Antipeptide Antibodies Recognizing Plasmin Sensitive Sites in Bovine β-Casein Sequence

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, 63122 Saint Genès-Champanelle, France

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 assays. 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 C-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.

INTRODUCTION

Casein proteolysis is a major contributor to textural changes and flavor development in ripened cheeses (*1*). Caseins are degraded into small peptides and free amino acids during a complex process, described by Grappin et al. (*2*) 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 ratios), 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, and starter and nonstarter microflora are active in most ripened cheese varieties. However, their relative contributions vary substantially depending on cheesemaking practices. For instance, in Swiss-type cheeses, cooking the curd extensively inactivates the coagulant (chymosin), and simultaneously enhances plasmin activity, which therefore becomes predominant (*3*).

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 (*4*). In a recycle reactor, Visser et al. (*5*) 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. (*6*) 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) (*7*). The peptide separation profiles can also be compared as cheese "fingerprints" 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, and 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. (*7*), using antibodies raised to single casein fractions for Western-blott assay. More selective antibodies have been produced as tentative probes specific for significant peptides, such as κ-CN (f106−169) released by chymosin (*8*), or β-CN (f1−28) released by plasmin (*9*). However, the antibodies raised against the peptides cross-detected

* To whom correspondence should be addressed (fax +33 3 84 37 37 81; e-mail dupont@poligny.inra.fr).
† Station de Recherches en Technologie et Analyses Laitières.
‡ Station de Recherches sur la Viande.

1572 *J. Agric. Food Chem.,* Vol. 49, No. 3, 2001

Senocq, et al.

Table 1. Design of the Peptide Immunogens

sequence	β-CN A^2 peptide	plasmin-sensitive bonds[a]	serum
CEESITRINKKIEKFQS^{b}EEQQ	(f20−39)	$K_{28}-K_{29} \gg R_{25}-I_{26}; K_{29}-I_{30}; K_{32}-F_{33}$	CEES
CEDELQDKIHPFAQTQ	(f42−56)	$K_{48}-I_{49}$	CEDE
CGVSKVKEAMAPKHKEMPFPK	(f94−113)	$K_{105}-H_{106}; K_{107}-E_{108} \gg K_{97}-V_{98}; K_{99}-E_{100}$	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 (*5*). ^{b} Nonphosphorylated serine residue.

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 toward 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 (*10*). Plasminogen was obtained from fresh bovine blood according to the procedure of Deutsch and Mertz (*11*). It was concentrated to 70 g/L by ultrafiltration at 17000*g*, for 50 min at 4 °C using MacroSep (50 kDa cutoff, Filtron Technology Corporation, Northborough, MA), 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 and Whitney (*12*).

Crude β-CN with ca. 90% purity was obtained in gram amounts by the batch-wise procedure of Leaver and Law (*13*), dialyzed against water, and lyophilized.

Homogeneous β-CN was obtained from crude β-casein by the FPLC ion exchange procedure of Andrews et al. (*14*), scaled to fit a HR16/10 column (Pharmacia Biotech., Uppsala, Sweden). Briefly, 600 mg of crude β-CN was 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, and 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. (*15*), casein fractions α_S and κ according to Collin et al. (*16*), and immunoglobulins G (IgG) according to Levieux (*17*).

β-Casein Hydrolysis. Homogeneous 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 h at 37 °C, in 75 mM ammonium acetate, pH 6.0. For further RP−HPLC separation of the hydrolysates (0.5 and 8 h), 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 h).

HPLC Fractionation of β-CN Hydrolysates. β-CN hydrolysates were separated on a Zorbax C18 column (4.6 × 150 mm, 300 Å, 3.5 μm, Agilent Technologies, Massy, France), as described by Beuvier et al. (*18*). 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. (*19*). 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 of 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. To increase their immunogenicity, fractions of the peptides were conjugated 2:1 w/w to Keyhole Limpet Hemocyanin by the N-terminal sulfydryl group.

Production of Anti-Peptide Polyclonal Antibodies. Each conjugated peptide was used to immunize two rabbits, except (f94−113) and (f193−209) which were each used for 3 rabbits, and (f20−39) which was used for 4 rabbits. Adult female rabbits were given monthly multiple-site subcutaneous injections of conjugate−adjuvant emulsion, prepared from 0.5 mL of conjugate at 0.4 g/L in saline, and 0.5 mL of Freund's complete adjuvant (initial injection) or incomplete adjuvant (booster injection) (Difco Laboratories, Detroit, MI). From the third month onward, blood was collected on the 6th and 9th 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. (*15*). 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 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 toward 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,

Antipeptide Antibodies to Bovine β-Casein

J. Agric. Food Chem., Vol. 49, No. 3, 2001 **1573**

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. Aliquots of 100 μL were incubated, for 2 h 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 according to the following procedure. Gelatin (250 μL) at 10 g/L (1.04070, Merck), 100 μL of anti-peptide antiserum at 1:10 000 v/v, and 100 μL of goat anti-rabbit immunoglobulin–alkaline phosphatase conjugate at 1:3000 v/v (Sigma) were incubated successively for 1 h periods in the wells, at 37 °C in PBS-T (PBS buffer made 0.55 g/L Tween 20 (Merck)). Between each of the incubation steps, wells were rinsed for 15 s with four changes of 250 μL of PBS-T. Following the last rinsing, 100 μL of *p*-nitrophenyl phosphate (Sigma) at 1 mg/mL in 1M diethanolamine-HCl, 1 mM $MgCl_2$, and 0.1 mM zinc acetate was incubated in the wells. After 1 h, 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 (*20*). Briefly, 7 μg of whole casein or β-CN hydrolysate were separated on an 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. (*21*).

Immunodetection was conducted according to the following procedure. The membrane was incubated at room temperature, for 1 h periods, in 10 mM Tris-HCl, 0.5 M NaCl, 0.5 mM DTT, 0.275 g/L Tween 20, and 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 Naphthol 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: 35 μL of a solution of 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 5 μL/min. Immobilization of β-CN on the activated surface was achieved by using undiluted hybridoma culture supernatant containing anti β-CN (f1–25) MAb, the serum of a nonsensitized rabbit at 1:10 v/v, and pure β-CN at 10 mg/L, which 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 of 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 successively injecting three antisera onto the immobilized β-CN without regeneration of the surface.
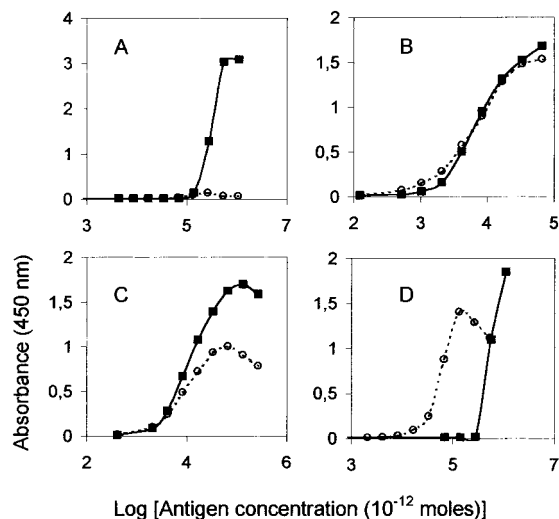

Figure 1. Reactivity of anti-peptide antiserum CDMP2 raised to β-CN (f184–202) (A), antiserum CEES1 raised to β-CN (f20–39) (B), antiserum CEDE1 raised to β-CN (f42–56) (C), antiserum CDMP1 raised to β-CN (f184–202) (D) toward β-CN (- -○- -) and their respective immunization peptides (–■–) in ACP–ELISA. Antisera diluted 1:10 000 v/v; procedure described in Materials and Methods.

RESULTS

Binding of Anti-Peptide Antisera to their Target Sequences. The reactivity of the antisera toward 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 three types of behavior: CEES1 displayed the same sensitivity and detection limit toward both antigens (Figure 1B), CEDE1 displayed a similar detection limit, but a lower sensitivity for β-CN than for the peptide (Figure 1C), and 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 toward β-CN was further investigated in Western-blott assay, 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 (R_f 0.190) agreed with that reported by McSweeney et al. (*21*). All anti-peptide antisera, including CDMP2, detected specifically β-CN (Lanes 2–7), 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 investiga-
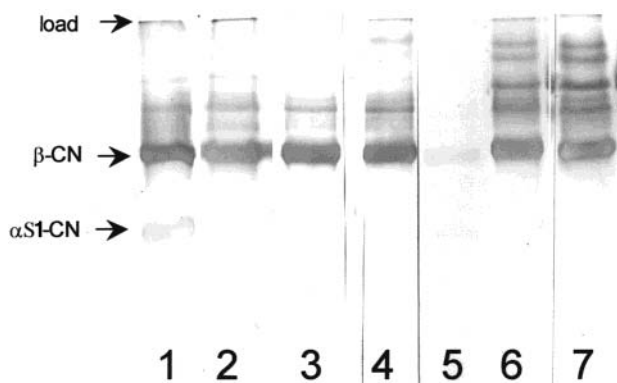
1574 *J. Agric. Food Chem.,* Vol. 49, No. 3, 2001

Senocq, et al.

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 and Methods.

Table 2. Reactivity of Anti-Peptide Antisera with Immunization Peptides and β-CN[a]

| | peptide | β-CN | | |
serum	ACP−ELISA	ACP-ELISA	Western-blott	biosensor
CEES1	+	+	+	+
CEDE1	+	+	+	+
CGVS1	+	+	+	+
CQSK1	+	−	±	−
CDMP1	+	+	+	+
CDMP2	+	−	+	+
CYQE1	+	+	+	+

[a] +/±/−, Antigenic/weakly antigenic/non antigenic in the assays described in Materials and Methods section.

tions. 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 Toward 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 concentrations. CEDE1 cross-reacted faintly with κ-CN, and CYQE1 cross-reacted with α_S-CN, but the nonspecific binding was kept below 10% of β-CN detection. In Western-blott, α_{S1}-CN migrated at R_f 0.295, as indicated by McSweeney et al. (*21*). 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 comigrated with reactive γ-caseins.

Localization of Determinants Recognized by Oligoclonal Anti-Peptide Antisera. 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). Following are the residual antigenicities of the peptides. 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

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

molecular mass (Da)		
measured[a]	theoretical[b]	peptide 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 (*28*).
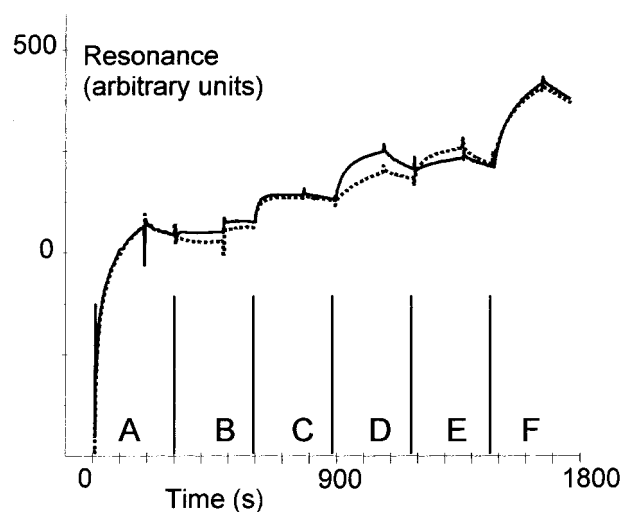

Figure 3. Specific capture (expressed as induced plasmonic resonance) of anti β-CN (f1−25) MAb (A), nonspecific rabbit IgG (B), β-CN (C), rabbit anti-peptide antiserum CDMP2 (−) and CDMP1 (− − −) raised to β-CN (f184−202) (D), rabbit anti-peptide antiserum CDMP1 (−) or CDMP2 (− − −) (E), and rabbit anti-peptide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

(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 neither did it 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 the 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
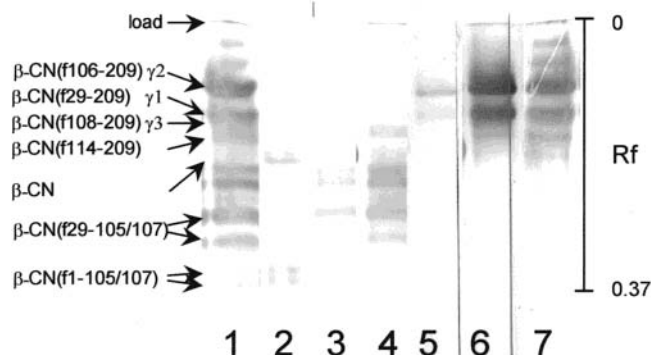
Antipeptide Antibodies to Bovine β-Casein

J. Agric. Food Chem., Vol. 49, No. 3, 2001 **1575**

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 and 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 h, pH 6.0) of plasmin (■) or chymosin (□) toward 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 1:10 000 v/v; detection of bound antibody as described in Materials and Methods.

apparently to the casein at two distinct and nonoverlapping 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 homologous to that of bovine. 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 positions 96, 101, and 103 of ovine and caprine sequences are mutated with respect to those of bovine sequence. 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 at least one 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 and Fox (*22*) 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 R_f 0.180 had mobility similar to that of the unknown component (f1-?) isolated by McSweeney et al. (*21*) 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 R_f 0.200 and 0.225 might be attributed to components (f29−97) and (f29−99), which are expected in the profile, but whose mobilities were not reported.
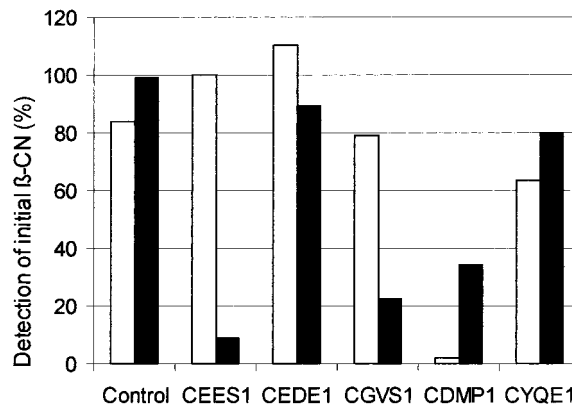
In addition, CEDE1 reacted faintly with minor bands at R_f 0.145 and 0.155. From their mobility and their reactivity with anti N-terminal antibodies, these components appeared to be clearly distinct from γ3-CN that migrated at R_f 0.140 (Lane 6). Detection using CGVS1 (Lane 4) revealed the same components 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-, and γ3-caseins and β-CN (f114−209), similar to the detection with CYQE1 (lane 7). In addition, CYQE1 reacted also with residual β-CN, and detected specifically two unidentified components at R_f 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 percent 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 toward 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

1576 *J. Agric. Food Chem.,* Vol. 49, No. 3, 2001

Senocq, et al.

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 those of 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 (*9*). 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 also did not 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 toward 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. (*23*), the carrier conjugated at the peptide N-terminus exerted no obvious negative influence, likely because of 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 subpopulations 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 that was similar for both antisera, whereas they were clearly different in the ELISA assay. It is likely that reducing the peptide's length would contribute to avoiding elicitation of 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, respectively) 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. 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 (*24, 25*). 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 in situ comparison of 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 toward 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 the C-terminal segments of β- and γ-caseins (*26*). In most cheeses, and particularly in Cheddar cheese, chymosin therefore contributes very little, if at all, to the breakdown of γ- and β-caseins (*21*). Peptides resulting from chymosin-like-specificity cleavage at this segment were however evidenced in mature Cheddar cheeses (*6*). *Lactococcus* starter cell envelope proteinases or cathepsin D might be responsible for these cleavages, but evidence is still needed. Release of these peptides cannot yet be 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 AND NOMENCLATURE USED

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. (*27*), 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 ionization−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; R_f, electrophoretic

Antipeptide Antibodies to Bovine β-Casein

J. Agric. Food Chem., Vol. 49, No. 3, 2001 **1577**

mobility ratio to the migration front; RPHPLC, reversed-phase high-pressure liquid chromatography.

DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid disodium; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*-[ethanesulfonic acid]; Tris, tris-(hydroxymethyl) aminomethane.

LITERATURE CITED

(1) Fox, P. F.; McSweeney, P. I. H. Proteolysis in cheese during ripening. *Food Rev. Int.* **1996**, *12*, 457−509.

(2) Grappin, R.; Rank, T. C.; Olson, N. F. Primary proteolysis of cheese proteins during ripening: A review. *J. Dairy Sci.* **1985**, *68*, 531−540.

(3) Ollikainen, P.; Kivela, T. The importance of plasmin in Swiss-type cheese ripening. *Milchwissenschaft* **1989**, *44*, 204−206.

(4) Andrews, A. T.; Alichanidis, E. Proteolysis of caseins and the proteose-peptone fraction of bovine milk. *J. Dairy Res.* **1983**, *50*, 275−290.

(5) Visser, S.; Noorman, H. J.; Slangen, C. J.; Rollema, H. S. Action of plasmin on bovine β-casein in a membrane reactor. *J. Dairy Res.* **1989**, *56*, 323−333.

(6) Singh, T. K.; Fox, P. F.; Healy, A. Isolation and identification of further peptides in the diafiltration retentate of the water-soluble fraction of Cheddar cheese. *J. Dairy Res.* **1997**, *64*, 433−443.

(7) Addeo, F.; Garbo, G.; Intorcia, N.; Pellegrino, L.; Resmini, P.; Chianese, L. Gel electrophoresis and immunoblotting for the detection of casein proteolysis in cheese. *J. Dairy Res.* **1995**, *62*, 297−309.

(8) Picard, C. *Production d'anticorps anti-peptides synthétiques pour le dosage du caséinomacropeptide de vache par une méthode ELISA d'inhibition.* Thesis, Université de Bourgogne, France, 1993.

(9) Pizzano, R.; Nicolai, M. A.; Addeo, F. Antipeptide antibodies in milk and cheese analysis. *Recent Res. Dev. Agric. Food Chem.* **1999**, *3*, 403−412.

(10) IDF. *Calf rennet and adult bovine rennet. Determination of chymosin and bovine pepsin contents (chromatographic method)*; Standard 110A; International Dairy Federation: Brussels, Belgium, 1987.

(11) Deutsch, D. G.; Mertz, E. T. Plasminogen: purification from human plasma by affinity chromatography. *Science* **1970**, *170*, 1095−1096.

(12) Wei, T. M.; Whitney, R. M. Batch fractionation of bovine caseins with DEA-cellulose. *J. Dairy Sci.* **1984**, *68*, 1630−1636.

(13) Leaver, J.; Law, A. J. R. Preparative scale purification of bovine caseins on a cation-exchange resin. *J. Dairy Res.* **1992**, *59*, 557−561.

(14) Andrews, A. T.; Taylor, M. D.; Owen, A. J. Rapid analysis of bovine milk proteins by fast protein liquid chromatography. *J. Chromatogr.* **1985**, *348*, 177−185.

(15) Jeanson, S.; Dupont, D.; Grattard, N.; Rolet-Répécaud, O. Characterization of the heat treatment undergone by milk using two inhibition ELISAs for quantification of native and heat denatured α-Lactalbumin. *J. Agric. Food Chem.* **1999**, *47*, 2249−2254.

(16) Collin, J. C.; Kokelaar, A.; Rolet-Répécaud, O.; Delacroix-Buchet, A. Dosage des caséines du lait de vache par électrophorèse et par chromatographie liquide rapide d'échange d'ions (FPLC): comparaison des résultats. *Lait* **1991**, *71*, 339−350.

(17) Levieux, D. Immunoglobulines bovines et brucellose. Purification des immunoglobulines et préparation de leurs antisérums spécifiques. *Ann. Rech. Vét.* **1974**, *5*, 329−342.

(18) Beuvier, E.; Berthaud, K.; Cegarra, S.; Dasen, A.; Pochet, S.; Buchin, S.; Duboz, G. Ripening and quality of swiss-type cheese made from raw, pasteurized or microfiltered milk. *Int. Dairy J.* **1997**, *7*, 311−323.

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

(20) Andrews, A. T. Proteinases in normal bovine milk and their action on caseins. *J. Dairy Res.* **1983**, *50*, 45−55.

(21) McSweeney, P. I. H.; Pochet, S.; Fox, P. F.; Healy, A. Partial identification of peptides from the water-insoluble fraction of cheddar cheese. *J. Dairy Res.* **1994**, *61*, 587−590.

(22) Lane, C. N.; Fox, P. F. The individual or combined action of chymosin and plasmin on sodium caseinate or β-CN in solution: effect of NaCl and pH. *Lait* **1999**, *79*, 423−434.

(23) Schaaper, W. M. M.; Lankhof, H.; Puijik, W. C.; Meloen, R. H. Manipulation of antipeptide immune response by varying of the coupling of the peptide with the carrier protein. *Mol. Immunol.* **1989**, *26*, 81−85.

(24) Collin, J. C.; Berdagué, J. L.; Dognin-Bergeret, M.; Grappin, R. Affinage et qualité du gruyère de Comté. IV. Etude de la protéolyse. *Lait* **1987**, *67*, 299−318.

(25) Roudot-Algaron, F.; Le Bars, D.; Kerhoas, L.; Einhorn, J.; Gripon, J. C. Phosphopeptides from Comté cheese: nature and origin. *J. Food Sci.* **1994**, *59*, 544−548.

(26) Exterkate, F. A.; Lagerwerf, F. M.; Haverkampf, J.; Van Schalkwijk, S. The selectivity of chymosin action on α_{S1}- and β-casein in solution is modulated in cheese. *Int. Dairy J.* **1997**, *7*, 47−54.

(27) Eigel, W. N.; Butler, J. E.; Ernstrom, C. A.; Farrell, H. M.; Harwalkar, V. R.; Jenness, R.; Whitney, R., McL. Nomenclature for proteins of cow's milk: fifth revision. *J. Dairy Sci.* **1984**, *67*, 1599−1631.

(28) Jimenez-Flores, R.; Kang, Y. C.; Richardson, T. Cloning and sequence analysis of bovine β-casein cDNA. *Biochem. Biophys. Res. Commun.* **1987**, *142*, 617−621.