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ELISA for monitoring the cleavage of β-casein at site Lys[28]–Lys[29] by plasmin during Comté cheese ripening

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Proteolysis of casein is the principal cause of textural changes and flavour development in ripened cheese (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-characterised fragments. This initial step, called primary proteolysis, is catalysed principally by the residual coagulant (chymosin and pepsin), and to a variable extent by endogenous milk proteases, such as plasmin, cathepsin D, and possibly somatic cell proteinases. Enzymes originating from either rennet or milk are active in most ripened cheese varieties. However, their relative contributions vary substantially depending on manufacturing practices. For instance, in Swiss-type cheeses, cooking the curd extensively inactivates the coagulant, and simultaneously enhances plasmin activity, which therefore becomes predominant (Ollikainen & Kivela, 1989).

A number of the fragments produced by primary proteolysis undergo intense secondary proteolysis, which is believed to be catalysed mainly by additional enzymes from the microflora. Simultaneous proteolysis by enzymes of different specificity leads to a complex mixture of partially overlapping peptides. For instance, more than 100 β-CN fragments have been isolated from Cheddar cheese (Singh *et al.* 1997).

Plasmin preferentially hydrolyses β-CN, α_{s2}-CN, and to a lesser extent α_{s1}-CN, but it does not hydrolyse κ-CN. Plasmin is highly specific for lysine-X type peptide bonds and, to a lesser extent, arginine-X. Indeed, plasmin cleaves all 15 potential sites in β-CN sequence in a recycle reactor (Visser *et al.* 1989). However, three bonds are predominantly cleaved in milk and cheese: Lys[28]–Lys[29], Lys[105]–His[106] and Lys[107]–Glu[108], cleavage of which releases the polypeptides β-CN f(29–209), f(106–209) and f(108–209), known as γ_1-CN, γ_2-CN and γ_3-CN, respectively. Their complementary peptides, β-CN f(1–28), f(1–105), f(1–107), f(29–105) and f(29–107), contribute to the proteose peptone fraction of milk (Andrews & Alichanidis, 1983).

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These events, as compulsory introductory steps of β-CN proteolysis, limit the rate of subsequent peptide degradation, thought to be entirely responsible for the cheese characteristics. Indeed, in Swiss-type cheese, plasmin concentration was significantly correlated with the organoleptic quality of the cheese (Bastian *et al.* 1997). Much effort has thus been devoted to specifically monitor the progress of plasmin activity in cheese. Analytical techniques, which successfully characterise the complex proteolytic pattern achieved in mature cheeses, are of limited value in tracking back to the early steps performed by plasmin.

In recent years, immunoassays, which are both highly sensitive and specific compared with classical biochemical techniques, have offered good prospects for achieving this goal. For instance, Addeo *et al.* (1995) used anti β-CN antibodies to detect specific β-CN proteolysis patterns in Western-Blott. In parallel, our team and others have tried more specific anti-peptide antibodies as tentative probes for peptides released during significant individual cleavage events, such as κ-CN f(106–169) (Picard, 1993) and β-CN f(1–28) (Pizzano *et al.* 2000). Both studies encountered the cross-detection of the target peptides' precursors as a major drawback.

Recently, we explored a different approach in order to overcome this problem: we produced anti-peptide antibodies against major cleavage sites of plasmin in β-CN sequence (Senocq *et al.* 2001). In particular, the anti-β-CN f(20–39) antibody CEES1 was specifically designed to recognise the β-CN Lys^{28}–Lys^{29} bond. This antibody was shown to recognise only β-CN and no other milk protein, and cross-reacted with β-CN from ewe and goat. Using this antibody, an indirect ELISA was developed that detected hydrolysis of β-CN in aqueous solutions by plasmin, but not by chymosin. Therefore, this technique was considered as a promising way to monitor β-CN hydrolysis into γ_1-CN by plasmin *in situ*.

In this study, we used this ELISA to monitor plasmin action at the β-CN cleavage site Lys^{28}–Lys^{29} during ripening of Comté, a Swiss-type cheese. Using this technique, it was demonstrated that the temperature of the warm room used for Comté cheese ripening strongly influenced hydrolysis of β-CN by plasmin at the Lys^{28}–Lys^{29} bond.

materials and methods

Purification of β-casein

Individual milk, found to be homozygous for β-CN A^2 by isoelectric focusing was obtained from the Coopérative Graindorge S.A. (F-14140 Livarot, France). The top fat layer was removed after centrifugation (1800 ***g***, 4 °C, 15 min). Whole casein was obtained from skim milk by isoelectric precipitation at pH 4·6 (Wei & Whitney, 1984).

Crude β-CN was obtained in gram amounts by batch-wise ion exchange (Leaver & Law, 1992), dialysed against water, and lyophilised. The purity determined by urea-PAGE was $\sim 90\,\%$.

Homogeneous β-CN was obtained from crude β-CN by fast protein liquid chromatography (FPLC) ion exchange (Andrews *et al.* 1985), scaled-down to fit a HR16/10 column (Pharmacia, S-751 82 Uppsala, Sweden). Briefly, 600 mg of crude β-CN were loaded onto a 20-ml column of Q Sepharose FF (Pharmacia), and eluted at 1 ml/min with a linear gradient of 0·09–0·32 M-NaCl in buffer (25 mM-Tris-HCl–4·5 M-urea–0·08 mM-DTT, pH 7·0). Fractions collected every 5 min were checked by urea-PAGE for homogeneity, pooled, dialysed against water, and lyophilised.

The γ-CN fraction was prepared according to Collin *et al.* (1991).

Table 1. *Ripening processes applied to Comté cheese, storage temperature and duration*

Process	Pre-ripening		Warm room	Cold room
1	13 °C, 2 weeks		17 °C, 5 weeks	6 °C, 10 weeks
2	13 °C, 7 weeks		17 °C, 5 weeks	6 °C, 9 weeks
3	13 °C, 3 weeks	10 °C, 5 weeks	17 °C, 5 weeks	6 °C, 9 weeks
4	13 °C, 7 weeks		19 °C, 5 weeks	6 °C, 9 weeks
5	13 °C, 3 weeks	10 °C, 5 weeks	17 °C, 5 weeks	6 °C, 4 weeks

Plasmin preparation

Inactive plasminogen was isolated from fresh bovine blood (Deutsch & Mertz, 1970), and kept frozen at –20 °C. Aliquots were activated to plasmin (E.C.3.4.21.7) immediately before use, by treating for 10 min at 37 °C with urokinase (E.C.3.4.21.73; Choay, Sanofi Winthrop, F-94250 Gentilly, France) at 10 IU/g.

Hydrolysis of β-CN by plasmin in solution

Purified bovine β-CN was hydrolysed by plasmin at 1 mg/g at 37 °C, in 75 mM-ammonium acetate buffer, pH 7·0. Hydrolysis was stopped by adding a specific irreversible inhibitor, N-α-*p*-tosyl-L-lysine chloromethyl ketane, at 0·67 mM after 0, 5, 10, 15, 20, 25, 60, 90 or 150 min incubation.

Production of antibodies

The antiserum CEES1 was raised against the peptide β-CN f(20–39) as described elsewhere (Senocq *et al.* 2001). Briefly, 90 % pure peptide CEESITRINKKIEKF-QSEEQQ was chemically synthesised, and modified by C-terminal amidation (Synt:em, F-30000 Nîmes, France). This sequence corresponds to β-CN f(20–39), with a Ser residue substituted for the phosphorylated residue $SerP^{35}$, and an additional N-terminal Cys residue. This peptide, conjugated 2:1 w/w to Keyhole Limpet Hemocyanin by the terminal sulphydryl group, was given monthly to a female rabbit by multiple site subcutaneous injections. Conjugate-adjuvant emulsion was 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, Detroit, MI 48232, USA). Antiserum obtained from a single bleeding on month 3, 6 d after injection, was used throughout this study.

Polyclonal anti-casein antiserum was likewise obtained from a rabbit given 0·5 mg aliquots of purified β-CN.

Cheese samples

Influence of ripening procedure on hydrolysis of the β-CN Lys^{28}–Lys^{29} *bond*. Four Comté cheeses, considered to be identical because they were made in the same vat from the same milk, were obtained from Comté cheese producers and ripened following four different ripening processes. This procedure was repeated 10 times for a total number of 40 cheeses. Each ripening process comprised a pre-ripening period, a warm-room period and eventually a cold-room period, and differed in duration and applied temperature (Table 1). The cheeses were sampled before the pre-ripening, during the warm-room and the cold-room periods and at full maturity.

Influence of Lactococcus lactis *proteinase activity on the results obtained using ELISA*. Comté is a Swiss-type cheese made from raw milk. Its manufacture starts by incubation of the milk with *Lc. lactis* for 30–120 min at 28–32 °C. Then, thermophilic

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starters (*Streptococcus thermophilus* and *Lactobacillus helveticus*) are added and the milk is coagulated by the action of calf rennet. In order to check whether the action of *Lc. lactis* proteinases, known to hydrolyse the Lys^{27}–Lys^{28} peptide bond (Juillard *et al.* 1995), was able to modify the percentage of β-casein intact at site Lys^{28}–Lys^{29} as determined by ELISA, the following experiment was carried out: two Comté-type cheeses were manufactured with a milk incubated 2 h at 32 °C with a cocktail of three strains of *Lc. lactis* previously isolated from Comté cheeses (Jeanson, 2000). In parallel, two Comté-type cheeses were made from the same milk without *Lc. lactis*. After manufacture, these four cheeses were ripened according to process 5, Table 1, and sampled for analysis by ELISA.

Cheese samples preparation

Samples (1 g) were finely grated and dispersed in 40 ml buffer (0·4 M-trisodium citrate–0·15 M-NaCl–75 mM-EDTA–10 mM DTT–8 M-urea, pH 7·8) for 1 h at 40 °C and then 45 min at room temperature. The solid fat layer was discarded after centrifugation at 1800 ***g*** at 4 °C for 15 min.

ELISA

Flat-bottomed ELISA plates (NUNC Maxisorp, Polylabo, F-67023 Strasbourg, France) were coated with 100 μl of either (1) serial dilutions of purified β-CN, from 6·4 μg/ml to 0·1 μg/ml in 0·1 M-sodium bicarbonate buffer, pH 9·6, or (2) β-CN treated with plasmin and diluted at 0·8 μg/ml in 0·05 M-sodium phosphate–0·15 M-NaCl buffer, pH 7·2 (PBS), or (3) cheese dispersion diluted at 50 μl/l in PBS. Each sample was coated simultaneously onto two separate wells.

Plates were then incubated for 2 h at 37 °C. Blocking of the remaining binding sites was performed by adding 250 μl gelatine (Sigma, St. Louis, MO 63178, USA) at 10 g/l PBS-T buffer (0·55 g Tween 20/l PBS) and incubating for 1 h at 37 °C. Then, anti-peptide antibody CEES1 1/15000 v/v in PBS-T was added to each well, and the plate was further incubated for 1 h at 37 °C. Bound immune complexes were revealed by incubating 100 μl goat anti-rabbit immunoglobulin–alkaline phosphatase conjugate (Sigma) 0·33 ml PBS-T/l for 1 h at 37 °C. Wells were rinsed between each incubation step, four times for 15 s with 250 μl PBS-T. Following the last rinsing, 100 μl *p*-nitrophenyl phosphate (Sigma), 1 g/l buffer (1 M-diethanolamine–HCl–1 mM-$MgCl_2$, 0·1 mM-zinc acetate) was added to the wells. After 1 h at 37 °C, 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. Each determination was carried out in triplicate on different days, except cheese dispersions which were assessed four times independently.

Urea-PAGE

Urea-PAGE was carried out on discontinuous gels according to the method of Andrews (1983). Briefly, 7 μg β-CN, either intact or treated with plasmin, 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 an intensity of 20 mA at 15 °C for 60 min. Bands were identified from their relative mobility (R_f) with reference to Mc Sweeney *et al.* (1994). γ_1-CN migrated at R_f 0·16. Staining was performed as described by Blakesley & Boezi (1977). Gels were scanned and analysed using GS-670 densitometric analyser and image analysis software Molecular Analyst (BioRad, Ivry F-94203, France). Densitometric profiles were manually integrated. Determinations were run in triplicates on different days.

SDS-PAGE

SDS-PAGE was carried out using precast Tricine gels (16% T, 2·6% C, pH 8·3) from Novel Experimental Tech (D-65929 Frankfurt, Germany) and 121 g Tris/l–179 g tricine/l–10 g SDS/l running buffer, pH 8·3.

Western-Blotting

Immediately after separation on SDS-PAGE gels, peptides were transferred from the gel onto a 0·2-μm nitrocellulose membrane (Whatman Biobind-NC, Polylabo; Mc Sweeney *et al.* 1994).

Immunodetection was as follows: the membrane was incubated at room temperature, for 1-h periods, in buffer (10 mM-Tris–HCl–0·5 M-NaCl–0·5 mM-DTT, 0·275 g Tween 20/l, pH 8·0), with, successively, heat inactivated horse serum (1/10 v/v), anti-peptide antibody CEES1 1 ml/l, and goat anti-rabbit immunoglobulin–alkaline phosphatase conjugate 0·33 ml/l (Sigma). The membrane was washed between each step by soaking four times for 5 min in the same buffer. After the last washing, the membrane was stained for ~ 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.

Statistical analysis

Median comparisons of residual intact β-CN, between cheeses ripened in a warm room at 17 °C or 19 °C, was carried out using a Wilcoxon rank sum test on S-plus® 3·4.

RESULTS

Specificity of anti-peptide antibody CEES1 toward β-CN hydrolysates in vitro

Kinetics of hydrolysis of β-CN by plasmin in aqueous solution was followed by western-blotting using as primary antibody, either the anti-peptide antibody CEES1 or a rabbit polyclonal antiserum raised against β-CN (Fig. 1). Control polyclonal antiserum detected β-CN as a band with an apparent Mw 23·6 kDa, that decreased regularly with time to almost disappear after 150 min hydrolysis. Meanwhile, two bands at 19·9 and 15·4 kDa, corresponding to γ_1- and co-migrating γ_2- and γ_3-caseins, respectively, increased to reach a maximum after 25 min hydrolysis and then decreased to eventually disappear after 150 min hydrolysis. Finally, a band around 8·6 kDa was revealed by control polyclonal antiserum between 0 to 150 min, and appeared, from both its Mw and its end-product status, to correspond to β-CN f(29–105/7).

Anti-peptide antibody CEES1 detected β-CN, but did not reveal $\gamma_2+\gamma_3$, nor γ_1-CN. It detected a band at 11·7 kDa that may correspond to proteose-peptone component 5. The observed antigenicity of β-CN peptides was in accordance with the theoretical specificity of the anti-peptide antibody CEES1, which is directed against an epitope centred on the Lys^{28}–Lys^{29} peptide bond.

Standard curve of indirect ELISA

The standard curve obtained using anti-peptide antibody CEES1 as primary antibody and purified β-CN as antigen in an indirect ELISA indicated a log-linear detection (R^2 0·985) between 200 and 1600 ng/ml β-CN.

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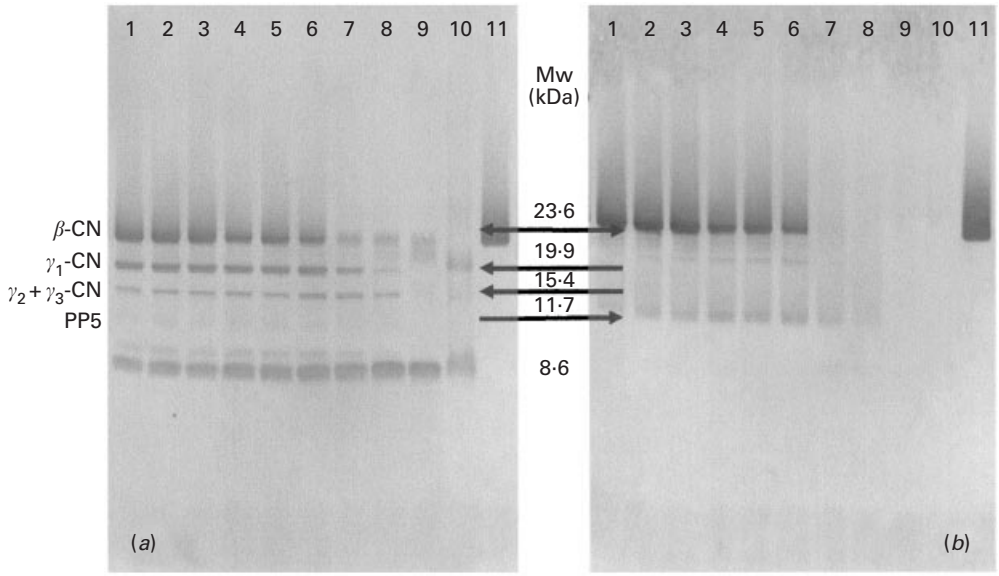

Fig. 1. Immunoblotting of β-casein hydrolysed by plasmin (ratio of 1/1000 w/w; Lanes 1 to 9), and purified γ- (Lane 10), and β-casein (Lane 11). Lanes 1 to 9 correspond to hydrolysis times of 0, 5, 10, 15, 20, 25, 60, 90 and 150 min, respectively. Staining with a control anti-β-casein polyclonal serum (a) or the anti-peptide antibody CEES1 (b) as primary antibody.

Monitoring of hydrolysis of β-CN Lys^{28}–Lys^{29} by plasmin in aqueous solution

The level of intact Lys^{28}–Lys^{29} bonds of β-CN during 150 min hydrolysis by plasmin was determined by ELISA using antibody CEES1. Simultaneously, the concentration of γ_1-CN was determined by urea-PAGE. Hydrolysis of the β-CN Lys^{28}–Lys^{29} bond increased over the 150-min period (not shown). In contrast, the concentration of γ_1-CN increased for the first 30 min hydrolysis to reach a maximum and then decreased to completely disappear after 90 min. These results clearly show that, while the cleavage of the Lys^{28}–Lys^{29} bond in β-CN is in progress, the concentration of the product γ_1-CN is controlled by the rate of its secondary degradation. In contrast, ELISA is independent of secondary degradation and reflects accurately the plasmin activity at the specific Lys^{28}–Lys^{29} bond.

Influence of Lc. lactis *proteinase activity on the results obtained using ELISA*

In order to check whether determination of β-CN intact at the Lys^{28}–Lys^{29} bond was influenced or not by the proteolytic activity of *Lc. lactis*, four Comté-type cheeses were manufactured from the same raw milk, two with *Lc. lactis* and two without. Percentages of β-CN intact at Lys^{28}–Lys^{29} bond after 4 months ripening were $67\% \pm 1$ and $69\% \pm 3$ for the cheeses made with or without *Lc. lactis*, respectively, showing that *Lc. lactis* proteinase activity has no significant influence on the percentages determined by ELISA in Comté-type cheeses.

Indirect ELISA for monitoring cleavage of β-CN Lys^{28}–Lys^{29} in Comté cheese during ripening

The ELISA test was applied to four identical Comté cheeses ripened according to four different processes (1–4, Table 1) in order to monitor the rate of degradation of β-CN by plasmin at the Lys^{28}–Lys^{29} bond. Figure 2 displays the estimated disappearance of this bond in its intact form. During the pre-ripening period, rates

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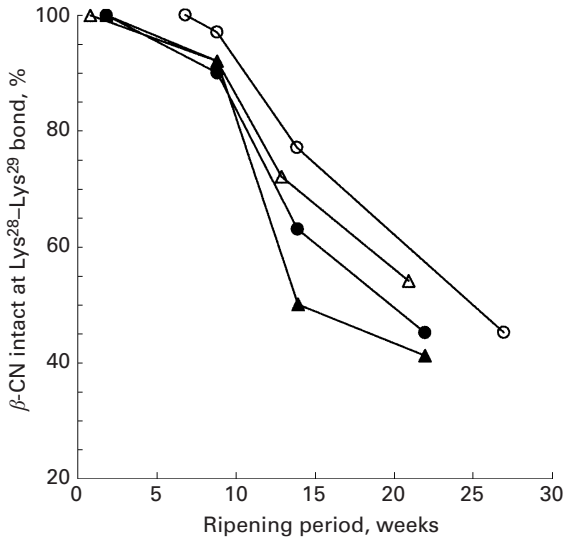

Fig. 2. Concentration of the β-CN peptide fraction containing intact epitope for the anti-peptide antibody CEES1 at the Lys^{28}–Lys^{29} bond in four identical Comté cheeses during the whole maturation processes 1 (–○–), 2 (–●–), 3 (–▽–) and 4 (–▲–). For details on the ripening processes, see the Material and Methods section (Table 1). All curves are shifted for simultaneous beginning of warm room period on week 8.

Table 2. *Percentage of residual β-CN determinant kept intact at the Lys^{28}–Lys^{29} bond after a 5-week period in a warm room at 17 or 19 °C. Cheeses were pairs produced from the same milk in the same vat*

(Values are means ± SD for $n = 4$)

	Residual β-CN (%)	
Cheese	17 °C	19 °C
1	73·0 ± 1·7	60·0 ± 0·8
2	86·6 ± 3·6	75·7 ± 1·4
3	85·4 ± 1·7	78·2 ± 0·9
4	74·5 ± 1·3	68·3 ± 0·8
5	79·2 ± 1·2	72·4 ± 4·7
6	79·6 ± 4·1	77·5 ± 0·9
7	89·5 ± 8·9	76·1 ± 1·9
8	95·5 ± 2·5	69·4 ± 1·7
9	75·2 ± 1·6	73·7 ± 2·3
10	79·7 ± 1·5	54·3 ± 1·5

Values at 19 °C were significantly higher than the corresponding value at 17 °C ($P < 0·01$).

of hydrolysis of β-CN Lys^{28}–Lys^{29} were identical for the four ripening processes. Process 1 eventually resulted in a slower degradation because the duration was shorter (2 weeks instead of 7–8). Transfer of cheeses to the warm room resulted in an acceleration of the hydrolysis of β-CN at the Lys^{28}–Lys^{29} bond for all of the ripening processes. Acceleration was higher for process 4, where holding temperature was 2 deg C higher than in processes 1 to 3. Finally, the rate of hydrolysis of β-CN Lys^{28}–Lys^{29} bond in the cold room was lower than in the warm room for all the ripening processes. After the ripening processes were completed, the percentage of intact β-CN Lys^{28}–Lys^{29} bonds ranged from 55% to 40% for processes 3 and 4, respectively, while values for processes 1 and 2 were intermediate.

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Effect of warm room temperature used for Comté cheese ripening on hydrolysis by plasmin of the Lys^{28}–Lys^{29} bond of β-CN

The influence of warm room temperature was further explored by monitoring, by ELISA, ten pairs of identical Comté cheeses (made from the same milk in the same vat), ripened using either process 2 or 4, during the 5-week warm-room period. Levels of intact β-CN Lys^{28}–Lys^{29} bond were determined before and after the warm-room period and percentage hydrolysis was calculated (Table 2). Hydrolysis was significantly higher ($P < 0{\cdot}01$) when warm room temperature was increased by only 2 degrees.

discussion

In this study, we have demonstrated that it was possible to monitor precisely β-CN hydrolysis specifically at the Lys^{28}–Lys^{29} bond during Comté cheese ripening. This new approach for monitoring enzymatic proteolysis uses an antipeptide antibody directed against an antigenic determinant that includes a peptidic bond susceptible to cleavage. The antibody binds on the targeted determinant of the protein only if the peptide bond is intact. This approach, with a wide potential application, is particularly relevant to the study of casein degradation during cheese ripening. Indeed, in most cheeses, rapid secondary proteolysis of all casein fragments generally impairs the quantitative detection of potential marker peptides. Monitoring of β-CN cleavage at Lys^{28}–Lys^{29} bond by means of marker peptide quantification proved impossible. Peptide β-CN f(1–28) is further degraded by bacterial proteases and peptidases, especially between residues 6/7 and 14/15 (Ferranti *et al.* 1997), while there is evidence that γ_1-CN is further shortened to γ_2-CN and γ_3-CN by plasmin itself. As a consequence, the concentrations in hard cheeses of both γ_1-CN (Collin *et al.* 1987; Delacroix-Buchet & Fournier, 1992) and β-CN f(1–28) (Pizzano *et al.* 2000) have been found to depend primarily on the rate of their catabolism rather than on their release by plasmin.

Using the ELISA test, we have observed that ripening conditions significantly influence hydrolysis of Lys^{28}–Lys^{29} bonds in cheese. It is generally accepted that elevating the ripening temperature toward the optimal temperature for both microbial proliferation and enzyme activity accelerates cheese maturation. Increasing the ripening temperature leads to a significant increase in proteolysis, as has been previously demonstrated for Cheddar (Aston *et al.* 1983, 1985; Fedrick *et al.* 1983), Serena (Gonzalez *et al.* 1990) and Montasio cheese (Innocente & Corradini, 1996). More recently, Chin & Rosenberg (1998) showed that increasing the ripening temperature from 5 to only 8 °C also resulted in notable changes in the rate and extent of proteolysis. Applying a detection method specific for the single cleavage site Lys^{28}–Lys^{29} in β-CN, we have similarly observed that increasing the warm-room temperature by two degrees significantly accelerates β-CN hydrolysis in Comté cheese. Since the Lys^{28}–Lys^{29} bond is hydrolysed mainly by plasmin in Comté cheese, we separated out the effect of temperature on a single enzyme at a very specific site.

The Lys^{28}–Lys^{29} peptide bond is the preferred cleavage site of plasmin in β-CN *in vitro*, and in cheeses. Therefore our method may be used to estimate *in situ* the activity of cheese plasmin. Alternatively, plasmin activity in cheese may be estimated by either measuring its cumulated activity on β-CN at all three major bonds: Lys^{28}–Lys^{29}, Lys^{105}–His^{106} and Lys^{107}–Glu^{108}, or by assessing *in vitro* the potential activity of extractable plasmin (Rollema *et al.* 1983). The former is performed by measuring the concentration of the resulting insoluble fraction γ-CN.

This method is not fully satisfactory since secondary proteolysis of γ-caseins has been shown. Ferranti *et al*. (1997) and Singh *et al*. (1995, 1997) demonstrated that in Grana-Padano and Cheddar cheeses respectively, γ-caseins were further cleaved between residues 192/193 and/or 193/194, identified cleavage sites of *Lactococcus* cell envelope proteinase (Singh *et al*. 1995). In addition, measuring activity of extracted plasmin on a synthetic substrate in a buffered medium is obviously a poor substitute for measuring plasmin activity in cheese, which is now possible using ELISA. Beside plasmin, other enzymes cleave the Lys^{28}–Lys^{29} bond of *β-CN in vitro*, and thus potentially compete with plasmin *in situ*. In Comté Cheese, only proteinase from *Lc. lactis* (Juillard *et al*. 1995) is acknowledged, but we demonstrated here that this protease had no significant influence on determination of the percentage of β-CN intact at the Lys^{28}–Lys^{29} bond. However, in some other cheese varieties, metalloproteinases of *Penicillium roqueforti* and *Penicillium camemberti* (Trieu-Cuot *et al*. 1982) will also occur. This possible competition would, if verified *in situ*, result in an overestimation of plasmin activity in cheese by all current methods, including ELISA. However, it is not possible to monitor enzymatic competition in cheese by current methods, and furthermore, there is theoretical and experimental evidence that enzymatic specificity assessed *in vitro* might not accurately reflect that prevalent *in situ* (Reid & Coolbear, 1998).

The anti-peptide antibody CEES1 is oligoclonal. We have previously observed minor cross-detection of peptide β-CN f(1–28) that may slightly bias assay of plasmin activity. This problem could be overcome by assessing the cheese insoluble fraction devoid of soluble β-CN f(1–28), assuming that an increase of the analysis time is acceptable. Alternatively, raising a monoclonal antibody with a sharper specificity centred on the Lys^{28}–Lys^{29} peptide bond would be preferable, especially if a routine application is envisaged. This work is currently in progress.

With this exploratory work focusing on the hydrolysis of β-CN at the Lys^{28}–Lys^{29} bond, we assessed the feasibility of this approach. Detailed study of proteolysis during cheese ripening, now requires development of a battery of antibodies specific to the major cleavage sites of different caseins by coagulant enzymes, indigenous enzymes, bacterial proteases or possibly peptidases.

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