Antipeptide Antibodies as Analytical Tools To Discriminate among Bovine α_{s1}-Casein Components

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Polyclonal antibodies raised against synthetic peptides reproducing sequence stretches of bovine α_{s1}-casein were used as probes to discriminate within the α_{s1}-casein fraction of bovine milk and cheese. A minor α_{s1}-casein component, selectively recognized by an antisera directed against the bovine 139−149 α_{s1}-casein sequence, was found to be a C-terminally truncated α_{s1}-casein form. This component coeluted with the main α_{s1}- and α_{s2}-casein by anion-exchange chromatography of whole casein, whereas by RP-HPLC it eluted with α_{s2}-casein only. Similarly to the main α_{s1}-casein, the C-terminally truncated form was hydrolyzed in vitro by chymosin and early in the cheese-making.

Keywords: *α_{s1}-Casein; polyclonal antibodies; peptide antigens; ELISA; immunoblotting*

INTRODUCTION

By high-resolution gel electrophoresis, each of the four fractions constituting whole casein, i.e. α_{s1}-, α_{s2}-, β-, and κ-casein, showed a high microheterogeneity. Genetic variants, discretely phosphorylated and glycosylated casein fractions, and peptides formed by limited hydrolysis under action of native milk proteinases have been claimed responsible for the electrophoretic heterogeneity (Swaisgood, 1992).

In addition to these factors, the copresence of nonallelic casein forms has been recently reported. Ovine α_{s2}-casein had been found to be constituted by a mixture of two forms produced from different mRNAs (Boisnard et al., 1991). Multiple forms of mature caprine α_{s1}-casein A, B, and C, resulting from alternatively spliced pre-mRNAs, were also identified (Ferranti et al., 1997a). At present, half the ovine α_{s1}-casein fraction is constituted by at least six nonallelic forms (Ferranti et al., 1997b).

We have previously reported that antipeptide antibodies may be used to selectively detect minor components of bovine casein: both anti-E8E and anti-N11L, raised, respectively, against bovine f141−148 and f139−149 α_{s1}-casein, recognized a minor component belonging to the bovine α_{s1}-casein family, whereas they failed to detect any of the main bovine α_{s1}-casein forms (Pizzano et al., 1997). Despite the strict homology existing among the casein fractions from the different species (Swaisgood, 1992), both anti-E8E and anti-N11L were also unable to recognize any ovine, caprine, or water buffalo casein component, thus indicating that the minor bovine α_{s1}-casein could represent a suitable marker of cow specificity (Pizzano et al., 1997).

In this work we have used antipeptide antibodies as analytical tools to determine the main structural features of this minor bovine α_{s1}-casein form. Whole bovine casein was separated by using the most effective chromatographic and electrophoretic methodologies, as reviewed by Strange et al. (1992). The major casein components were identified according to the procedure indicated by Chianese et al. (1995) by using polyclonal antibodies against FPLC-purified casein fractions, whereas the minor bovine α_{s1}-casein was selectively labeled by anti-N11L. Polyclonal antibodies, raised against synthetic peptides reproducing specified sequence stretches of bovine α_{s1}-casein, were used to detect along chromatograms and electrophoretic patterns all of the casein components carrying the considered α_{s1}-casein regions, thus providing an overall picture of the minor bovine α_{s1}-casein structure.

To exploit the anti-N11L antibodies for detecting bovine milk in adulterated ovine, caprine, and water buffalo cheeses, the stability of the anti-N11L reactive bovine α_{s1}-casein during cheese-making and cheese ripening was also evaluated.

MATERIALS AND METHODS

Synthetic Peptides, Conjugation to the Carrier, and Production of the Antisera. The peptide carrying the sequence 139−149 of bovine α_{s1}-casein, NQELAYFYPEL (N11L), and anti-N11L antisera were previously described (Pizzano et al., 1997). All other peptides were synthesized and HPLC purified by Primm (Milano, Italy). The purity grade was guaranteed >95%, and the chemical structure of each peptide has been verified by MALDI-TOF mass spectrometry analysis. The first peptide reproduces the N-terminal sequence (residues 1−22) of the bovine α_{s1}-casein (Mercier et al., 1971), to which one cysteine residue was added to its C terminus: RPKHPIKHQGLPQEVLNENLLRC. The second carries the C-terminal sequence (residues 186−199) of the B variant of the protein (Grosclaude et al., 1972), with one cysteine residue added to its N terminus: CIGSQNSEKTTMPLW. The third is the sequence 23−34 of bovine α_{s1}-casein, which is the N-terminal sequence of the α_{s1}-I peptide (Mulvihill and Fox, 1979), lengthened by two glycine residues and one cysteine residue at its C terminus: FFVAPFPEVFGKGGC. The cysteine residues in the peptides were included to link the correctly oriented peptides to ovoalbumin, through the

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heterobifunctional reagent, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Pierce, Rockford, IL), according to the cross-linking procedure reviewed by Mattson et al. (1993). The coupled products were used to immunize two rabbits, and the collected antisera were respectively named anti-Nterm, anti-Cterm, and anti-α_{s1}I, to denote that in the immunogen peptides were respectively contained the N terminus of bovine α_{s1}-casein, the C terminus of bovine α_{s1}-casein, and the N terminus of the α_{s1}-I peptide.

All of the antisera were filtered on 0.45 μm (Millipore, Bedford, MA), aliquoted, and stored at −20 °C.

Milk Collection and Casein Preparation. Individual bovine milk containing only the B variant of α_{s1}-casein, identified as previously described (Addeo et al., 1983), was drawn from a local private herd according to sanitary regulations. Fat was removed from the freshly sampled milk by centrifugation at 4500 rpm for 10 min at 4 °C. Whole casein was immediately obtained from defatted milk by isoelectric precipitation at pH 4.6 according to the procedure of Aschaffenburg and Drewry (1959). The pellet was suspended in water, the pH was brought to neutrality with 2 N NaOH, and the casein solution was lyophilized. The sodium caseinate was weighed and dissolved in phosphate-buffered saline (PBS; 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136 mM NaCl, 2.7 mM KCl, pH 7.4) at the desired concentration.

Cheese Sample Preparations. Parmigiano Reggiano cheese samples of different ages were from a commercial source. For electrophoretic analysis, cheese (100 g/L) was dissolved in 8 M urea solution containing 2-mercaptoethanol (10 mL/L) and manually defatted after centrifugation at 4500 rpm for 10 min at 4 °C (Addeo et al., 1995). For ELISA analysis, samples were dissolved in 50 mM ammonium carbonate, pH 8.5, and defatted as described above. Protein concentration was determined according to the method described by Bradford (1976), using Bio-Rad protein assay dye reagent (Bio-Rad, Hercules, CA) as reagent and lyophilized whole bovine casein as standard proteins. In the ELISA tests serial dilutions of the samples (1:4^n, $n = 1-6$) were assayed from 8 mg/mL.

Ion-Exchange Chromatography of Whole Bovine Casein. Chromatography was carried out on whole bovine casein on Q-Sepharose Fast Flow anion exchanger (Pharmacia, Uppsala, Sweden), scaling up the analytical procedure described by Andrews et al. (1985). Briefly, 1 g of lyophilized bovine casein was incubated for 1 h at 37 °C in 25 mL of 20 mM Tris-Cl, 4.5 M urea, 0.8 mM DTT, pH 7, and then applied to a column (26 × 300 mm) containing 150 mL of Q-Sepharose Fast Flow equilibrated at room temperature at a flow of 1 mL/min in the run buffer (25 mM Tris-Cl, 4.5 M urea, 0.08 mM DTT, pH 7). After the column was washed with 200 mL of the run buffer, elution was carried out by a 800 mL linear NaCl gradient from 0 to 0.35 M for 8 h. Fractions of ∼5 mL were collected with a FRAC-100 collector (Pharmacia LKB), while absorbance of the eluted proteins was determined at 280 nm with a Uvicord S II detector (LKB) and registered on a two-channel REC-482 recorder (Pharmacia). Pooled fractions were extensively dialyzed against deionized water, adjusted to pH 7 with diluted NaOH, and then lyophilized.

This procedure is based on classical techniques of DEAE liquid chromatography of caseins (Mercier, 1968).

Reversed-Phase HPLC of Whole Bovine Casein. Whole bovine casein was fractionated using a Vydac C_4 column (214TP1010: 10 × 250 mm, 10 μm) with Kontron equipment (Kontron Instruments, Milan, Italy), consisting of two model 420 pumps, a Rheodyne sample injector (80 μL loop), and a model 491 solvent programmer. Protein detection was carried out at 220 nm using a Kontron variable-wavelength detector (model 430).

The preparative procedure developed by Jaubert and Martin (1992) for caprine casein was adapted for the bovine one. The mobile phase was obtained by mixing solvent A (0.1% trifluoroacetic acid in water) and solvent B (0.1% trifluoroacetic acid in acetonitrile). Lyophilized bovine casein was dissolved at a concentration of 50 mg/mL in 7.8 M urea containing 10 mM β-mercaptoethanol and incubated at 37 °C for 1 h. The sample was filtered through a 0.45 μm filter (Millipore, Bedford, MA), and aliquots of 80 μL were injected. Elution was achieved at a flow rate of 2 mL/min by using a linear gradient from 35 to 50% solvent B over 40 min after a 10 min hold at 35% solvent B. The column effluent was collected manually in six fractions according to monitoring at 220 nm and freeze-dried.

Reversed-Phase HPLC of the pH 4.6 Soluble Fraction of Chymosin-Hydrolyzed Whole Bovine Casein. The in vitro chymosin hydrolysate of whole bovine casein (0.5 g) was carried out as previously described (Addeo et al., 1995). After 1 h of incubation, the reaction was stopped at 100 °C for 5 min, and then the pH was brought to 4.6 by using the procedure of Aschaffenburg and Drewry (1959). Supernatant was collected by centrifugation at 4500 rpm for 10 min, freeze-dried, and redissolved in 3 mL of water. A 500 μL aliquot was fractionated on a Vydac C_{18} column (201HS1010, 10 × 250 mm, 10 μm) at a flow rate of 1.5 mL/min by using the HPLC equipment as described above and the same solvents. After a 15 min hold at 25% solvent B, elution was performed by a linear gradient from 25 to 55% B over 45 min. Throughout the chromatography the column eluate was automatically collected, and the 1 min fractions were vacuum-dried and redissolved in 1 mL PBS, and 1:6 dilutions (v/v) were tested in competitive ELISA assays.

Competitive ELISA Assays. Microtiter plates (Falcon, cod. 3911) were coated with whole bovine casein 5 μg/mL PBS when anti-N11L was used and 2 μg/mL PBS when anti-Nterm, anti-Cterm, and anti-α_{s1}I antisera were used.

After an overnight incubation at 4 °C, the wells were washed twice with PBS and filled with blocking buffer (10% v/v heat-inactivated horse serum in PBS). Microplates were placed at 37 °C for at least 1 h; they were then emptied and kept at −20 °C, ready for immediate utilization. To determine the presence and the level of the antigen in a sample, 50 μL aliquots were introduced in each well and then 50 μL of antisera diluted in the blocking buffer was added. The analysis of each diluted sample was performed at least in triplicate, and antisera were used at the following final dilution values: anti-N11L, 1:500; anti-Nterm and anti-Cterm, 1:2000; and anti-α_{s1}I, 1:1000 (v/v).

After 1 h at 37 °C in a humid atmosphere, the plates were emptied and washed five times with PBS. One hundred microliter aliquots of a 1:2000 (v/v) diluted goat antibody anti-rabbit IgG labeled with horseradish peroxidase (Bio-Rad) were then added. Following incubation for 1 h at 37 °C in a humid atmosphere and five washings with PBS, 100 μL amounts of a solution of 0.1 mg/mL tetramethylbenzidine (Sigma) in 50 mM citrate−phosphate buffer, DMSO 10% v/v, pH 5.0, containing 0.01% (v/v) hydrogen peroxide were added to each microtiter well. After 10−15 min at room temperature, the reaction was stopped by adding 100 μL of 1 M H_2SO_4. The resulting absorbance was measured automatically at 450 nm with a model 450 microplate reader (Bio-Rad).

Electrophoresis and Immunoblotting. Vertical polyacrylamide gel electrophoresis (disc-PAGE) was carried out at pH 8.6 in the presence of 6.1 M urea, as described by Chianese et al. (1992).

After gel electrophoresis, the proteins were carefully transferred by capillary diffusion from the gel onto nitrocellulose paper. Immunodetection was carried out using rabbit polyclonal antipeptide antisera as primary antibodies and horseradish peroxidase-labeled rabbit immunoglobulins as secondary antibodies. The procedure was essentially that already described by Chianese et al. (1992), but the blocking solution was replaced with PBS containing heat-inactivated horse serum at 10% in volume.

RESULTS AND DISCUSSION

Anion-Exchange Chromatography of Whole Bovine Casein. Fractionation of whole bovine casein on a Q-Sepharose column using a linear NaCl gradient gave five major peaks, as shown in Figure 1. The disc-PAGE patterns at alkaline pH of the separated fractions

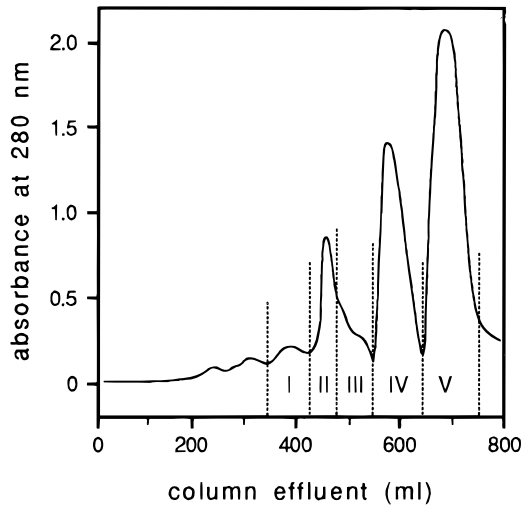

Figure 1. Anion-exchange chromatography of whole bovine casein on a Q-Sepharose Fast Flow matrix. Details about the procedure are given under Materials and Methods. Fractions were gathered into five pools, named I−V, as schematically reported over the 280 nm absorbance profile, dialyzed against distilled water, and freeze-dried.

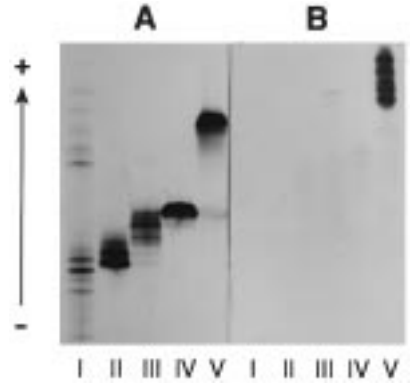

Figure 2. Disc-PAGE at pH 8.6 in denaturing conditions of 50 μg amounts of the casein pools obtained from the anion-exchange chromatography reported in Figure 1: (A) Coomassie Brilliant Blue R-250 stained gel; (B) immunodetection with 1:1000 (v/v) diluted anti-N11L antisera as primary antibodies and 1:2000 (v/v) diluted horseradish peroxidase labeled goat anti-rabbit IgG antibodies as secondary antibodies. The pools, specifically stained according to the procedure reported by Chianese et al. (1995), chiefly contained (I) γ-casein, (II and III) κ-casein, (IV) β-casein, and (V) α_{s1}- + α_{s2}-casein.

stained by Coomassie Brilliant Blue R-250 are shown in Figure 2A. The identification of the casein fractions, made according to the method proposed by Chianese et al. (1995), indicated an order of elution of the caseins similar to that reported by Andrews et al. (1985). By immunoblotting, anti-N11L recognized in fraction V some reactive components, migrating clearly ahead of the major α_{s1}- and α_{s2}-casein (Figure 2B). The multiple anti-N11L-detected components might even be differently phosphorylated forms of a single casein species, considering that each main casein fraction is constituted by protein species at different phosphorylation degrees (Swaisgood, 1992). However, on the whole, the anti-N11L reactive components represented a minor casein fraction, since they were not detected by Coomassie Brilliant Blue R-250 staining.

A bovine α_{s1}-casein preparation obtained from anion-exchange chromatography was also found to be reactive toward antisera against the bovine f140−149 α_{s1}-casein by Rolland et al. (1993, 1995). The present results

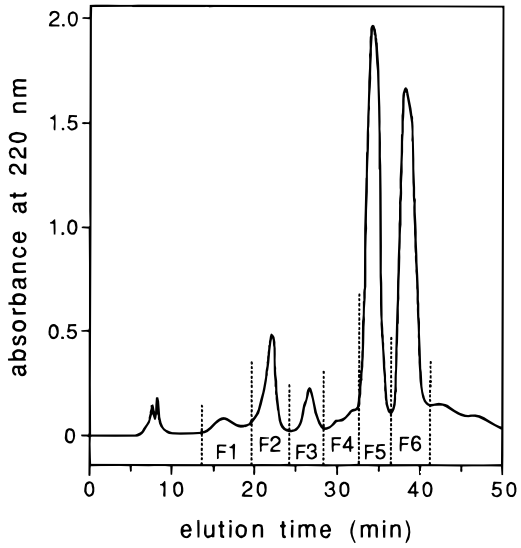

Figure 3. Reversed-phase HPLC fractionation of whole bovine casein on a semipreparative Vydac C_4 column (protein injected 4 mg). Details about the sample preparation, the HPLC equipment, and the elution conditions are given under Materials and Methods. According to the 220 nm absorbance monitoring, the column effluent was manually collected into the six consecutive fractions indicated over the chromatogram as F1−F6.

clearly demonstrate that the positive response could have originated only from a minor casein fraction, coeluting with the main bovine α_{s1}-casein.

HPLC of Whole Bovine Casein. The reversed-phase HPLC procedure for the separation of caprine α_{s1}-casein variants (Jaubert and Martin, 1992) was used to improve the resolution of bovine casein components obtained by the anion-exchange chromatography. By RP-HPLC seven fractions were collected, as shown in Figure 3, and analyzed by disc-PAGE at alkaline pH. The Coomassie Brilliant Blue R-250 stained patterns (panel A) and the immunoblotting prints using anti-N11L (panel B), anti-Nterm (panel C), and anti-Cterm (panel D) are reported in Figure 4. Only the HPLC fraction F3, estimated until now to be constituted by α_{s2}-casein (Visser et al., 1986), presented bands reactive to anti-N11L. Both the anti-N11L immunostained bands and the main α_{s1}-casein components were recognized by anti-Nterm (Figure 4C), confirming that anti-N11L reactive components belonged to the bovine α_{s1}-casein family, as already reported (Pizzano et al., 1997). Interestingly, the minor α_{s1}-casein components eluted within the fraction F3, at variance with the major α_{s1}-casein, were not immunostained by anti-Cterm.

These results indicated that the C-terminal chain extension of the anti-N11L reactive minor bovine α_{s1}-casein was shorter than that of the main α_{s1}-casein. As previously reported (Pizzano et al., 1997), anti-N11L had detected identical bands in milk samples containing B or C α_{s1}-casein variant, differing for the single amino acid substitution Glu^{192} (B) → Gly^{192} (C) (Grosclaude et al., 1972), whereas Coomassie Brilliant Blue staining had revealed different α_{s1}-casein patterns, according to the α_{s1}-casein variant assortment of each milk sample. In compliance with these findings, the short α_{s1}-casein chain may contain, at the most, up to the 191st residue of the entire α_{s1}-casein sequence.

Most likely, this short α_{s1}-casein is a proteolytic degradation of α_{s1}-casein, and arises, similarly to γ-caseins, by the action of plasmin, an endogenous

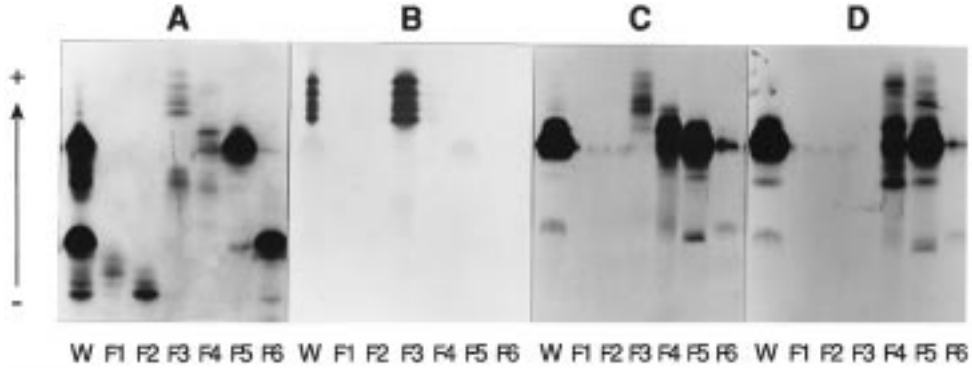

Figure 4. Disc-PAGE at pH 8.6 in denaturing conditions and immunoblotting analysis of 50 μg amounts of whole bovine casein (lane W) and HPLC separated caseinic fractions as named in Figure 3 (lanes F1−F6): (A) Coomassie Brilliant Blue R-250 stained gel; immunodetection using 1:1000 (v/v) dilutions of (B) anti-N11L, (C) anti-Nterm, and (D) anti-Cterm as primary antibodies and secondary antibodies as indicated for Figure 2.

proteinase of bovine milk freshly drawn from healthy animals (Grufferty and Fox, 1988). Nevertheless, the truncated protein might originate from an abnormal splicing of the bovine α_{s1}-casein mRNA, occurring beside the correct process leading to the synthesis of the main bovine α_{s1}-casein, as suggested by McKnight et al. (1989).

In any case, the same production mechanism, i.e., proteolysis or alternative splicing, is always acting since the short α_{s1}-casein was detected in all of the individual bovine milks we have tested, as a constitutive component (Pizzano et al., 1997). This α_{s1}-casein component might have proven to be evolutionarily advantageous by playing a cohesive role in the stabilization of the bovine casein micelles, as well as other minor components also recovered in milk (Paquet, 1989).

Reassemblage of α_{s1}-Casein from the C-Terminal Truncated α_{s1}-Casein and the C-Terminal α_{s1}-Casein Peptide. The finding that the 139−149 α_{s1}-casein region can be detected by anti-N11L antibodies only in the truncated C-terminal α_{s1}-casein form suggests that the C-terminal sequence in the 199-residue-long α_{s1}-casein might prevent the access of anti-N11L antibodies to their target region. The α_{s1}-casein carboxyl-terminal moiety (residues 100−199) is the hydrophobic domain considered responsible for the self-association of the α_{s1}-casein monomer in aqueous solution (Schmidt, 1982). According to the three-dimensional molecular model of the protein of Kumosinski et al. (1991), the folding of the chain brings the C-terminal tail into close proximity with the 139−149 region.

To assess whether the C-terminal sequence plays a role in the α_{s1}-casein packaging, the effect of the free C-terminal α_{s1}-casein peptide on the anti-N11L binding to its α_{s1}-casein antigenic form was assayed (Table 1). This was carried out by preincubating a given amount of whole bovine casein with a large excess on molar basis of either free C-terminal or N-terminal peptide and then recording the anti-N11L binding in competitive ELISA tests. In comparison with the control samples, anti-N11L reactive α_{s1}-casein, contained in the whole casein, in the presence of the C-terminal peptide, appeared less able to compete for the antibodies with the antigens immobilized onto the solid support. In fact, whole casein in mixture with the C-terminal peptide gave rise to an inhibition 3-fold lower than that obtained by testing the same amount of casein alone, similar to that obtained from a 5-fold diluted casein sample. On the contrary, the N-terminal peptide in mixture with bovine casein had no significant effect on the inhibitory activity of the anti-N11L reactive species since a binding signal similar to that of bovine casein alone was obtained.

Table 1. Competitive ELISA Results of Whole Bovine Casein Preincubated with either the C-Terminal or N-Terminal Bovine α_{s1}-Casein Peptide[a]

sample	net absorbance at 450 nm[b]	% inhibition[c]
whole casein, 250 μg/mL	0 ± 0.01	100
whole casein, 50 μg/mL	0.18 ± 0.01	89
whole casein, 10 μg/mL	0.90 ± 0.03	46
whole casein, 2 μg/mL	1.42 ± 0.04	15
whole casein, 0.4 μg/mL	1.63 ± 0.05	2
buffer, PBS	1.66 ± 0.04	0
whole casein, 10 μg/mL + C-terminal peptide, 1 mg/mL	1.41 ± 0.04	15
whole casein, 10 μg/mL + N-terminal peptide, 1 mg/mL	0.88 ± 0.03	47

[a] Experimental details: Samples containing 10 μg/mL whole bovine casein and 1 mg/mL C-terminal or N-terminal bovine α_{s1}-casein peptide were incubated at 37 °C for 1 h in PBS and then tested in a competitive ELISA assay. For comparison, five scalar dilutions ranging from 250 to 0.4 μg/mL whole bovine casein were tested in the same plate; 1:500 (v/v) diluted anti-N11L was used as primary antibody. Plate coating and antibody detection were as described under Materials and Methods. Triplicates of each sample were analyzed and the mean values ± standard deviations ("s") were considered, as indicated by Skoog and West (1963). [b] Net absorbance, obtained by subtracting from the actual ELISA signals that of the sample containing the highest concentration of whole bovine casein (250 μg/mL, ELISA test absorbance value: 0.092 ± 0.008). [c] % inhibition = (max − x) × 100/max, where max is the net signal obtained by testing only the PBS buffer and x is the net signal obtained from the corresponding sample.

These results indicated either that the C-terminal sequence of α_{s1}-casein directly interacted with the 139−149 region of truncated α_{s1}-casein forms or that it was able to stabilize conformations which hid the anti-N11L target region. In any case, the anti-N11L reactive species was found to resemble the entire protein in the presence of the free C-terminal peptide, and consequently, the antigenicity of its 139−149 region was decreased.

It is noteworthy that the C-terminal peptide had been unable to compete in control ELISA assays with anti-N11L antibodies for the binding to the antigenic casein coated on the plate wall (results not shown). Most likely the immobilized antigens had undergone such conformational alterations that no interaction between the peptide and the immobilized protein could occurr.

Anti-N11L Antigenicity in Bovine Cheese. To detect anti-N11L antigenic species in bovine cheese and

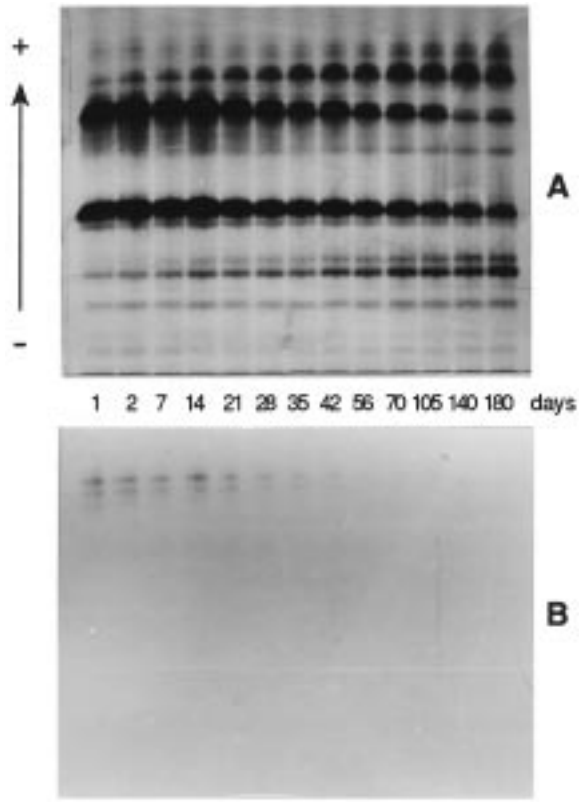

Figure 5. Parmigiano Reggiano cheese samples, ranging from 1 to 180 days old, analyzed by using disc-PAGE at pH 8.6 in denaturing conditions and blotted onto nitrocellulose: (A) Coomassie Brilliant Blue R-250 stain; (B) immunodetection using 1:1000 (v/v) dilutions of anti-N11L as primary antibodies and secondary antibodies as indicated for Figure 2.

to assay their stability during cheese ripening, samples of hard cheese of different ages (from 24 h to 6 months) were analyzed by disc-PAGE at alkaline pH. The Coomassie Brilliant Blue R-250 patterns (Figure 5A) were compared with those stained by anti-N11L sera (Figure 5B). Only a few faint bands were recognized by anti-N11L in cheese samples, starting from the 24-h-old one. Furthermore, the intensity of the immunodetected bands progressively decreased with cheese ripening.

On the contrary, by using competitive ELISA assays, constant amounts of anti-N11L reactive products, irrespective of the ripening age of the cheese, were detected in the same cheese samples analyzed by immunoblotting (experiments not shown). Interestingly, constant levels of anti-N11L reactive species were also detected when only the pH 4.6 soluble fractions of cheese were tested instead of the entire cheese samples (experiments not shown).

On the whole, ELISA and immunoblotting results obtained from cheese analysis indicated that the short α_{s1}-casein was hydrolyzed in the early phases of cheese-making, giving rise to pH 4.6 soluble anti-N11L reactive peptides undetectable by immunoblotting, probably owing to a poor ability to bind to the nitrocellulose sheet as a consequence of their low molecular size. Nevertheless, these hydrolysis products had proven to be stable during cheese ripening, at least with regard to the anti-N11L antigenic region.

Anti-N11L Antigenicity in the Chymosin Hydrolysate of Whole Bovine Casein. To identify the primary degradation products of the short α_{s1}-casein during cheese-making, the in vitro chymosin hydrolysate of whole bovine casein was used as a simplified model of early made cheese and its pH 4.6 soluble fraction was taken into consideration, according to the indications from the preliminary analysis of anti-N11L antigenicity in bovine cheese. The RP-HPLC chromatogram of the pH 4.6 soluble fraction of the chymosin digest is reproduced in Figure 6. Here the results of the competitive ELISA assays analyzing each fraction with anti-N11L, anti-Nterm, anti-Cterm, and anti-α_{s1}I are also reported. The chymosin-mediated products of anti-N11L reactive α_{s1}-casein were clearly localized in a single peak along the RP-HPLC chromatogram. According to the ELISA results, the short α_{s1}-casein, upon chymosin action, was not detectable any longer by anti-Nterm but it was still recognized by anti-α_{s1}I, thus indicating a similar susceptibility of minor and main α_{s1}-casein to chymosin (Mulvihill and Fox, 1979). The f1−23 α_{s1}-casein peptide released from the different α_{s1}-casein forms contained in whole casein was eluted in the first major peak, as resulted from anti-Nterm recognization. As expected, the anti-Cterm antibodies were not able to detect any species among the pH 4.6 soluble products resulting from chymosin hydrolysis of

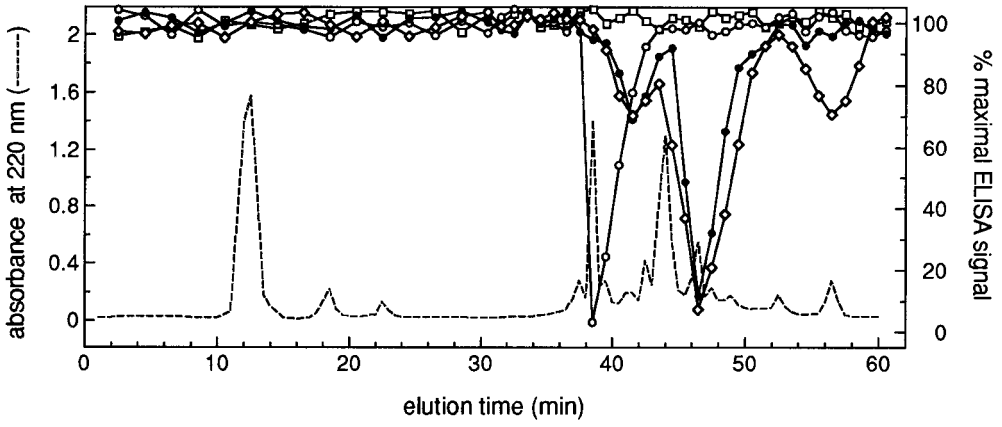

Figure 6. Analysis of the pH 4.6 soluble fraction of in vitro chymosin hydrolysate of whole bovine casein separated by using reversed-phase HPLC on a semipreparative Vydac C_{18} column. Details about the sample preparation, the HPLC equipment, and the elution conditions are given under Materials and Methods. One minute fractions were collected, vacuum-dried, and redissolved in 1 mL of PBS; 1:6 (v/v) dilutions in PBS were tested in competitive ELISA assays by using 1:500 (v/v) anti-N11L (●), 1:2000 (v/v) anti-Nterm α_{s1}-casein (○), 1:2000 (v/v) anti-Cterm α_{s1}-casein (□), and 1:1000 (v/v) anti-α_{s1}I (◇). ELISA methodology was described under Materials and Methods.

whole bovine casein, thus confirming that the C-terminal α_{s1}-casein region was not affected by the enzyme action.

The combination of HPLC as a separative technique with ELISA tests for the screening of the eluted species has proven to be a very powerful analytical methodology for the determination of a specific product within very complex mixtures. Furthermore, the possibility of assaying each fraction from a single separation by using several antipeptide antisera has enhanced our ability to search for and identify the different casein molecular species.

ABBREVIATIONS USED

PBS, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136 mM NaCl, 2.7 mM KCl, pH 7.4; ELISA, enzyme-linked immunosorbent assay; disc-PAGE, vertical polyacrylamide gel electrophoresis; N11L, the peptide NQELAYFYPEL, identical to the sequence 139−149 of bovine α_{s1}-casein; anti-N11L, antisera raised against the N11L peptide conjugated to keyhole limpet hemocyanin; anti-Nterm, anti-Cterm, and anti-α_{s1}I, antisera, respectively, raised against peptides carrying the following bovine α_{s1}-casein sequences: 1−22 (N-terminal), 186−199 (C-terminal), and 23−34 (internal), conjugated to ovoalbumin.

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