.* (1999). Immunisation with 20 μg

21 of purified β-CN was carried out by the footpad route (Mirza *et al.*, 1987). For each experiment, popliteal lymph nods

22 from two immunised mice were pooled. Lymphocytes were fused in a ratio of 5:1 to Sp2/O-Ag-14 myeloma according

23 to two procedures. Essentially, in mass fusion (Köhler & Milstein, 1975) polyethylene glycol (PEG 1000, Merck) (450g/l

24 water) was drop-wise added onto pellets of mixed cells, whereas in filter fusion (Buttin *et al.*, 1978), it was allowed to

25 permeate through monolayers of mixed cells adsorbed on membrane filters. Supernatant media of log phase

26 hybridoma culture were screened in enzyme linked immunosorbent assay (ELISA) for Ig binding to β-CN.

27

28 *ELISA*

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

30 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,

31 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,

32 human, ewe, goat and mare) (1 mg/l CB) were coated simultaneously. For antigenicity mapping, peptides initially

1   dissolved in 1 ml 0·05 M-phosphate buffer–0·15 M-NaCl, pH 7·2 (PBS), were coated at the optimised dilution that

2   produced similar detection signal as β-CN (0·5 mg/l CB). Aliquots of 100 µl antigens were incubated, for two hours at

3   37°C, in wells of a flat-bottomed 96-well microtitration plate (Nunc Maxisorp, Polylabo 67023 Strasbourg, France). The

4   remaining binding sites were blocked by incubating 250 µl gelatine (Merck) (10 g/l PBS-T buffer, i.e. Tween 20 (Merck)

5   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

6   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.

7   For the following assays, dilution was optimised whenever possible as to produce a ~2·0 units absorbance increase in

8   screening conditions. Bound Ig were detected by incubating 100 µl of goat anti mouse immunoglobulin alkaline

9   phosphatase conjugate (0·33 ml/l PBS-T) for one hour at 37°C in the wells (Jackson Immunoresearch, Interchim).

10   Wells were rinsed between each incubation step for 15 s with four changes of 250 µl PBS-T. Following the last rinsing,

11   100 µl *p*-nitrophenyl phosphate (1 g/l 1 M-diethanolamine-HCl–1 mM-MgCl$_2$–0·1 mM-Zinc acetate) (Sigma) were

12   incubated in the wells. After one hour, the absorbance at 405 nm was read against a blank, and corrected for

13   background signal in the absence of antigen. Threshold for alien casein recognition was set at 4% of bovine signal. All

14   assays, except screening, were performed in duplicate

15   *Isotyping.* Light and heavy Ig chains were specifically detected in screening conditions using secondary antibodies

16   picked from the Kit 37502 from Pierce (Interchim).

17

18   *Biosensor assay using BIAcore 3000*

19   IgG MAbs were assayed for reactivity with β-CN using BIAcore 3000 (Fägerstam *et al.*, 1992). Kinetics

20   measurement were performed from single runs. Additive assays were performed without labelling antibodies using on-

21   line detection (Daiss & Scalice, 1994).

22   *Capture assay.* The carboxy-methylated dextran surface (CM5) was conditioned for immobilisation of IgG MAbs as

23   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

24   rabbit anti-mouse Fc γ (30 mg/l 10 mM-sodium acetate buffer, pH 4·8), and 20 µl of 1 M-ethanolamine hydrochloride,

25   pH 8·5 solution were injected in sequence at 5 µl/min. Undiluted supernatants were injected at 10 µl/min for 3 min.

26   Unoccupied binding sites of the matrix were blocked with IgG1, IgG2 and IgG3 from mixed mice ascitic fluids non-

27   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-

28   NaCl, pH 7·4) was injected at 10 µl/min for 3 min. The total mass immobilised on the sensor surface was recorded on-

29   line throughout all steps as a proportional increase of the surface plasmonic resonance. For comparison of β-CN

30   capture, resonance was read at 3 min after injection of IgG and β-CN. The mass ratios of bound antigen and antibody

31   were corrected to molar ratio using the following Mw values: 146 kDa for IgG1 and G2, 170 kDa for IgG3 and 24 kDa

32   for β-CN.

1   *Pair-wise sandwich assay.* MAbs were pair-wise assayed within the reactivity groups established for tryptic

2   mapping (Table 2), with single MAb IV pooled with group V. Primary MAb was assayed for capture of β-CN, then

3   secondary MAb was injected for binding to captured casein in the same conditions.

4

5   *Sequence analysis*

6   Primary sequence of β-CN from ewe, goat, human, cow-buffalo, cow, and zebra (*Equus grevyi*) (respectively n°

7   P11839, P33048, P05814, Q9T5I0, P02666, and Q28401 in SwissProt-trEMBL) were compared with free software

8   Multalin 5.4.1 (INRA, 31326 Toulouse, France) set on default parameters. Sequence for bovine β-CN was corrected

9   according to Jimenez-Flores *et al.* (1987). Percentage homology was calculated as aligned residues matching

10   bovine's. Scales for hydrophilicity (Hopp & Woods), solvent accessibility (Boger *et al.*) and antigenicity (Parker *et al.*)

11   were calculated from primary structure using free release ANTHEPROT V4.9 (IBCP-CNRS UPR 412, 69367 Lyon,

12   France) with default parameters and five residues window.

13

1 **RESULTS**

2 *Production of hybridoma*

3 Table 1 features the outcome of three independent fusion experiments carried each with a pair of mice. 21

4 hybridoma secreting anti β-CN Ig were produced. All carried κ light chains, whereas several heavy chain classes (G1,

5 G2a, G3, M and A) were found. One experiment (filter protocol) yielded IgG MAbs of sub-classes exclusively 2a and 3,

6 whereas other experiments yielded a marked majority of sub-classes 1.

7 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

8 displaying the highest levels of cross-reactivity were found directed at β-CN C-terminus (group V). They frequently

9 recognised multiple test antigens, which suggested non-specific binding rather than casual sequence homology.

10 Systematic cross-reaction with β-Lg originated apparently from contamination of the test antigen solution with β-CN.

11 *Mapping of β−casein antigenicity*

12 The MAbs were assigned to five groups (I to V) according to their reactivity with 5 fragments of β-CN in ACP-ELISA

13 (Table 2). HPLC fractions containing peptides shorter than 25 residues were not detected. The epitopes recognised by

14 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)

15 respectively. MAbs designated as II and IV did not recognised these peptides, but recognised respectively β-CN (f33-

16 97) and β-CN (f177-209). Tentative determinants appeared as 33-48 and 177-184 respectively. However, it is possible

17 that the residues 49-52 and 184-187 had been too involved in their epitopes.

18 *Reactivity with alien sequence variants*

19 In ACP-ELISA, no MAb discriminated the casein from goat and sheep, nor that from cow and cow-buffalo. Each MAb

20 detected casein from at least one non-bovine specie. They were discriminated into eight classes by their specificity

21 towards ovine, equine and human casein (Fig. 1). The number of MAbs cross-reacting with ovine (20), human (7), and

22 with both (6) decreased with decreasing sequence homology (respectively 91%, 53% and 51%). MAb I-1 reacted only

23 faintly with equine and human casein.

24 *Epitope delineation*

25 The sequences of cow, buffalo, ewe and human β-CN were compared for the substituted residues within the

26 plasmin-cleaved peptide antigens. The sequence for equine β-CN has not been reported, but a partial sequence for

27 Zebra is available, which is likely very close. Four determinants, 4-8, 14-24, 177-183 and 184-190 could be delineated

28 without ambiguity, and probable sites of binding were refined for other MAbs (Fig. 2). Residues critical to the epitope of

29 MAb IV were precisely identified at position 180-181. Similarly to MAb IV, MAb V-1 also recognised horse sequence,

30 and was thus attributed an adjacent epitope, i.e. 184-190. MAb I-2 was assigned to the sequence 4-8, common to

31 ruminants. MAbs I-4,6 and MAbs I-3 and I-7 were attributed to the conserved domain 14-24, common to human and

1 ruminants, and partially shared by equine. MAbs I-3 and I-7 recognised however an epitope not common to equine

2 casein, and thus included at least one unidentified mutated residue.

3 *BIAcore epitope mapping*

4 In BIAcore assay, capture of β-CN by any MAb from group I, II or III prevented totally the further binding of all MAbs

5 from the same group. Within the remaining MAbs IV and V, assayed pair-wise, non-interfering binding to β-CN was

6 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).

7 *Capture of β-CN by immobilised IgG monoclonal antibodies*

8 The MAbs assayed in biosensor were rated for their ability to capture β-CN in solution (Table 4). MAb I-1 achieved

9 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-

10 ELISA screening. These MAbs were apparently directed against β-CN epitopes specifically exposed upon conformation

11 changes subsequent to coating. The contrasted capture ability discriminated markedly sub-groups of MAbs within the

12 groups I to V.

13

14 **DISCUSSION**

15 Previous constructions of hybridoma against β-CN relied on intra-peritoneal immunisation, and each highlighted

16 apparently a single dominant epitope, though clear data often lack. MAb 1C3 recognises β-CN (f193-202) (Nagaune *et*

17 *al.*, 1988), whereas three MAbs CAS-OT# recognise redundantly β-CN (f105-193) (Oudshorn *et al.*, 1994). In addition,

18 MAb AH4 recognises an unidentified bovine-specific epitope (Anguita *et al.*, 1995), and five MAbs named 58-#

19 recognise an identical unknown epitope (Kuzmanoff *et al.*, 1991). According to Mirza *et al.* (1987), footpad route

20 increases the positive clone rate (100% *vs* 8%), the specificity spectrum, and particularly emphasises conserved

21 epitopes. Using this protocol, we achieved only a low positive clone rate, possibly owing to low intrinsic

22 immunogenicity of β-CN, but we elicited an unprecedented panel of specificity. A single IgA was obtained. IgA may be

23 obtained in mice specifically upon oral immunisation route and/or collection of gut associated lymphocytes, and

24 seldom result from other protocols. Oral immunisation probably occurred, either previously to the primary injection, *via*

25 the diet, or simultaneously, by casual ingestion of the immunogenic mixture. Dietary β-CN digests enhances class-

26 switch towards IgA in mice, possibly *via* cytokine(s) stimulation (Otani *et al.*, 2000).

27 All MAbs recognised β-CN fragments, which indicated they recognised continuous epitopes. Typically 10% of MAbs

28 raised to globular proteins recognise peptides (Pellequer *et al.*, 1991), which was observed for bovine β-Lg (~36 kDa)

29 (Venien *et al.*, 1997). The striking difference for β-CN (100% *vs* 10%) accounts probably for the high proportion of

30 aperiodic structure (70% *vs* 45%), and the lack of stable tertiary structure in β-CN. Owing to the generalised cross-

31 reactivity with peptides, all the continuous epitopes could be circumscribed.

32 Seven antigenic areas were defined in ELISA assays, including three individual epitopes, tentatively delineated as

33 4-8, 178-183 and 185-190, and four larger determinants, defined as 14-24, 33-48, 49-94 and 184-209. The

1 determinant 14-24 was also found to contain two epitopes at least, one common to all assayed species, and the other

2 differing in equine sequence. The eight corresponding groups of MAbs were further split into 13 epitopic classes

3 according to (i) the additivity assays and (ii) the contrasted rates of β-CN capture, in BIAcore assays. The determinants

4 14-24 and 49-94 were found to contain at least respectively 4 and 2 distinct but probably overlapping epitopes. The

5 determinant 184-209 contained three distinct epitopes probably overlapping and one non-overlapping one (V-2).

6   The fusion process immortalises only a restricted population of the collected lymphocytes that constitute the

7 individual immune response, typically 10^{-5}-10^{-6}. Moreover, Otani *et al.* (1987) have demonstrated that individual

8 responses of rabbits immunised with β-CN varied dramatically. The specificity panel we achieved sums the responses

9 of six mice and thus features a cross section of β-CN immunogenic determinants. Otani *et al.* have investigated β-CN

10 immunogenicity by the analysis of individual rabbit polyclonal antibodies raised either to bovine β-CN (1984; 1987) ot to

11 β-CN digest (1990). They have identified discrete antigenic determinants at the sequences 1-25, 26-60, 61-93, 94-102,

12 103-109, 110-144 and 157-185. In addition, they have found the peptide β-CN (f186-209) immunogenic. Our

13 experiments failed to detect determinants in the sequence 94-144. This discrepancy may/might be originated in the

14 different host we used (mouse *vs* rabbit). The comparison of the sequence homology for the three species at 94-144

15 does/does not support this hypothesis. Alternatively, a bias should be considered in our collection. The screening step

16 relied exclusively on ACP-ELISA with coated β-CN, and we may have failed to detect some antibodies specific for a

17 native form. As much as 7% of specific MAbs non-reactive in ACP ELISA with β-Lg have been reported by Venien *et*

18 *al.* (1997). β-CN is dramatically less organised than β-Lg, as was illustrated by the lower rate of ELISA specific MAbs we

19 obtained (10% *vs* 25%). It is thus likely that a lesser rate, if any, of such MAbs would be elicited against β-CN.

20   The determinant 14-24, recognised by MAbs I-3,7, coincides with the phosphorylated cluster. This sequence has

21 been found a potent determinant common to α_S- and β- casein in immunised mice, and in allergic humans, and its

22 reactivity has been shown to depend upon proper state of phosphorylation (Bernard *et al.*, 2000). No cross-reactivity

23 with α_S-CN was observed for MAbs I-3,7. The determinant 3-12 has not been found dependant upon phosphorylation

24 (Pizzano *et al.*, 2000). It would be interesting to investigate the reactivity of MAbs I-1,7 with dephosporylated species,

25 since phosphatases are acting in cheese (Ferranti *et al.*, 1997a).

26   β-CN terminal sequences 1-25 and 184-209 represented each ~12% of the sequence in length. They carried each 5

27 out of the 13 distinct determinants (~38%) we identified, and particularly carried all the epitopes conserved in two or

28 more non-bovine species. Stewart *et al.* (1987) reported that the N-terminal domain 1-50 was highly conserved in most

29 species (including cow, human, rat, ewe), in particular at the phosphorylated cluster, but that the C-terminus was

30 highly variable. It is now a widespread affirmation that protein termini are highly immunogenic. This opinion arises

31 partly from the confusion of immunogenicity for the reactivity towards antipeptide antibodies. This characteristic implies

32 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).

REFERENCES

ADDEO, F., GARBO, G., INTORCIA, N., PELLEGRINO, L., RESMINI, P. & CHIANESE, L. 1995 Gel electrophoresis and immunoblotting for the detection of casein proteolysis in cheese. *Journal of Dairy Research* **62** 297-309

ANDREWS, A. T., TAYLOR, M. D. & OWEN, A. J. 1985 Rapid analysis of bovine milk proteins by fast protein liquid chromatography. *Journal of Chromatography* **348** 177-185

ANDREWS, A., T. & ALICHANIDIS, E. 1983 Proteolysis of caseins and the proteose-peptone fraction of bovine milk. *J. Dairy Res.* **50** 275-290

ANDREWS, A., T. 1983 Proteinases in normal bovine milk and their action on caseins. *Journal of Dairy Research* **50** 45-55

ANGUITA, G., MARTIN, R., GARCIA, T., MORALES, P., HAZA, A. I., GONZALES I., SANZ, B. & HERNANDEZ, P. 1995 Indirect ELISA for detection of cow's milk in ewe's and goat's milks using a monoclonal antibody against bovine β-casein. *Journal of Dairy Research* **62** 655-659

BERNARD, H., MEISEL, H., CREMINON, C. & WAL, J.M. 2000. Post-translational phosphorylation affects the IgE binding capacity of caseins. *FEBS Letters* **467** 239-244

BEUVIER, E., BERTHAUD, K., CEGARRA, S., DASEN, A., POCHET, S., BUCHIN, S. & DUBOZ, G. 1997 Ripening and quality of swiss-type cheese made from raw, pasteurised or microfiltered milk. *International Dairy Journal* **7** 311-323

BUTTIN, G., LEGUERN, G., PHALENTE L., LIN, E. C. C., MEDRANO, L. & CAZENAVE, P. A.01978 Production of hybrids lines secreting antiidiotypic antibodies by cell fusion on membrane filters. *Current Topics in Microbiology and Immunology* **81** 27-36

COLLIN, J. C., KOKELAAR, A., ROLET-REPECAUD, O. & DELACROIX-BUCHET, A. 1991 [Determination of cow's milk caseins by electrophoresis and fast protein liquid ion-exchange chromatography (FPLC®): comparative results] *Lait* **71** 339-350

DAISS, J. L. & SCALICE, E. R. 1994 Epitope mapping on BIAcore: theoretical and practical considerations. *METHODS : a companion to Methods in Enzymology* **6** 143-156

DEUTSCH, D. G. & MERTZ, E. T. 1970 Plasminogen: purification from human plasma by affinity chromatography. *Science* **170** 1095-1096

EIGEL, W. N., BUTLER, J. E., ERNSTROM, C. A.; FARRELL, H. M., HARWALKAR, V. R., JENNESS, R. & WHITNEY, R. McL. 1984 Nomenclature for proteins of cow's milk : fifth revision. *Journal of Dairy Science* **67** 1599

FÄGERSTAM, L. G., FROSTELL-KARLSSON, Å., KARLSSON, R., PERSSON, B. & RÖNNBERG, I. 1992 Biospecific interaction analysis using surface plasmon resonance detection aplied to kinetic, binding site and concentration analysis. *Journal of Chromatography* **597** 397-410

FERRANTI, P., BARONE, F., CHIANESE, L., ADDEO, F., SCALONI, A., PELLEGRINO, L. & RESMINI P. 1997a Phosphopeptides from Grana Padano cheese: nature, origin and changes during ripening. *Journal of Dairy Research* **64** 601-615

FERRANTI, P., ITOLLI, E., BARONE, F., MALORNI, A., GARRO, G., LAEZZA, P., CHIANESE, L., MIGLIACCO, F., STINGO, V. & ADDEO, F. 1997 b Combined high resolution chromatographic techniques (FPLC and HPLC) and mass spectrometry-based identification of peptides and proteins in Grana Padano cheese. *Lait* **77** 683-697

JEANSON, S., DUPONT, D., GRATTARD, N. & ROLET-REPECAUD, O. 1999 Characterisation of the heat treatment undergone by milk using two inhibition ELISAs for quantification of native and heat denatured α–Lactalbumin. *Journal of Agricultural and Food Chemistry* **47** 2249-2254

JIMENEZ-FLORES, R., KANG, Y. C. & RICHARDSON, T. 1987 Cloning and sequence analysis of bovine β–casein cDNA. *Biochemical and Biophysical Research Communications* **142** 617-621

KOBAYASHI, T., AMETANI, A., YAMAUCHI, K. & KAMINOGAWA, S. 1991 Differences in defining residues relevant to antibody binding by ELISA and proteolysis protection at the level of peptide antigenic determinant. *Biochimica et Biophysica Acta* **1077** 11-18

KÖHLER, G. & MILSTEIN, C. 1975 Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256** 495-497

KUMOSINSKI, T. F., BROWN, E. M. & FARELL, H. M. Jr. 1993 Three-dimensional molecular modeling of bovine caseins: an energy-minimized β-casein structure. *Journal of Dairy Science* **76** 931-945

KUZMANOFF, M. K., ANDRESEN, J. & BEATIE, C. W. 1991 Isolation and characterisation of monoclonal antibodies monospecific for bovine α-casein and β–CN. *Journal of Dairy Science* **74** 803-810

1   Leaver, J. & Law, A. J. R. 1992 Preparative scale purification of bovine caseins on a cation-exchange resin. *Journal of Dairy Research* **59** 557-561

2   Léonil, J., Mollé, D., Gaucheron, F., Arpino, P., Guénot, P. & Maubois, J. L. 1995 Analysis of major bovine milk proteins by on-line high
3   performance liquid chromatography and electrospray ionization-mass spectrometry. *Lait* **75** 193-210

4   Levieux, D. 1974 [Bovine Immunoglobulins and Brucellosis. Purification of immunoglobulins and preparation of their respective specific antisera]
5   *Annales de Recherches Vétérinaires* **5** 329-342

6   Mirza, I.H., Wilkin, T.J., Cantarini, M. & Moore, K. 1987 A comparison of spleen and lymph node cells as fusion partners for the raising of
7   monoclonal antibodies after different routes of immunisation. *Journal of Immunological Methods* **105** 235-243

8   Nagaune, S. I., Kaminogawa, S., Enomoto, A., Kobayashi, T., Kurisaki, J. I. & Yamauchi, K. 1988 Preparation of anti bovine β-casein monoclonal
9   antibody and analysis of the interaction between the antibody and β-casein fragment. *Agricultural and Biological Chemistry* **52** 2577-2581

10   Otani, H., Dong, X. Y. & Hosono, A. 1990 Antigen specificity of antibodies raised in rabbits injected with a chymotryptic casein digest with molecular
11   weight less than 1000. *Japanese Journal of Dairy and Food Science* **39** A31-A37

12   Otani, H., Higashiyama, S. & Tokita, F. 1984 Studies on the antigenic structure of bovine □□casein. IV. Antigenic activities of peptides obtained by
13   cyanogen bromide cleavage of 94-209 region. Milchwissenschaft 39 469-472

14   Otani, H., Kihara, Y. & Park M. 2000 The immunoenhancing property of a dietary casein phosphopeptide preparation in mice. Food and Agricultural
15   Immunology 12 165-173

16   Otani, H., Mine, Y. & Hosono, A. 1987 Studies on the antigenic structure of bovine □□casein. V. Antigenic activities of some peptides derived from
17   residues 1-93. Milchwissenschaft 42 505-508

18   Oudshoorn, P., Hiemstra, P. & Hessing, M. 1994 Preparation and characterization of monoclonal antibodies directed against bovine □□casein.
19   International Dairy Journal 4 671-678

20   Pellequer, J. L., Westhof, E. & Van Regenmortel, M. H. V. 1991 Predicting location of continuous eitopes in proteins from their primary structures.
21   Methods in enzymology 203 176-201.

22   Pizzano, R., Nicolai, M. A. & Addeo, F. 1999 Antipeptide antibodies in milk and cheese analysis. Recent Results and Developments in Agricultural &
23   Food Chemistry 3 403-412

24   Pizzano, R., Nicolai, M. A., Padovano, P., Ferranti, P., Barone, F. & Addeo, F. 2000 Immunometrical evaluation of bovine □-casein and its 1-28
25   phosphopeptide in cheese during ripening. Journal of Agricultural and Food Chemistry ASAP Article 10.1021, Web realease: September 23.

26   Stewart, A. F., Bonsing, J., Beattie, C. W., Shah, F., Willis, I. M., Mackinlay, A. G. 1987 Complete nucleotide sequences of bovine α_{S2}- and β-
27   casein cDNAs : comparisons with related sequences in others species. *Molecular Biology and Evolution* **4** 231-241

28   Swaisgood, H. E. 1992 Chemistry of the caseins. In *Advanced Dairy Chemistry-1: Proteins*, 63-110 (Ed. P.F. Fox). London, UK & New York, USA:
29   Elsevier Applied Science

30   Venien, A., Levieux, D., Astier, C., Briand, L., Chobert, J. M. & Haertle, T. 1997 Production and epitopic characterization of monoclonal
31   antibodies against bovine β-Lactoglobulin. *Journal of Dairy Science* **80** 1977-1987

32   Wei, T. M. & Whitney, R. M. 1984 Batch fractionation of bovine caseins with DEA-cellulose. *Journal of Dairy Science* **68** 1630-1636

33

1 **FIGURE LEGENDS**

2

3 **Fig. 1**. Distribution of 21 anti bovine β-casein MAbs according to their reactivity with ovine, human and equine whole

4 casein in ACP-ELISA. Group size when >1 is given in brackets.

5

6 **Fig. 2.** Tentative epitopes of specific anti bovine β-casein MAbs from 7 reactivity classes as determined from their

7 reactivity with tryptic fragments, buffalo, ovine and human sequence variants in ACP-ELISA.

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

fusion	growing /lymphocytes	positive /hybrids	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

1 **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

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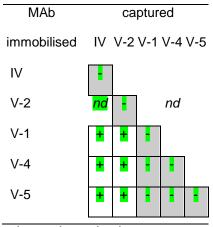
3 detection of hydrolysed β-CN

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

5 § classification of reactive MAbs in ACP-ELISA

6

1   **Table 3.** *Interfering (greyed) or non-interfering pair-wise binding to bovine β-casein of specific MAbs with close*

2   *epitopes. Binding is expressed as a molar % of maximal capture of the secondary MAb.*

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

3   *nd* : non determined

4

1 **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		

2

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		

3

4 **FIGURES**