Immunochemical Evaluation of Bovine β-Casein and Its 1−28 Phosphopeptide in Cheese during Ripening

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Polyclonal antibodies raised against the plasmin-released 1−28 phosphopeptide from bovine β-casein [i.e., β-CN(f1−28)4P] specifically recognized the tryptic β-casein 1−25 and 2−25 peptides, whatever the degree of phosphorylation, but were unresponsive to the shortened β-casein 16−22 phosphopeptide. These antibodies were able to recognize the parent bovine β-casein as well as the homologous water buffalo protein, but they could not detect the homologous counterparts from ovine and caprine milks. Such antibodies were used in competitive enzyme-linked immunosorbent assays to monitor the plasmin-mediated release of the 1−28 phosphopeptide from β-casein and to evaluate the residual native β-casein in bovine cheese sampled during ripening. Applications of these polyclonal antibodies are suggested mainly for estimating the age of hard cheeses and, possibly, for tracing the presence of bovine casein in fresh ovine and caprine cheeses.

Keywords: *Cheese; ripening; quality certification; phosphopeptides; antipeptide antibodies; ELISA; immunoblotting*

INTRODUCTION

Casein proteolysis is regarded as one of the major events in the determination of typical sensory characteristics and quality of mature cheeses. The crucial steps of proteolysis occurring during the ripening phase of cheese-making were identified over a decade ago (Grappin et al., 1985). A significant role is played by plasmin (EC 3.4.21.7), the most important of the proteinases generally found in milk, acting in the breakdown of β- and α_{s2}-caseins in stored milk and cheese (Grufferty and Fox, 1988). Plasmin hydrolysis of β-casein leads to the formation of pH 4.6 insoluble γ-caseins, that is, γ_1-, γ_2-, and γ_3-caseins, corresponding to the β-casein fragments 29−209, 106−209, and 108−209, respectively (Eigel et al., 1979). The peptides complementary to γ-caseins contribute to the so-called proteose−peptone fraction of milk (Andrews and Alichanidis, 1983) and were found to give rise to most of the β-casein low molecular mass peptides in long-term-ripened cheeses (Addeo et al., 1992, 1994; Roudot-Algaron et al., 1994; Ferranti et al., 1997b; Singh et al., 1995, 1997).

For quality certification of matured cheeses, objective parameters for estimating the age of cheese are required. γ_3-Casein, isolated from a 24-month-aged cheese sample, was found to be a reliable indicator of ripening in Parmigiano-Reggiano cheese (Addeo et al., 1995). An analytical method, based on the densitometric evaluation of the γ-casein fractions along the isoelectric focusing profile of a Grana Padano cheese sample, was proposed to determine cheese age (Restani et al., 1996). More recently, an electrophoretic ripening index for the deduction of the age of Grana Padano cheese samples has been established (Mayer et al., 1998). However, electrophoretic methodologies, requiring tedious and cumbersome procedures, appear to be somewhat unsuitable for routine control. On the other hand, analysis of cheese samples using conventional immunochemical techniques is complex, owing to the simultaneous presence of the closely related products arising from each casein fraction. Nevertheless, antipeptide antibody technology has provided especially valuable analytical tools when selected sequences must be targeted (Groome, 1994).

In this paper an immunochemical methodology for monitoring cheese ripening and identifying parameters that are well-correlated with the age of cheese is reported. The study focused on the proteolytic cleavage of the Lys^{28}−Lys^{29} peptide bond in β-casein produced by plasmin. To monitor the reaction course, antipeptide antibodies raised against the bovine β-casein 1−28 peptide containing four phosphorylated Ser residues, briefly designated β-CN(f1−28)4P, were used. The antibodies' binding specificity toward the phosphorylated peptides from the other bovine casein fractions as well as toward degradation products of β-CN(f1−28)4P was investigated using competitive ELISA assays. Their reactivity toward the parent protein from different species was evaluated through immunoblotting. These antibodies were ultimately used in competitive ELISA assays to detect the 1−28 phosphopeptide released from β-casein in the pH 4.6 soluble fraction of Parmigiano-Reggiano cheese samples at different ripening ages. The residual β-casein, retaining its N terminus, was monitored in the pH 4.6 insoluble fraction of the same samples.

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MATERIALS AND METHODS

Preparation of Bovine β-Casein. Electrophoretically pure bovine β-casein was obtained by fractionating whole casein from skimmed raw bulk bovine milk on a Q-Sepharose Fast Flow anion exchanger (Pharmacia, Uppsala, Sweden), as already reported (Pizzano et al., 1998).

Plasmin Hydrolysis of Bovine β-Casein. Bovine β-casein (100 mg), purified as above-reported, was dissolved in 2 mL of 50 mM NH_4HCO_3, pH 8.5, and 10 μL of 5 units/mL plasmin suspension (EC 3.4.21.7, Boehringer Mannheim GmbH, Mannheim, Germany) was added. After 16 h of incubation at 37 °C, the mixture reaction was kept at 100 °C for 5 min to inactivate plasmin and then the pH was slowly brought to 4.6 with HCl under pH meter control. Supernatant was recovered by centrifugation at 4500 rpm for 10 min, filtered on 0.22 μm (Millipore, Bedford, MA), and stored at 4 °C.

RP-HPLC Purification of the Bovine β-CN(f1—28)4P. Aliquots (100 μL) of the pH 4.6 soluble fraction of plasmim-hydrolyzed β-casein, obtained as above-described, were fractionated on a Vydac C_{18} column (201HS1010, 10 × 250 mm, 10 μm) (Vydac, Hesperia, CA) attached to a Kontron HPLC equipment (Kontron Instruments, Milan, Italy), consisting of two model 420 pumps, a Rheodyne sample injector, and a model 491 solvent programmer. Elution was followed at 220 nm using a Kontron variable-wavelength detector (model 430). The mobile phase was obtained by mixing 0.1% trifluoroacetic acid in water (solvent A) and 0.07% trifluoroacetic acid in acetonitrile (solvent B). A total flow rate of 2.5 mL/min was applied. After a 10 min hold at 20% solvent B, elution was performed by a linear gradient from 20 to 26% B over 30 min. A further 10 min hold at 95% solvent B allowed collection of all of the retained species. Peaks were manually collected, and 10 μL aliquots were analyzed by electrospray mass spectrometry (ES/MS). The fraction corresponding to the bovine β-CN(f1−28)4P was dried by flushing under nitrogen and stored at −20 °C.

Conjugation of the Bovine β-CN(f1—28)4P to the Carrier and Production of the Antiserum. The RP-HPLC-purified bovine β-CN(f1−28)4P was linked to keyhole limpet hemocyanin (KLH) using glutaraldehyde as coupling agent, according to the procedure reported by Reichlin (1980). Finally, the KLH-conjugated phosphopeptide was used to immunize two rabbits. The antiserum, called anti-βPP, was filtered on 0.45 μm (Millipore, Bedford, MA), split up in aliquots of 1 mL, and stored at −20 °C.

Analysis of Tryptic Phosphopeptides from Whole Bovine Casein. A commercial preparation of tryptic phosphopeptides from whole bovine casein (MD Foods, Viby J., DK), produced according to the procedure described by Ellegård et al. (1999), was fractionated by RP-HPLC according to the method of Ferranti et al. (1997a). Aliquots (200 μL) of 2.5 mg of dried sample dissolved in 1 mL of water were injected onto the column for five consecutive runs. Peaks were manually collected and pooled according to their retention time. A 10 μL aliquot of each fraction was analyzed by ES/MS while the remaining part was dried one by one by flushing under nitrogen and then redissolved with 1 mL of 50 mM NH_4HCO_3, pH 8.5. The absorbance of each sample solution was measured at 220 nm with a model Uvikon 930 spectrophotometer (Kontron) and then brought to 0.48, the lowest of the recorded values, by dilution with the appropriate volume of 50 mM NH_4HCO_3, pH 8.5. The resulting dilutions were analyzed by ELISA.

ES/MS Analysis. ES/MS spectra were recorded on a Platform single-quadrupole instrument, equipped with an electrospray ion source (Micromass, Manchester, U.K.). Samples drawn from HPLC fractions were injected directly into the ion source (kept at 120 °C) via a loop injecton at a flow rate of 10 μL/min. The spectra were scanned from *m*/*z* 1800 to 400 at 5 s/scan, acquired and elaborated using Mass-Linx software (Micromass). Mass scale calibration was performed by a separate injection of myoglobin (16951.5 Da). Mass values are reported as average masses. To identify peptides, the measured mass values were compared with the molecular weights of the expected components, determined with the aid of the Peptide Tools software (Hewlett-Packard, Palo Alto, CA).

Preparation of Grana Padano Cheese Phosphopeptides. Peptides containing multiple SerP residues from Grana Padano cheese samples were prepared by using selective precipitation with barium nitrate in aqueous ethanol, according to the procedure of Ferranti et al. (1997a). Bovine β-casein 16−22 peptide, designated β-CN(f16−22)3P, was obtained through RP-HPLC fractionation of Grana Padano cheese phosphopeptides, as previously described (Ferranti et al., 1997a), and its purity was checked by ES/MS.

Parmigiano-Reggiano Cheese Sample Fractionation. Parmigiano-Reggiano cheese samples of different ages were supplied by the Consorzio di Tutela del Formaggio Parmigiano-Reggiano (Reggio Emilia, Italy). Grated cheese samples (200 mg) were freeze-dried and defatted with diethyl ether in a Soxhlet apparatus. The dry residue of each cheese sample was suspended in 10 mL of 10 mM ammonium acetate, pH 4.6, and homogenized with an Ultra-Turrax (model T25, IKA Labortechnik, Staufen, Germany) at 12000 rpm for 2 min at room temperature. The resulting suspension was centrifuged at 4500 rpm for 20 min and the supernatant, the pH 4.6 soluble fraction of the cheese sample, was carefully removed with a Pasteur pipet from the tube containing the pellet, the pH 4.6 insoluble fraction. A defined aliquot of each supernatant (5 mL) was dried by flushing under nitrogen and redissolved with the same amount (5 mL) of 50 mM NH_4HCO_3, pH 8.5, for ELISA analysis. The casein pellets were washed twice with 10 mL of 10 mM ammonium acetate, pH 4.6, and then were dissolved in 40 mL of 50 mM NH_4HCO_3, pH 8.5, the sample buffer for ELISA assaying.

Competitive ELISA Assay. As specified in the figure legends, RP-HPLC-purified β-CN(f1−28)4P or bovine β-casein, purified as above-described (2 μg/mL in 50 mM NH_4HCO_3, pH 8.5), was used to coat microtiter plates (cod. 3911, Falcon, Oxnard, CA). After an overnight incubation at 4 °C and the saturation step, performed as described elsewhere (Pizzano, 1988), 50 μL aliquots of serial dilutions of the samples in 50 mM NH_4HCO_3, pH 8.5, were introduced into each well and then 50 μL of a 1:2000 (v/v) dilution of the antiserum was added. The subsequent steps of washing, anti-rabbit enzyme-linked antibody reaction, and absorbance measurements were as described previously (Pizzano, 1988). As reported in the figure legends, β-CN(f1−28)4P (200 μg/mL in 50 mM NH_4HCO_3, pH 8.5) or bovine β-casein (2 mg/mL in 50 mM NH_4HCO_3, pH 8.5) was used as standard solution. Each dilution of the samples and of the appropriate standard solution was assayed at least in triplicate, and the mean absorbance value was reported or used for data analysis. The observed coefficient of variation of the ELISA response (%CV, that is, %CV = 100 × SD/mean value, where SD is the standard deviation of the replicate measurements) was usually <6%. Sample concentrations were calculated by interpolation on the standard curve using the Microplate Manager III Data Analysis software (Bio-Rad, Hercules, CA).

PAGIF Analysis and Immunoblotting of Whole Casein from Different Species. Whole casein was obtained from the raw milk of cow, ewe, goat, and water buffalo by precipitation at pH 4.6, according to the currently used procedure [for details, see Pizzano et al. (1998)]. Isoelectric focusing on thin-layer polyacrylamide gel (PAGIF), transfer of electrophoretic profiles onto nitrocellulose paper by capillary diffusion, and immunostaining were as already reported (Pizzano et al., 1998).

RESULTS AND DISCUSSION

Purification of the Peptide Antigen and Binding Specificity of the Antipeptide Antibodies. A one-step RP-HPLC procedure was set up to isolate β-CN(f1−28)4P from the pH 4.6 soluble fraction of plasmin hydrolysate of bovine β-casein. Experimental conditions were described under Materials and Methods. The peak, eluted at the retention time of 25.2 min of the RP-HPLC

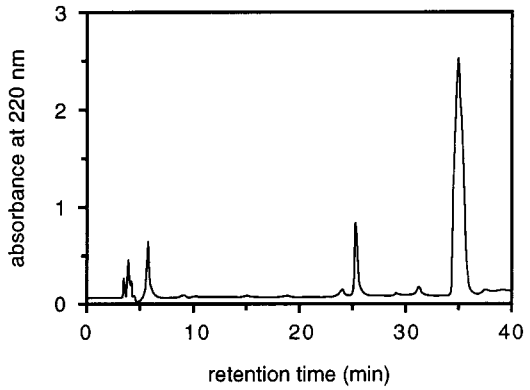

Figure 1. RP-HPLC fractionation on a Vydac C_{18} column of the pH 4.6 soluble plasmin hydrolysate of bovine β-casein. Details about the sample preparation, the HPLC equipment, and the elution conditions are given under Materials and Methods. By ES/MS analysis, the peak at retention time 25.2 min was found to contain a single component with a molecular mass of 3476.0 ± 0.5, corresponding to the bovine β-CN(f1−28)4P.

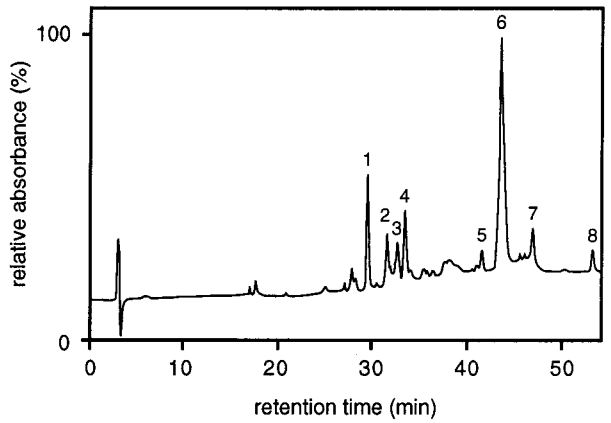

Figure 2. RP-HPLC fractionation on a Vydac C_{18} column of tryptic phosphopeptides from whole bovine casein. Experimental conditions are described under Materials and Methods. ES/MS analysis of the peaks indicated over the chromatogram as 1−8 is given in Table 1.

chromatography shown in Figure 1, gave a single signal at molecular mass 3476.0 ± 0.5 Da by ES/MS analysis, corresponding to β-CN(f1−28)4P.

To define the binding specificity of the anti-βPP antiserum, raised against the RP-HPLC-purified β-CN(f1−28)4P, tryptic phosphopeptides from whole bovine casein were fractionated by RP-HPLC, as shown in Figure 2, and the single peaks, indicated in the figure by the numbers 1, 2, ..., 8, were used for ELISA and ES/MS analysis. To obtain comparable results, peak solutions with the same absorbance at 220 nm were prepared, as described under Materials and Methods, and the same aliquot of each solution was assayed by competitive ELISA. Similar amounts of antibody-reactive phosphopeptides were found in the sample solutions prepared from peaks 5−8 (results not shown). According to the composition of each RP-HPLC peak deduced from ES/MS results, reported in Table 1, all of the tryptic phosphopeptides originating from the 1−28 region of bovine β-casein, regardless of the content of phosphorylated Ser residues, were recognized by the anti-βPP antiserum. On the contrary, tryptic phosphopeptides generated from bovine casein fractions other than β-casein, as well as that from β-casein, but not located in the 1−28 region, were not detected by the anti-βPP antiserum, even though they contain closely related phosphorylated sequence stretches. These results proved that the anti-βPP antibodies were effectively directed toward the proper amino acid sequence, quite far from the phosphorylation degree.

Table 1. Identification by ES/MS Analysis of Tryptic Phosphopeptides from Whole Bovine Casein Separated by RP-HPLC

RP-HPLC peak[a]	molecular mass (Da) measured[b]	theoretical[c]	assigned peptide[d,e]
1	2720.9 ± 0.2	2720.9	α_{s1}-CN(f59−79)5P
	2061.4 ± 0.3	2061.4	β-CN(33−48)1P
2	3008.8 ± 0.3	3008.8	α_{s2}-CN(f46−70)4P
3	1927.0 ± 0.2	1926.7	α_{s1}-CN(f43−58)2P
4	2746.8 ± 0.2	2747.0	α_{s2}-CN(f1−21)5P
	2618.9 ± 0.3	2618.9	α_{s2}-CN(f2−21)5P
5	2723.9 ± 0.3	2724.0	β-CN(f4−25)4P
6	3121.9 ± 0.3	3122.3	β-CN(f1−25)4P
	2965.8 ± 0.3	2966.2	β-CN(f2−25)4P
7	3041.8 ± 0.3	3042.2	β-CN(f1−25)3P
	2885.9 ± 0.2	2886.1	β-CN(f2−25)3P
8	2961.8 ± 0.4	2962.1	β-CN(f1−25)2P
	2805.9 ± 0.6	2806.0	β-CN(f2−25)2P

[a] Peak numbers refer to the RP-HPLC separation shown in Figure 2. [b] The molecular mass values of the components were determined by ES/MS analysis of each RP-HPLC peak, as described under Materials and Methods, and reported as average mass ± SD. [c] By considering the known amino acid sequence of bovine caseins (Swaisgood, 1992) and the enzyme specificity, phosphopeptides occurring in the tryptic hydrolyzate of whole bovine casein were predicted and their molecular weights were calculated using the Peptide Tools software (Hewlett-Packard). [d] Assigning each component to the indicated peptide was based on comparison of the measured molecular mass to the theoretical values, as calculated and reported above. [e] CN, casein; 1P, 2P, ..., 5P, number of phosphate groups included in the peptide.

Anti-βPP Reactivity toward Phosphopeptides Developed in Grana Padano Cheese during Ripening. The water soluble extract of 12-month-old Comté cheese (Roudot-Algaron et al., 1994) and the casein phosphopeptides developed in a 14-month-old Grana Padano cheese (Ferranti et al., 1997a) were found to contain degraded forms of the β-CN(f1−28)4P generated from the primary hydrolysis of β-casein by plasmin, but the parent peptide was absent. In particular, in 14-month-old Grana Padano cheese, Ferranti et al. (1997a) identified the β-casein 7−28 phosphopeptide, abbreviated β-CN(f7−28)4P, and a set of peptides, each arising from the progressive hydrolysis of the β-CN(f7−28)4P by aminopeptidases and/or carboxypeptidases of microbial origin. To evaluate the amount of species recognized by anti-βPP in the phosphopeptide fraction isolated from Grana Padano cheese, competitive ELISA assays were carried out (Figure 3). Cheese samples at different ages were considered, to account for the composition changes observed in the phosphopeptide fraction during ripening of Grana Padano cheese (Ferranti et al., 1997a). Anti-βPP reactive species strongly decreased from a 4-month-old to a 14-month-old cheese and gradually disappeared in the older cheese samples. Considering that some peptides from β-casein degradation, namely, β-CN(f1−20)4P, β-CN(f2−20)4P, β-CN(f3−20)4P, β-CN(f4−20)-4P, and β-CN(f5−20)4P, identified in the fraction soluble in 120 g/L trichloroacetic acid of a 6-month-old Parmigiano-Reggiano cheese (Addeo et al., 1994), were not found any longer in a 14-month-old Grana Padano

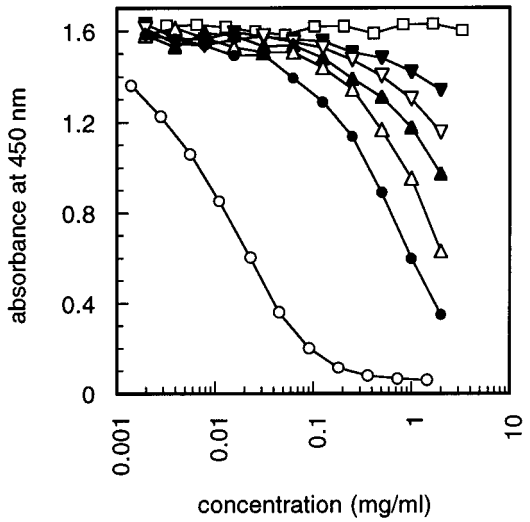

Figure 3. Competitive ELISA assays of the phosphopeptide fraction isolated from Grana Padano cheese ripened for 4 months (●), 8 months (△), 14 months (▲), 18 months (▽), and 38 months (▼) and of RP-HPLC-purified β-CN(f16−22)3P (□). As positive reference sample RP-HPLC-purified β-CN(f1−28)-4P was assayed (○). The ELISA assay procedure was described under Materials and Methods. Serial dilutions (1:2^n, n from 1 to 12) of each sample were analyzed in triplicate, and the mean values of the obtained ELISA signals were reported.

cheese (Ferranti et al., 1997a), the loss of antibody reactive species observed from a 4-month-old to a 14-month-old cheese sample may be related to the disappearance of those intermediate β-casein peptides little hydrolyzed at their N terminus, thus indicating that the first amino acid residues of β-CN(f1−28)4P are primarily involved in the antibody recognition. The residual anti-βPP recognition observed in the phosphopeptide fraction from the older cheese samples may have been caused by the presence either of trace amounts of β-CN-(f1−28)4P or of degraded oligopeptides originating from the parent β-CN(f1−28)4P, which retain any ability to bind to the antibodies. No anti-βPP reactivity was found toward the β-CN(f16−22)3P, purified according to the procedure of Ferranti et al. (1997a). This peptide was identified as a product resistant to further hydrolysis accumulating in the cheese at various ages (Ferranti et al., 1997a). This result suggested that the C-terminal region of β-CN(f1−28)4P, containing a very hydrophilic cluster of Ser phosphorylated residues, was not targeted for antibody production. Actually, this finding contradicts current opinion, according to which hydrophilic regions are expected to be antigenic because of their likely surface location (Harlow and Lane, 1988).

Interestingly, polyclonal antibodies raised against a synthetic peptide carrying the 1−28 sequence of bovine β-casein containing no phosphorylated residues had given results quite similar to those obtained throughout this work using anti-βPP (not shown). This finding is not enough to attribute intrinsic immunodominant features to the N-terminal region of the peptide. According to Schaaper et al. (1989), the peptide region mostly affecting the antipeptide antibody specificity is generally opposite to that engaged for coupling with the carrier in the conjugate used for the antibody production. Considering that both the polyclonal antibody preparations had been raised against the respective peptides linked to the carrier protein through their C terminus, the immune response, in both cases, might

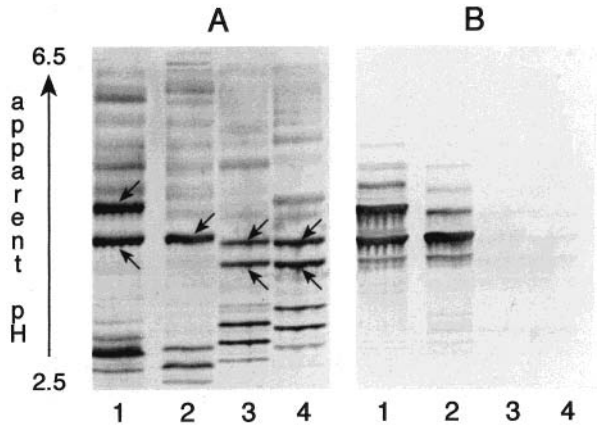

Figure 4. PAGIF analysis of 100 μg amounts of whole casein from cow (lane 1), water buffalo (lane 2), sheep (lane 3), and goat (lane 4) milks: (A) Coomassie Brilliant Blue G-250 stained gel; (B) immunodetection using 1:1000 (v/v) diluted anti-βPP as primary antibodies and 1:2000 (v/v) diluted horseradish peroxidase labeled goat anti-rabbit IgG antibodies as secondary antibodies. All of the protein bands indicated by the arrows in the panel A were recognized by polyclonal antibodies raised against the entire bovine β-casein in a parallel experiment, not shown in this figure.

have been developed mainly against the common N-terminal sequence stretch of the two peptides.

Anti-βPP Reactivity toward β-Casein from Different Species. PAGIF analysis of whole casein samples from the different species was carried out to test the ability of the antiserum to recognize the parent bovine β-casein and the homologous protein from water buffalo, ovine, and caprine milks (Swaisgood, 1992). The Coomassie Brilliant Blue G-250 stained gel (panel A) and the immunoblotting print using the antiserum (panel B) are reported in Figure 4. The bands relative to both bovine and water buffalo β-casein, identified by immunostaining the electrophoretic profiles using polyclonal antibodies against the entire bovine β-casein, were specifically immunodetected by anti-βPP, whereas no antibody reactive band was found along the profiles relative to both ovine and caprine whole casein.

The selective antibody recognition of bovine and water buffalo whole casein was confirmed in competitive ELISA analysis of whole casein from milks of the four considered species (not shown). Accordingly, preliminary experiments have indicated that these antibodies are suitable reagents for the detection of bovine casein in ovine and caprine fresh dairy products by immunochemical assays. Substitution of ewes' and goats' milk by cows' milk is a well-known fraudulent practice due to differences in price between them. Consequently, a number of analytical methods had been proposed for the detection of cows' milk in ewes' and goats' milk and cheese (Ramos and Juárez, 1986). Recently, a reference method, based on isoelectric focusing of γ-caseins, has been established by the European Commission (*Official Journal of the European Communities*, 1996). However, there is an increasing interest in the development of immunoassays for food analysis arising from the need for basic screening tests that are quick, economic, accurate, sensitive, and easy to use for routine control (Allen, 1990).

The bovine β-casein antibody recognition is consistent with the generally accepted fact that peptides which carry either the C- or N-terminal sequence serve as good targets to induce antibodies capable of detecting parent proteins (Harlow and Lane, 1988).

Table 2. 1−28 Sequence Stretch of β-Casein from Different Species[a]

	1				5					10					15					20					25			
cow	R	E	L	E	E	L	N	V	P	G	E	I	V	E	S	L	S	S	S	E	E	S	I	T	R	I	N	K
water buffalo	R	E	L	E	E	L	N	V	P	G	E	I	V	E	S	L	S	S	S	E	E	S	I	T	H	I	N	K
ewe	R	E	Q	E	E	L	N	V	V	G	E	T	V	E	S	L	S	S	S	E	E	S	I	T	H	I	N	K
goat	R	E	Q	E	E	L	N	V	V	G	E	T	V	E	S	L	S	S	S	E	E	T	I	T	H	I	N	K

[a] The amino acid differences are boxed.

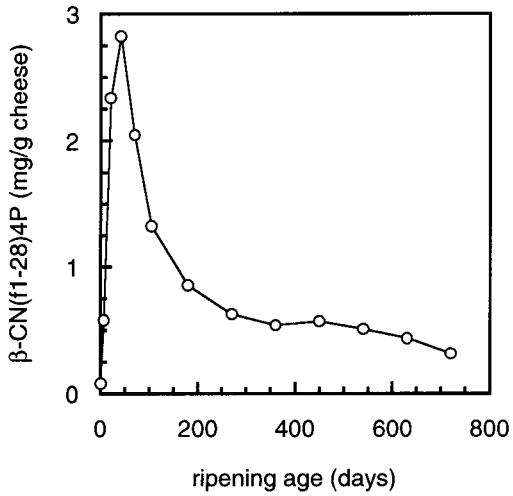

Figure 5. Competitive ELISA assays of the pH 4.6 soluble fraction of Parmigiano-Reggiano cheese samples, ranging from 1 day to 24 months. Microtiter plates were coated by 2 μg/mL RP-HPLC-purified β-CN(f1−28)4P and serial dilutions (1:4^n, n from 1 to 6) of 200 μg/mL RP-HPLC-purified β-CN(f1−28)-4P were used to obtain the standard curve. The samples preparation, the ELISA assay procedure, and data analysis were described in detail under Materials and Methods. Three aliquots of serial dilutions (1:4^n, n from 1 to 3) of each sample were tested, and the mean values of the ELISA signals were considered for determining sample concentrations. The anti-βPP reactivity of each cheese sample was reported as β-CN-(f1−28)4P content determined in the sample and referred to 1 g of cheese.

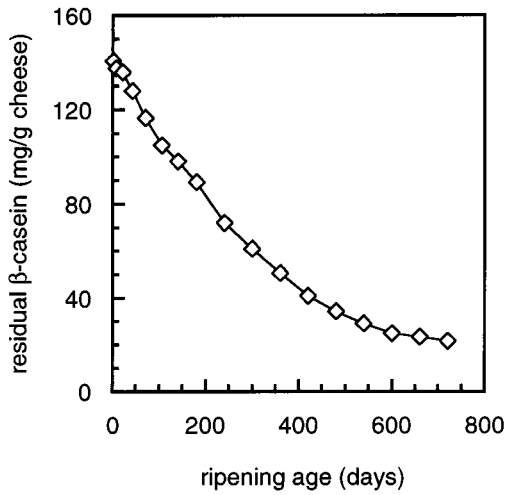

Figure 6. Competitive ELISA assays of the pH 4.6 insoluble fraction of Parmigiano-Reggiano cheese samples, ranging from 1 day to 24 months. Microtiter plates were coated by 2 μg/mL bovine β-casein, and serial dilutions (1:4^n, n from 1 to 6) of 2 mg/mL bovine β-casein were used to obtain the standard curve. The samples preparation, the ELISA assay procedure, and data analysis were reported in detail under Materials and Methods. Three aliquots of serial dilutions (1:4^n, n from 1 to 3) of each sample were tested, and the mean values of the ELISA signals were considered for determining sample concentrations. The anti-βPP reactivity of each cheese sample was reported as β-casein content determined in the sample and referred to 1 g of cheese.

Assuming that the considered β-casein region is exposed at the same extent in the different species, owing to the overall close similarity of their primary structure, the observed antibodies' species specificity may refer to the small differences in the β-casein 1−28 sequence stretch of the different species, shown in Table 2. Some interesting indications concerning the role played by single amino acid residues in the antibodies' recognition may be drawn. Arg^{25} of the bovine β-casein is not significant for binding to the antipeptide antibodies, because water buffalo β-casein, carrying in the same position a His residue, is recognized by the antibodies as well as the bovine one. The substitution of Ser^{22} with Thr^{22} in the caprine β-casein cannot be solely blamed for the unsuccessful antibody cross-reactivity, because ovine β-casein, although containing Ser^{22} just as the bovine, was not detected. Consequently, the species specificity revealed by antibodies could be related to the amino acid residues located in positions 3, 9, and 12 of the bovine β-casein. In particular, Pro^9, influencing the conformational arrangement of the protein region, might be relevant in the selective antibody recognition of both bovine and water buffalo β-casein.

Anti-βPP Monitoring of Plasmin Action on the Bond Lys^{28}—Lys^{29} of Bovine β-Casein during Cheese Ripening. To monitor the plasmin-mediated release of the 1−28 phosphopeptide from β-casein during cheese ripening, competitive ELISA methodology, using anti-βPP, was employed and the Parmigiano-Reggiano cheese was chosen as a model sample.

The ELISA signals obtained from the pH 4.6 soluble fractions of cheese samples ripened from 1 day to 24 months were compared to that of a standard solution of the HPLC-purified bovine β-CN(f1−28)4P. Sample concentrations were calculated by interpolation on the standard curve using the data analysis software as described under Materials and Methods, and the amounts of the extracted peptide with respect to the processed cheese samples are reported in Figure 5.

Principally, two occurrences, acting in opposite directions, may affect the amount of the β-CN(f1−28)4P in cheese samples: (i) its production by plasmin action and (ii) its enzymatic degradation by microbial proteinases. Figure 5 indicates that β-CN(f1−28)4P was formed early and that the effects of the proteolytic activity from microbial origin became relevant in the advanced stages of cheese ripening, practically balancing that of the further peptide formation due to the Lys^{28}−Lys^{29} hydrolysis of β-casein by plasmin.

The corresponding pH 4.6 insoluble fractions of the aforementioned Parmigiano-Reggiano cheese samples were also assayed in competitive ELISA, using anti-βPP as primary reagent and HPLC-purified β-casein as reference protein. As shown in Figure 6, a low but

progressive decrease of the amount of the antiβPP reactive species was found, reflecting a constant plasmin action on β-casein throughout cheese ripening. Data analysis had indicated that the bond Lys^{28}−Lys^{29} of β-casein in Parmigiano-Reggiano cheese had a half-time of ~8 months. On the basis of electrophoretic tecniques, a comparable rate of β-casein breakdown was observed in Grana Padano cheese during ripening (Mayer et al., 1998), thus indicating that the contribution of plasmin to proteolysis occurring in the two Italian hard cheese varieties is very similar.

On the whole, these results strongly suggested that the ripening age of the Parmigiano-Reggiano cheese samples can be unambiguously evaluated by the ELISA determination of the residual β-casein in the pH 4.6 insoluble fractions of the samples. The procedure may be further simplified by assaying whole defatted cheese samples instead of their pH 4.6 insoluble fractions, thus eliminating the fractionation at pH 4.6 for the cheese sample preparation. In fact, ELISA analysis of unfractionated cheese samples gave results similar to those obtained by testing the corresponding pH 4.6 insoluble fractions, except with respect to the first period of ripening, for which residual β-casein, as determined as the species recognized by anti-βPP, was slightly overestimated, owing to the simultaneous presence of the pH 4.6 soluble β-casein phosphopeptides (experiments not shown).

Clearly, the analysis of a significant number of long-term-ripened cheese samples of known age is required to validate such a procedure.

ABBREVIATIONS USED

β-CN(f1−28)4P, bovine β-casein 1−28 peptide containing four phosphorylated Ser residues; β-CN(f16−22)3P, bovine β-casein 16−22 peptide containing three phosphorylated Ser residues; β-CN(f7−28)4P, bovine β-casein 7−28 peptide containing four phosphorylated Ser residues; RP-HPLC, reversed-phase high-performance liquid chromatography; ES/MS, electrospray mass spectrometry; KLH, keyhole limpet hemocyanin; anti-βPP, antiserum raised against β-CN(f1−28)4P conjugated to KLH; ELISA, enzyme-linked immunosorbent assay; PAGIF, polyacrylamide gel isoelectric focusing.

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