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Daniel Senocq,[†] Didier Dupont,[†]* Odile Rolet-Répécaud,[†] Francis Faurie,[†] and Didier Levieux[‡]

[†]INRA-Station de Recherches en Technologie et Analyses Laitières, B.P. 89, F-39801 Poligny, France and [‡] INRA-Station de Recherches sur la Viande

* Author to whom correspondence should be addressed (fax +33 3 84 37 37 81; e-mail dupont@poligny.inra.fr)

INRA-Station de Recherches sur la Viande 63122 Saint Genès-Champanelle, France

ABSTRACT

In order to investigate plasmin activity in cheese, we produced antibodies to bovine β–casein with controlled specificity, suitable as markers of the integrity of the major bonds involved in its initial breakdown. Sixteen rabbits were immunized with synthetic substitutes for six plasmin-sensitive peptides. Antisera raised to the peptides (f20-39), (f40-56), (f94-113), (f184-202), and (f193-209) recognized β–casein in ACP-ELISA, Western-blott and biosensor. Casein *in vitro* hydrolysis by plasmin or chymosin reduced the detection of these determinants in ACP-ELISA, in agreement with the enzymatic sensitivity of bonds included within the binding sites, or in their neighborhood. Antiserum to (f20-39) in particular allowed the specific detection of plasmin cleavage at the bond generating γ1–CN. Antisera to N-terminus preferentially detected the cleavage by chymosin. Immunoassays using these antibodies would allow *in situ* monitoring of significant proteolysis events without bias originated in the secondary degradation of the released peptides.

Keywords: *bovine β–casein; plasmin; proteolysis; polyclonal antibody; peptide immunogen; immuno-blotting; ELISA; biosensor*

INTRODUCTION

Casein proteolysis is a major contributor to textural changes and flavor development in ripened cheeses (Fox & McSweeney, 1996). Caseins are degraded into small peptides and free amino acids during a complex process, described by Grappin *et al.* (1985) as a two-step scheme. Caseins are initially broken down into large, well-characterized fragments. This primary proteolysis is catalyzed essentially by the residual coagulant (chymosin and pepsin in various ratio), and to a lesser extent by endogenous milk proteases, such as plasmin, cathepsin D and other somatic-cell proteinases. One part of these fragments undergoes secondary proteolysis, for a large part catalyzed by additional enzymes from the microflora. The main enzymes originating from rennet, milk, starter and non-starter microflora are active in most ripened cheese varieties. However, their relative contributions vary substantially following cheesemaking practices. For instance, in Swiss-type cheeses, cooking the curd inactivates extensively the coagulant (chymosin), and simultaneously enhances plasmin activity, which therefore becomes predominant (Ollikainen & Kivela, 1989).

Plasmin preferentially hydrolyzes β-CN, α_{S2}-CN, and to a lesser extent α_{S1}-CN, whereas it does not hydrolyze κ-CN. It is highly specific to the peptidic bonds lysine-X and to a lesser extent arginine-X. Three bonds are predominantly cleaved in milk and cheese: cleavage of Lys_{28}-Lys_{29}, Lys_{105}-His_{106} and Lys_{107}-Glu_{108} releases the polypeptides β–CN (f29-209), (f106-209) and (f108-209), known as γ1–CN, γ2–CN and γ3–CN respectively. Their complementary peptides, β–CN (f1-105) and (f1-107), β–CN (f29-105) and (f29-107), and β–CN (f1-28) contribute to the proteose peptone fraction of milk (Andrews & Alichanidis, 1983). In a recycle reactor, Visser *et al*. (1989) elicited further cleavage at virtually all 15 potential sites, thus releasing 16 peptides. In cheese, limited proteolysis by the combined action of plasmin and other enzymes leads to a larger number of peptides: for instance, Singh *et al*. (1997) isolated more than 100 β–CN fragments in Cheddar cheese.

Assessment of the extent and progress of proteolysis is of major interest, as an objective index of cheese maturity and quality. The extent of proteolysis can be estimated from the accumulation of a few end-products chosen as quantitative markers, such as γ3–CN or α_{S1}-CN (f80-199) (Addeo *et al*., 1995). The peptide separation profiles can also be compared as cheese "finger prints", for individual or intervarietal differences. Finally, complete characterization of cheese proteolysis requires the isolation and identification of individual peptides. Owing to their number and their biochemical parenthood, isolation of most peptides requires successive application of chemical or physical fractionation, of electrophoretic and chromatographic separation, whereas their identification requires expensive techniques, such as mass-spectroscopy or peptide sequencing. None of these techniques allows monitoring of the action of one particular enzyme on its substrates during ripening.

In recent years, immunoassays have challenged classical techniques with detection methods that are both sensitive and specific. The proteolysis patterns in PAGE were made specific for one substrate by Addeo *et al*. (1995), using antibodies raised to single casein fractions for Western-blott. More selective antibodies have been produced as tentative probes specific for significant peptides, such as κ–CN (f106-169) released by chymosin (Picard, 1993), or β–CN (f1-28) released by plasmin (Pizzano *et al*., 1999). However, the antibodies raised against the peptides cross-detected systematically the original intact casein, which did not allow one-step detection. We investigated a new strategy to overcome this drawback in detection of proteolysis events.

The objective of the study was to produce antibodies to monitor the integrity of plasmin-sensitive bonds in β–CN. To this end, we immunized 16 rabbits with six synthetic peptides mimicking β–CN A^2 peptides (f20-39), (f42-56), (f94-113), (f167-179), (f184-202) and (f193-209), which included or bordered 14 plasmin sensitive bonds. The specificity of the anti-peptide antisera towards intact β–CN and isolated fragments was assessed using ELISA, Western-blott and biosensor assays. The reactivity of hydrolyzed β–CN with the characterized antisera was evaluated as a specific marker of plasmin-mediated proteolysis.

MATERIALS AND METHODS

Chemicals. Unless otherwise stated, chemicals of analytical grade were purchased from Prolabo (Merck eurolab, Darmstadt, Germany).

Enzymes. Chymosin (E.C. 3.4.23.4) was obtained from bovine rennet by reference technique (IDF, 1987). Plasminogen was obtained from fresh bovine blood according to the procedure of Deutsch & Mertz (1970). It was concentrated to 70 g/l by ultrafiltration at 17000 g, for 50 min at 4°C using MacroSep (50 kDa cut-off, Filtron Technology Corporation, Northborough, MA, USA), and kept frozen at -20°C. Immediately before use, aliquots were treated for 10 min at 37°C with urokinase (E.C.3.4.21.73) (Choay, Sanofi Winthrop, Gentilly, France) at an enzyme/substrate ratio of 1/100 IU/mg, and thus activated to plasmin (E.C. 3.4.21.7).

β-Casein Purification. Individual milk from an homozygous cow for β–CN was purchased from the Coopérative Graindorge (Livarot, France). Fat was removed by centrifugation at 1800 g for 15 min at 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 with ca. 90% purity was obtained in gram amounts by the batch-wise procedure of Leaver & Law (1992), dialyzed against water, and lyophilized.

Homogenous β–CN was obtained from crude β-casein by the FPLC ion exchange procedure of Andrews *et al.* (1985), scaled to fit a HR16/10 column (Pharmacia Biotech., 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 buffer composed of 25 mM Tris-HCl, 4.5 M urea, 0.08 mM DTT, pH 7.0. Fractions (5 ml) were collected, checked for homogeneity, pooled, dialyzed against water, and lyophilized.

Other Test Antigens. Whole casein from cow's milk was purchased from Merck, that from ewe, goat, and human milk from Sigma (Sigma-Aldrich, St.Quentin-Fallavier, France), and Bovine Serum Albumin (BSA) from Pierce (Interchim, Montluçon, France). Other bovine proteins were obtained 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).

β–Casein Hydrolysis. Homogenous bovine β–CN was hydrolyzed by either plasmin or chymosin under the same conditions. Hydrolysis was carried out at an enzyme/substrate ratio of 1/100 w/w for 0.5, 8 or 14 hours at 37°C, in 75 mM ammonium acetate, pH 6.0. For further RP-HPLC separation of the hydrolysates (0.5 and 8 hours), the reaction was stopped by boiling for 10 min at 100°C, whereas urea was added at a final concentration 4 M to the other hydrolysates (14 hours).

HPLC Fractionation of β–CN Hydrolysates. β–CN hydrolysates were separated on a Zorbax C18 column (4.6x150 mm, 300 Å, 3.5 µm, Agilent Technologies, Newport, USA), as described by Beuvier *et al.* (1997). Prior to injection, hydrolysates were diluted with an equal volume of HPLC buffer A containing 8 M urea and filtered through 0.45 µm. Detection was performed simultaneously by UV absorbance at 214 nm and electrospray ionization mass-spectroscopy (ESI-MS), as described by Léonil *et al.* (1995). Resolved peaks comprising single peptides were collected from further injections of 0.5 mg of hydrolyzed β–CN, and vacuum-dried at room temperature.

Peptide Immunogens. The chosen peptide immunogens, their sequences, the localization of their counterpart residues within β–CN A^2 sequence, and the plasmin sensitive bonds they included are reported in Table 1. The additional bonds Lys_{113}-Tyr_{114} and Arg_{183}-Asp_{184} bordered two peptides, of which (f184-202) was an end-product of plasmin induced cleavage. Peptides (f184-202) and (f193-209) overlapped each other.

The peptides were chemically synthesized by Synt:em (Nîmes, France), with a minimum purity 75%. A serine residue was substituted for the phosphorylated serine residue at position 35. The peptides were modified by C-

terminal amidation, and addition of a cysteine residue at N-terminus. In order to increase their immunogenicity, fractions of the peptides were conjugated 2:1 w/w to Keyhole Limpet Hemocyanin by the N-terminal sulphydryl group.

Production of Anti-Peptide Polyclonal Antibodies. Each conjugated peptide was used to immunize two rabbits, except (f94-113) and (f193-209) each used for 3 rabbits, and (f20-39) used for 4 rabbits. Adult female rabbits were given monthly multiple site intradermal injections of conjugate-adjuvant emulsion, prepared from 0.5 ml conjugate at 0.4 g/l in Saline, and 0.5 ml Freund's Complete Adjuvant (initial injection) or Incomplete Adjuvant (booster injection) (Difco Laboratories, Detroit, MI, USA). From the third month onwards, blood was collected on the 6^{th} and 9^{th} days after injection. Antisera were labeled from the initial N-terminal residues of their peptide immunogens, as reported in Table 1, and numbered according to the animals.

Production of Anti β–CN Antibodies. *Monoclonal antibodies* were raised against pure β–CN, according to the procedure described by Jeanson *et al*. (1999). Prior to lymph nodes collection, polyclonal antisera were collected by bleeding the mice. *Polyclonal antiserum* was also obtained from a rabbit immunized with 0.5 mg aliquots of β–CN according to the procedure used for peptide antigens.

ACP-ELISA. Antisera were tested using an antigen coated on plate enzyme-linked immunosorbent assay procedure (ACP-ELISA) for possible cross-reactivity with, on the one hand, α_S-CN and κ–CN, and on the other hand, whole casein from cow, ewe, goat and human milk, and for their reactivity towards their own immunization peptide and purified β–CN. For determination of possible cross-reactions, all antigens were prepared at 0.5 µg/ml in carbonate buffer (0.1 M carbonate/bicarbonate, pH 9.6). For comparative reactivity assays, serial dilutions from approximately 2.10^{-9} M to $0.25.10^{-6}$ M were prepared following the dilutions: for β–CN, $50 * 2^n$ ng/ml in carbonate buffer, and for the peptides, $5 * 2^n$ ng/ml in Phosphate Buffered Saline (PBS= 0.05 M phosphate buffer, 0.15 M NaCl, pH 7.2), where n= 0 to 7. One hundred microliters aliquots were incubated, for 2 hours at 37°C, in duplicate wells of a flat-bottomed 96-well microtitration plate (NUNC F96 Maxisorp, Polylabo, Merck eurolab). The detection of bound antigens was as follows: 250 µl gelatin at 10 g/l (1.04070, Merck), 100 µl anti-peptide antiserum at 1/10000 v/v and 100 µl goat anti-rabbit immunoglobulin–alkaline phosphatase conjugate at 1/3000 v/v (Sigma) were incubated successively for one hour periods in the wells, at 37°C in PBS-T (PBS buffer made 0.55 g/l Tween 20 (Merck)). Between each incubation steps, wells were rinsed for 15 s with four changes of 250 µl PBS-T. Following the last rinsing, 100 µl *p*-nitrophenyl phosphate (Sigma) at 1 mg/ml in 1M diethanolamine-HCl, 1 mM $MgCl_2$, 0.1 mM Zn-acetate were incubated in the wells. After one hour, the absorbance at 405 nm was read against

a blank. The mean background signal in the absence of antigen was subtracted from mean absorbance calculated from duplicate wells.

Western-Blott Assay. *Urea-polyacrylamide gel electrophoresis* (PAGE) was carried out on discontinuous gels according to the method of Andrews (1983). Briefly, 7 µg whole casein or β–CN hydrolysate were separated on a 80*80 mm separation gel (12.5 % T, 3.3 % C, 4.5 M urea, pH 8.9). Migration was carried out at 20 mA/gel for 60 min at 15°C. Immediately after separation, peptides were transferred from the gel onto a 0.2 µm nitrocellulose membrane (Whatman Biobind-NC, Polylabo, Merck eurolab) as described by McSweeney *et al.* (1994).

Immunodetection was as follows: the membrane was incubated at room temperature, for one hour periods, in 10 mM Tris-HCl, 0.5 M NaCl, 0.5 mM DTT, 0.275 g/l Tween 20, pH 8.0 buffer, with, successively, heat inactivated horse serum at 1/10 v/v, anti-peptide antisera at 1/1000 v/v, and goat anti-rabbit immunoglobulin–alkaline phosphatase conjugate at 1/3000 v/v (Sigma). The membrane was washed between each step by soaking for five min in four changes of the same buffer. After the last washing, the membrane was stained for about 7 min at room temperature with Fast-Red TR salt, and Naphtol AS-MX phosphate disodium salt (Sigma), mixed according to the manufacturer's instructions.

Biosensor flow assay. Biacore 3000 was used with registered chemicals and procedures (Biacore AB, Uppsala, Sweden). The antisera were assayed for binding to β–CN immobilized on the sensor surface by means of a monospecific MAb directed against β–CN (f1-25). The carboxy-methylated dextran surface (CM5) was conditioned for immobilization of mouse IgG as follows: 35 µl of a solution 0.05 M N-hydroxy-succinimide, 0.2 M N-ethyl-N'-(3-dimethyl-aminopropyl), then 35 µl of rabbit anti-mouse Fc γ at 30 µg/ml in 10 mM sodium acetate, pH 4.8, and 20 µl of 1 M ethanolamine hydrochloride at pH 8.5 were injected in sequence at 5µl/min. Immobilization of β–CN on the activated surface was as follows: undiluted hybridoma culture supernatant containing anti β–CN (f1-25) MAb, the serum of a non-sensitized rabbit at 1/10 v/v, and pure β–CN at 10 mg/l were injected in sequence for 3 min at 10 µl/min in HEPES buffered Saline (HBS= 10 mM HEPES, 3 mM EDTA, 0.005% v/v polysorbate 20, 0.15 M NaCl, pH 7.4). Optimal binding of test antisera was obtained by single injections of dilutions 1/100 v/v in HBS buffer for 3 min at 10 µl/min. The mass coated at the surface of the sensor was recorded throughout all steps as a proportional increase of the surface plasmonic resonance.

Additive binding assay was carried out by injecting successively three antisera onto the immobilized β–CN without regeneration of the surface.

RESULTS

Binding of Anti-Peptide Antisera to their Target Sequences. The reactivity of the antisera towards their respective immunogen and β–CN at similar molarity was determined in ACP-ELISA. Antigen concentration dependent curves are displayed in Figure 1, for four representative antisera. All antisera detected their respective immunogen, however antisera CDMP2 (Figure 1A), CQSK1 and CQSK2 did not bind β–CN, even at the maximal concentration tested (6.4, 25.6, and 25.6 µg/ml respectively). Reactive antisera displayed 3 types of behavior: CEES1 displayed the same sensitivity and detection limit towards both antigens (Figure 1B), CEDE1 displayed a similar detection limit, but a lower sensitivity for β–CN than for the peptide (Figure 1C), finally CDMP1 (Figure 1D), CGVS1, and CYQE1 displayed a higher detection limit for the peptide than for β–CN.

The reactivity of anti-peptide antisera towards β–CN was further investigated in Western-blott, by immuno-staining of bovine whole casein separated using urea-PAGE (Figure 2). β–CN was identified as a major band in the profile stained with control rabbit anti β–CN antiserum (Lane 1). Its observed mobility (Rf 0.190) agreed with that reported by McSweeney *et al*. (1994). All anti-peptide antisera, including CDMP2, detected specifically β–CN (Lane 2 through 7), however, only very low detection was achieved with CQSK1 (Lane 5).

The antiserum CDMP2 was shown to detect β–CN in Western-blott, but not in ACP-ELISA. To elucidate its contrasted reactivity, CDMP2 was assayed, together with reference antisera raised to each of the 6 peptides, for binding to β–CN immobilized on the biosensor by specific capture at the remote site (f1-25). CQSK1 did not bind to β–CN, whereas all other antisera did, including CDMP2. The reactivity assessed in the three complementary techniques, ELISA, Western-blott and biosensor, is summarized in Table 2. The antisera CEES1, CGVS1, CEDE1, CQSK1, and CYQE1 were representative for the antisera raised to five peptide immunogens, and were selected for further investigations. In addition, CDMP1 and CDMP2, which were obviously directed to different determinants within a common immunogen, were studied in parallel.

Reactivity of Anti-Peptide Antisera Towards Other Caseins. The anti-peptide antisera were assayed in ACP-ELISA for binding to the related β–, κ– and α_S- casein fractions at equal concentration. CEDE1 cross-reacted faintly with κ–CN, and CYQE1 with α_S-CN, but the non-specific binding was kept below 10% of β–CN detection. In Western-blott, α_S-CN migrated at Rf 0.295, as indicated by McSweeney *et al*. (1994). It was faintly detected with control rabbit anti β–CN antiserum (Figure 2, Lane 1), but not with any anti-peptide antiserum. In particular, no cross detection was observed with CYQE1 (Lane 7), which suggested that the cross-detection observed in

ELISA may have arisen from contamination of the test antigen. The putative cross-detection of κ–casein could not be investigated because it co-migrated with reactive γ– caseins.

Localization of Determinants Recognized by Oligoclonal Anti-Peptide Antisera. In order to determine whether the antisera were directed against the N or C-terminal side of the plasmin sensitive bonds, the β–CN fragments released in hydrolysates were isolated to individually assess their reactivity in ACP-ELISA. The collected peptides were identified according to their masses (Table 3). The residual antigenicity of the peptides was as follows: CEES1 bound β–CN (f1-28), but not β–CN (f29-99). CEDE1 bound β–CN (f29-99), whereas it did not bind the complementary fragments β–CN (f29-48) and β–CN (f49-99). CGVS1 bound β–CN (f49-99) and β–CN (f49-105), and not β–CN (f106-209). CQSK1 recognized none of the peptides β–CN (f170-209) and (f177-209), and didn't either recognize the peptide β–CN (f106-209) that displayed its intact immunogenic sequence.

Peptides arising from cleavage at position 202 could not be isolated from the hydrolysates to test CYQE1 and CDMP antisera. However, the peptide β–CN (f184-209), together with synthetic peptides (f184-202) and (f193-209), allowed the antigenic mapping of β–CN C-terminus. In ACP-ELISA, CYQE1 recognized all three peptides, and was therefore at least partially directed at the common stretch (f193-202). CDMP1 recognized β–CN (f184-209) and (f184-202), and not β–CN (f193-209), whereas CDMP2 recognized specifically (f184-202). This indicated that the residues 184 to 193 contributed primarily to the determinants of both CDMP1 and CDMP2. To assess whether CDMP1, CDMP2 and CYQE1 were directed to distinct or overlapping epitopes, all three antisera recognizing β–CN in biosensor assay were further assayed for additive binding (Figure 3). When injected in sequence, CDMP1 inhibited the further binding of CDMP2, whereas in the reciprocal order, they bound additively. CDMP1 bound apparently to the casein at two distinct and non-overlapping epitopes simultaneously, one of which was competitively bound by CDMP2. Moreover, CYQE1 bound additively to each CDMP antisera (Figure 3E), and was thus specific for a third distinct determinant.

Finally, the 6 antisera reactive to bovine β–CN were assayed for binding to casein of related species with known sequence homology to bovine's one. All antisera recognized in ACP-ELISA whole casein from cow, ewe and goat, but not from human, except CGVS1 which was bovine specific. The residues at position 96, 101 and 103 of ovine and caprine sequences are mutated with respect to the bovine's one. At least one of these residues is a critical contributor to the determinant of CGVS1.

Application of Anti-Peptide Antisera to the Detection in Western-Blott of β–Casein Fragments Released by Plasmin. The reactive species in β–CN hydrolysate were investigated in Western-blott by staining

urea-PAGE separated digests with anti-peptide antisera as primary antibodies (Figure 4). Control detection with mouse anti β–CN antiserum (Lane 1) revealed 13, all of which were also detected by one at least anti-peptide antisera. Anti-peptide antisera revealed additionally residual β–CN, γ3-CN, and unidentified components with γ–casein-like mobility. These latter bands were poorly resolved from γ– caseins, and therefore visible only when specifically stained. Seven polypeptides were identified with reference to Lane & Fox (1999) who reported the analysis of a similar β–CN digest with this method and direct Coomassie Blue G 250 stain.

The distinct bands detected in the hydrolysate using CEES1 (lane 2) included residual β–CN and two bands accounting for β–CN (f1-105/107). Finally, a band detected at Rf 0.180 had similar mobility than the unknown component (f1-?) isolated by McSweeney *et al*. (1994) from the water insoluble fraction of a Cheddar cheese. These authors suggested that this component could arise from plasmin action, which was supported by its isolation in the hydrolysate.

Detection using CEDE1 (Lane 3) revealed two bands accounting for β–CN (f29-105/107). Two additional bands at Rf 0.200 and 0.225 might be attributed to components (f29-97) and (f29-99), which are expected in the profile, but which mobility was not reported. In addition, CEDE1 reacted faintly with minor bands at Rf 0.145 and 0.155. From their mobility and their reactivity with anti N-terminal antibodies, these components appeared to be clearly distinct from γ3-CN that migrated at Rf 0.140 (Lane 6). Detection using CGVS1 (Lane 4) revealed the same components recognized by CEDE1. In addition, CGVS1 reacted faintly with γ1-CN. Perceptible differences in CGVS1 and CEDE1 detection patterns may originate from a higher overall reactivity of CGVS1 under the assay conditions, rather than in genuine specificity.

Detection using CQSK2 (Lane 5) was faint, and the extensive staining required by its lack of reactivity resulted in excessive background noise. Detection with CDMP1 (lane 6) revealed γ1–, γ2– , γ3– caseins and β–CN (f114-209), similarly to the detection with CYQE1 (lane 7). In addition, CYQE1 reacted also with residual β–CN, and detected specifically two unidentified components at Rf 0.030 and 0.055. The lack of reactivity of these components with CDMP1 might reflect a lack of accessibility of the determinant of this antiserum rather than its actual removal.

Application of Anti-Peptide Antisera to the Quantitative Monitoring of β–Casein Proteolysis. The detection of β–CN extensively hydrolyzed by either plasmin or chymosin was quantified in ACP-ELISA and expressed as a % of intact β–CN detection (Figure 5). Control detection with anti β–CN (f1-25) MAb yielded 84 and 99% recovery for plasmin and chymosin hydrolysis respectively.

Detection of the determinants for CEES1, CGVS1 and CDMP1 was definitely inhibited by plasmin-induced hydrolysis. Only the detection with CEES1 was specifically sensitive to plasmin. In contrast, the detection with CDMP1 and CYQE1 was preferentially sensitive to chymosin. Detection with CEDE1 was only faintly affected by any enzyme under the conditions used. No antigenicity towards CQSK1 was generated upon cleavage.

DISCUSSION

In this study, we focused the immunogenic response on plasmin cleavage sites in β–CN sequence, by using synthetic peptides as substitute immunogens. This strategy relied on the hypothesis that the peptides mimicked the antigenic determinants exposed by the casein. It was successful for five peptides, and the corresponding antibodies cross-detected the peptide and the casein. In contrast, antibodies raised to (f167-179) and (f184-202) revealed that the casein adopted locally structural features differing from the peptide, which were not antigenic. The cleavage of the parent protein near such a potential determinant might ease the constraints and thus restore the antigenic cross-detection. Indeed, anti-peptide antibodies may be specific for a truncated form of the parent protein, e.g. α_{S1}-I casein (Pizzano *et al.*, 1999). Antibodies raised in the same study against β–CN (f176-185) failed to detect specifically γ– caseins. In the present work, antisera CQSK raised against the overlapping sequence β–CN (f167-179) did not recognize the casein, but did not either recognize any peptides truncated by plasmin or chymosin. The antiserum CDMP2 recognized the casein, except in ACP-ELISA where its determinant appeared to be involved in, or modified by, the adsorption onto the plate. Both antibodies CQSK and CDMP2 were ineffective for proteolysis monitoring.

All five antisera recognizing β–CN, except CEDE1, were partly directed towards the N-terminal half of the peptide immunogen. In the case of CEES1, CGVS1, and CDMP antisera, no such determinants were found in the C-terminal halves. In contrast with the observations of Schaaper *et al.* (1989), the carrier conjugated at the peptide N-terminus exerted thus no obvious negative influence, likely due to the addition of a spacer residue. Some N-terminal determinants of CEES1 and CGVS1 survived the cleavage by plasmin. It was not determined whether they were shortened fragments of the initial main epitope, thus recognized with lesser affinity, or residual determinants recognized only by marginal by sub-populations of the oligoclonal antisera. The detection in Western-blott qualitative assay of the sequence (f29-105) at each termini by either CEDE1, or by a part of CGVS1 antibodies, indicated an apparent specificity similar for both antisera, whereas they were clearly different in ELISA assay. It is likely that reducing the peptide's length would contribute in avoiding to elicit antibodies against such marginal determinants, but would also drastically reduce the peptide's immunogenicity, as was observed for (f167-179) (13

residues), and for (f23-34) and (f94-109) (12 and 14 residues) in preliminary experiments (not shown). Alternatively, adequate monoclonal anti-peptide antibodies would be advantageously raised against the peptide immunogens.

Binding of these five antisera to their target sequences was inhibited unequally by the hydrolysis of the sensitive bonds that were either included in the determinant, or bordering them. The quantitative detection of the residual binding in ACP-ELISA enabled monitoring of localized proteolysis events. The cleavage of the most significant bonds at position 28 and 105/107 could be detected using CEES1 and CGVS1 respectively. The binding of CEES1 was specifically inhibited by the plasmin-induced cleavage of the bond Lys_{28}-Lys_{29}, which releases the phosphopeptide β–CN (f1-28) and complementarily γ1-CN when the initial substrate is β–CN. The monitoring of these peptides is a poor index of plasmin activity at this site. This classical approach is biased by the subsequent degradation of the measured peptides, which are both prone to rapid proteolysis in cheese (Collin *et al.*, 1987; Roudot-Algaron *et al.,* 1994). Moreover, it requires the preliminary definition of the substrates and products to be monitored, whereas the immunological method we propose relies on a built-in specificity for the plasmin sensitive substrates that need not be identified or isolated. The strategy developed in the present work enables the direct monitoring of cleavage events, which is otherwise impossible, and is specific for predetermined cleavage sites of major significance. The parallel detection with CGVS1 and CEES1 could enable to compare *in situ* the actual cleavage rates for the two sites involved in the release of γ1 and γ2/γ3- caseins.

The detection of proteolysis at the C-terminus of β–CN with CYQE1 and CDMP1 was not specific for plasmin or chymosin activity. The respective cleavage sites of these enzymes, at positions 184 and 202 for plasmin, and 189 and 192 for chymosin, are very near. Moreover, the cleavage at any site affected the detection of the adjacent determinants: the determinants of CDMP1 and CYQE1 contained virtually no bonds sensitive to plasmin and chymosin respectively, but they nonetheless displayed reduced antigenicity towards these antisera upon treatment with both enzymes. In the conditions prevalent in cheese (low moisture, and high NaCl in moisture), the cleavage of these bonds is reduced by intermolecular hydrophobic interactions between C-terminal segments of β– and γ– caseins (Exterkate *et al.*, 1997). In most cheeses, and particularly in Cheddar cheese, chymosin contributes therefore very little, if at all, to the breakdown of γ– and β– caseins (McSweeney *et al.*, 1994). Peptides resulting from chymosin-like specificity cleavage at this segment were however evidenced in mature Cheddar cheeses (Singh *et al.*, 1997). *Lactococcus* starter cell envelope proteinases or cathepsin D might be responsible for these cleavages, but evidences are still needed. The release of these peptides cannot be yet routinely investigated by LC-

MS, nor can it be performed by monitoring γ– caseins that are their probable origin substrates. Immunoassays based on the antibodies CYQE1 and CDMP1 may provide convenient and sensitive detection of proteolysis in this segment of β– and γ– caseins. Assessing the stage of the ripening process when the cleavage of this segment is initiated would allow a more efficient use of the cumbersome peptide isolation procedures, and provide clues as to the responsible enzymes.

To investigate the status of other major sites known to participate in the breakdown of caseins, additional anti-peptide antibodies will have to be raised against selected stretches of α_{s1}-, α_{s2}- and κ- caseins.

ABBREVIATIONS USED AND NOMENCLATURE

CEES, CEDE, CGVS, CQSK, CDMP and CYQE, rabbit antisera raised to synthetic peptides (Table 1) conjugated to Keyhole Limpet Hemocyanin.

Proteins and fragments are labeled according to Eigel *et al.* (1984), except for: α_{S1}-I= α_{S1}-CN (f24/25-199); γ1-CN= β–CN (f29-209); γ2-CN= β–CN (f106-209); γ3-CN= β–CN (f108-209). Synthetic peptides and sequences are labeled omitting "β–CN".

ACP-ELISA, antigen coated on plate enzyme linked immunosorbent assay; ESI-MS, electrospray ionisation–mass spectroscopy; Fc, complement-binding domain of IgG; HBS, (10 mM HEPES, 3 mM EDTA, 0.005% v/v polysorbate 20, 0.15 M NaCl, pH 7.4); MAb, monoclonal antibody; PBS, (0.05 M phosphate buffer, 0.15 M NaCl, pH 7.2); PBS-T, (0.55 g/l Tween 20-PBS); PAGE, polyacrylamide gel electrophoresis; Rf, electrophoretic mobility ratio to the migration front; RP-HPLC, reverse phase–high pressure liquid chromatography.

Chemicals: DTT, dithiothreitol; EDTA, ethylene diaminetetraacetic acid disodium; HEPES, N-[2-hydroxyethyl]piperazine-N'-[ethanesulfonic acid]; Tris, tris-(hydroxymethyl) aminomethane.

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FIGURE CAPTIONS

Figure 1. Reactivity of anti-peptide antiserum CDMP2 raised to β–CN (f184-202) (A), antiserum CEES1 raised to β–CN (f20-39) (B), antiserum CEDE1 raised to β–CN (f42-56) (C), antiserum CDMP1 raised to β–CN (f184-202) (D) towards β-CN (--o--) and their respective immunization peptides (—■—) in ACP-ELISA. Antisera diluted 1/10000 v/v, procedure described in Materials & Methods.

Figure 2. Bovine whole casein separated by urea-PAGE and blotted onto nitrocellulose. Immuno-staining with, as primary antibody, rabbit anti β–CN antiserum (lane 1) or rabbit anti-peptide antisera: CEES1 raised to β-CN (f20-39) (2), CEDE1 raised to β-CN (f42-56) (3), CGVS1 raised to β-CN (f94-113) (4), CQSK1 raised to β-CN (f167-179) (5), CDMP1 raised to β-CN (f184-202) (6), CYQE1 raised to β-CN (f193-209) (7). All antisera diluted 1/1000 v/v. Detection of bound antibody as described in Materials & Methods.

Figure 3. Specific capture (expressed as induced plasmonic resonance) of anti β-CN (f1-25) MAb (A), non-specific rabbit IgG (B), β-CN (C), rabbit anti-peptide antiserum CDMP2 (—) and CDMP1 (- - -) raised to β–CN (f184-202) (D), rabbit anti-peptide antiserum CDMP1 (—) or CDMP2 (- - -) (E), rabbit anti-peptide antiserum CYQE1 raised to β–CN (f193-209) (F) onto the activated biosensor chip in multiple-site binding assay.

Figure 4. Bovine β-CN hydrolyzed by plasmin (1/100 w/w ratio, 37°C, 30 min, pH 6.0) separated by urea-PAGE and blotted onto nitrocellulose. Immuno-staining with, as primary antibody, mouse anti β–CN antiserum (lane 1) or rabbit anti-peptide antisera: CEES1 raised to β-CN (f20-39) (2), CEDE1 raised to β-CN (f42-56) (3), CGVS1 raised to β-CN (f94-113) (4), CQSK1 raised to β-CN (f167-179) (5), CDMP1 raised to β-CN (f184-202) (6), CYQE1 raised to β-CN (f193-209) (7). All antisera diluted 1/1000 v/v. Detection of bound antibody as described in Materials & Methods.

Figure 5. Residual reactivity of 100 µl of bovine β–CN 0.5 µg/ml extensively hydrolyzed by the action (1/100 w/w ratio, 37°C, 14 hours, pH 6.0) of plasmin (■) or chymosin (□) towards mouse anti β-CN (f1-25) MAb and 5 rabbit anti-peptide antisera raised to plasmin-sensitive determinants in ACP-ELISA. Mouse MAb at 1/100 v/v, rabbit antisera 1/10000 v/v. Detection of bound antibody as described in Materials & Methods.

TABLES

Table 1. Design of the Peptide Immunogens

sequence	β-CN A^2 peptide	plasmin sensitive bonds[a]	serum
CEESITRINKKIEKFQS[b]EEQQ	(f20-39)	K_{28}-K_{29}>>R_{25}-I_{26};K_{29}-I_{30};K_{32}-F_{33}	CEES
CEDELQDKIHPFAQTQ	(f42-56)	K_{48}-I_{49}	CEDE
CGVSKVKEAMAPKHKEMPFPK	(f94-113)	K_{105}-H_{106};K_{107}-E_{108}>>K_{97}-V_{98};K_{99}-E_{100}	CGVS
CQSKVLPVPQKAV	(f167-179)	K_{169}-V_{170};K_{176}-A_{177}	CQSK
CDMPIQAFLLYQEPVLGPVR	(f184-202)	-	CDMP
CYQEPVLGPVRGPFPIIV	(f193-209)	R_{202}-G_{203}	CYQE

[a] in order of decreasing sensitivity (Visser *et al*., 1989)

[b] non-phosphorylated serine residue

Table 2. Reactivity of Anti-Peptide Antisera with Immunization Peptides and β–CN

serum	peptide	β–CN		
	ACP ELISA	ACP ELISA	Western-blott	bio-sensor
CEES1	+	+	+	+
CEDE1	+	+	+	+
CGVS1	+	+	+	+
CQSK1	+	-	±	-
CDMP1	+	+	+	+
CDMP2	+	-	+	+
CYQE1	+	+	+	+

+/±/- : antigenic/weakly antigenic/non antigenic in the assays described in Materials & Methods section.

Table 3. Identification by ESI-MS of Tryptic Peptides from Bovine β–CN Separated using RP-HPLC

molecular mass (Da)		peptide
measured[a]	theoretical[b]	β–CN
3478.8 ± 0.4	3479.4	-4P (f1-28)
8073.4 ± 1.1	8072.1	-1P (f29-99)
2560.8 ± 0.4	2561.6	-1P (f29-48)
5529.3 ± 0.4	5529.5	(f49-99)
6156.8 ± 0.3	6157.3	(f49-105)
11826.0 ± 1.5	11825.0	(f106-209)
4484.9 ± 0.2	4484.4	(f170-209)
3722.6 ± 0.1	3722.5	(f177-209)
2910.9 ± 0.9	2910.5	(f184-209)

[a] average molecular mass ± SD calculated using a Sciex version Mac Spec 3.2 from measured m/z of multiprotonated ions.

[b] average mass calculated according to the amino acid sequence (Jimenez-Flores *et al.,* 1987).