Proteolysis of α_s-Casein as a Marker of Grana Padano Cheese Ripening

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ABSTRACT

Since casein proteolysis has a critical role in defining the typical characteristics of Grana Padano cheese, we evaluated the hydrolysis of α_s-casein during the ripening process. Thanks to the high specificity of the anti-$\alpha_s(\alpha_{s1}+\alpha_{s2})$-casein monoclonal antibody and amino acid sequence determination, it was possible to identify three main α_s-casein-derived polypeptides in cheese: α_a, α_b, and α_c. Their production by the three enzymes most involved in cheese proteolysis (pepsin, chymosin, and plasmin) was evaluated by performing in vitro digestions. Data showed that α_a was released in cheese mainly by the chymosin attack, while α_b and α_c were due to the action of plasmin. A significant correlation between the abundance of some polypeptides and ripening process was shown.
(**Key words:** α_s-casein, cheese, proteolysis, ripening)

Abbreviation key: R_f = relative mobility.

INTRODUCTION

Grana cheese is a well-known Italian cheese, appreciated both for its organoleptical qualities and for its nutritional value. Grana Padano is produced in a restricted area of Italy including certain provinces of Piedmont and Lombardy (Italian Presidential Decree, 1955). In 1996, Grana Padano obtained the internationally recognized Denominazione di Origine Protetta (a quality-control guarantee; CE Regulation n. 1107/96, 1996).

The cheeses are cylindrical and weigh from 24 to 40 kg. The principal stages of the standard industrial procedure for the production of Grana Padano cheese are as follows: after partial skimming in stainless steel vats lasting 6 to 8 h, the milk is transferred to copper cauldrons and heated to 32°C. A natural whey culture is added as a starter. Calves' rennet is used for coagulation, and the resulting curd is heated to 54 to 55°C. The warm "pasta" is transferred to special molds, then immersed in salted solution for 20 to 22 d, and finally ripened for 1 to 2 yr.

In grana cheeses (Grana Padano and Parmesan), the most important biochemical event during ripening is the proteolysis of caseins, which represent 80% of cow's milk proteins. During the proteolytic processes, the casein content decreases; in commercial cheese, it corresponds to 10 to 15% of the total proteins (Addeo et al., 1992, 1994). Proteolysis is produced by endo- and exopeptidases having different origins: milk derived (plasmin), rennet derived (pepsin and chymosin), and microbial enzymes. Their presence in a well-balanced ratio is important for the final quality of the cheese.

There are four major caseins produced by mammary epithelial cells: α_{s1}-, α_{s2}-, β, and κ-caseins (Mepham et al., 1985).

The α_{s1}-casein (199 AA) is a mixture of α_{s0} and α_{s1}-caseins with nine and eight phosphate groups, respectively. The α_{s2}-casein (207 AA) is composed of five proteins that differ in the number of bound phosphate groups (from 10 to 13 per molecule) (Grappin et al., 1985).

Several authors have reported how α_s-casein is susceptible to proteolysis both in vitro by purified enzymes and during cheese ripening (Eigel; 1977; Fox, 1989; Grappin et al., 1985; Green and Foster, 1974; Le Bars and Gripon, 1993; McSweeney et al., 1993; Mulvihill and McCarthy, 1993; Scherze et al., 1994; Sienkiewicz et al., 1994).

Since proteolysis is a significant indicator of cheese quality, we studied the profile of α_s-casein during the ripening of Grana Padano cheese, using electrophoretic techniques and specific monoclonal antibodies. As shown in previous studies, these techniques can provide a useful tool to follow cheese proteolysis (Addeo et al., 1995; Restani et al., 1996).

MATERIALS AND METHODS

Purified Proteins and Enzymes

Proteolytic enzymes and purified α_s-casein were purchased from SIGMA Aldrich (Milan, Italy). They had

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the following characteristics: α-casein: lyophilized powder, chromatographically purified; pepsin (EC 3.4.23.1): lyophilized powder, purified by crystallization followed by chromatography, activity = 3200 to 4500 units/mg of protein; plasmin (EC 3.4.21.7): lyophilized powder, activity = 2 to 4 units/mg of protein; chymosin (EC 3.4.23.4): from calf stomach, crystallized and lyophilized.

Cheese Samples

In this study, 100 different Grana Padano cheese samples (at least 10 samples for each time of ripening) were analyzed. They were kindly supplied by the Consorzio per la Tutela del Formaggio Grana Padano (Consortium for the Protection of Grana Padano cheese), Milan, Italy, with the following specifications: month of production, production site, and date of sample collection.

In Vitro Enzymatic Digestions

The in vitro hydrolyses were performed as follows: a) pepsin: enzyme/protein ratio 1/500 (wt/wt) in 0.01N HCl (pH 2) at 37°C, and in 50 m*M* acetate buffer (pH 5) at 37°C; b) chymosin: enzyme/protein ratio 1/100 (wt/wt) in 50 m*M* acetate buffer (pH 5) at 25°C; c) plasmin: enzyme/protein ratio 1/800 (wt/wt) in 50 m*M* phosphate buffer (pH 6.6) at 37°C. The final concentration of α_s-casein was always 3.0 mg/ml. The reaction was stopped at different times by diluting the digestion mixture with the same volume of sample buffer (0.25 *M* Tris-HCl buffer, pH 6.8, containing 7.5% glycerol, 2% SDS, and 5% β-mercaptoethanol) and then heating for 10 min at 100°C. Every in vitro digestion was repeated at least three times.

Preparation of Monoclonal Antibodies

Monoclonal antibodies were prepared according to the method of Galfré et al. (1977), with some modifications. Female Balb/c mice (4 to 6 wk old) were injected intraperitoneally with 50 μg of total casein solution (a mixture of α_s, β, and κ caseins; Sigma Aldrich), mixed with Freund's complete adjuvant and then with two boosters, at 14-d intervals. An antigen booster was administered 3 d before the spleen was removed; the spleen cells were then hybridized with Ag8.X63 myeloma cells in the presence of 50% (wt/wt) polyethylene glycol, molecular weight 1000. The fusion mixture was then diluted to 100 ml with hypoxanthine, aminopterin, thymidine medium and distributed on feeder layers of normal Balb/c mouse peritoneal exudate cells in a 24-well plate. Wells showing anti-α_s(α_{s1} and α_{s2})-casein activity in their supernatant were cloned by limiting dilution. The cloned cells (5 to 19 $\times$ 10^5 cells) were injected into Balb/c mice 1 wk after pretreatment with 0.5 ml of pristane. The monoclonal antibodies harvested from the peritoneal fluid were then assayed and selected.

SDS-PAGE

Cheese samples were separated using a polyacrylamide gel with the following characteristics. Gradient running gel: 9 to 19% acrylamide; 0.08 to 0.17% bis-acrylamide; 0.36 *M* Tris-HCl buffer, pH 8.8; 35% glycerol; 0.1% SDS; 0.02% ammonium persulfate; and 0.15% N,N,N′,N′-tetramethylenediamine. Stacking gel: 3.5% acrylamide, 0.09% bis-acrylamide, 0.125 *M* Tris-HCl buffer pH 6.8; 0.1% SDS; 0.02% ammonium persulfate; and 0.15% N,N,N′,N′-tetramethylenediamine. Running buffer: 25 m*M* Tris, 0.19 *M* glycine and 0.1% SDS (wt/vol), pH 8.8.

Purified α_s-casein was suspended in sample buffer at a final concentration of 1 mg/ml.

After the electrophoretic run (90 V at room temperature, for approximately 6 h) the gels were dyed with Coomassie brilliant blue G-250 according to the method of Neuhoff et al. (1988). All materials and instruments were purchased from Bio-Rad (Richmond, CA). Prestained molecular weight marker solution (broad range, Bio-Rad) contained: myosin (208-kDa), β-galactosidase (115 kDa), BSA (79.5 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (34.8 kDa), soybean trypsin inhibitor (28.3 kDa), lysozyme (20.4 kDa), and aprotinin (7.2 kDa).

Quantitative analyses were performed with a gel scanner (Sharp JX-330, Pharmacia Biotech, Sweden) and the Image Master 1D Software. This allowed the quantification of proteins on the gel, calculating the average density of pixels across the band length and integrating over the bandwidth. To calculate the amount of protein in each sample (g/100 g of cheese) we used a calibration curve generated by plotting the known value of purified protein loaded onto the gel versus the corresponding area obtained by integration. α_s-Casein (α_{s1} + α_{s2}-casein) derived fragments were quantified by the calibration curve obtained with purified α_s casein (we assumed that the fragments had approximately the same affinity for Coomassie blue). Every sample was analyzed at least five times; the coefficient of variation for the values of each sample was always below 5%.

Immunoblotting

After SDS-PAGE, proteins from the gel were transferred onto the polyvinylidene difluoride (Immobilon P;

Millipore, Bedford, MA) membrane by electrophoretic elution (wet transfer). The transfer buffer was: 25 m*M* Tris, 193 m*M* glycine, and 20% methanol. To verify the protein transfer, the absence in blotted gel was assayed by the usual staining procedure. The polyvinylidene difluoride membranes were blocked with 1% gelatin (120 min at 45°C) and washed three times for 2 min with 0.25% gelatin (Bio Rad) solution (in 150 m*M* NaCl, 5 m*M* EDTA, 50 m*M* Tris, 0.05% Triton-X) to prevent nonspecific adsorption of the immunological reagents. The membranes were then immersed in 10 ml of 0.25% gelatin solution, containing 10 μl of anti-$\alpha_s(\alpha_{s1} + \alpha_{s2})$-casein monoclonal antibodies. Antigen-IgG complex was detected with goat anti-mouse IgG antibodies (Sigma Aldrich), labeled with alkaline phosphatase; the secondary antibody commercial stock was diluted 1:1000 (vol:vol) in 0.25% gelatin solution. After incubation, 4 h at room temperature with agitation, the membranes were washed twice with 0.25% gelatin solution (2 min each) and once with Tris buffer solution (TBS; 20 m*M* Tris and 0.5 *M* NaCl) for 5 min.

Finally, after incubation in the bromochloroindolyl phosphate and nitro blue tetrazolium solution, an intense black-purple precipitate developed at the site of enzyme binding. The developing solution contained 15% bromochloroindolyl phosphate and 30% nitro blue tetrazolium in alkaline phosphatase buffer (100 m*M* Tris, 100 m*M* NaCl, 5 m*M* $MgCl_2$, pH 9.5). Unless otherwise specified, chemicals were from Merck (Darmstadt, Germany).

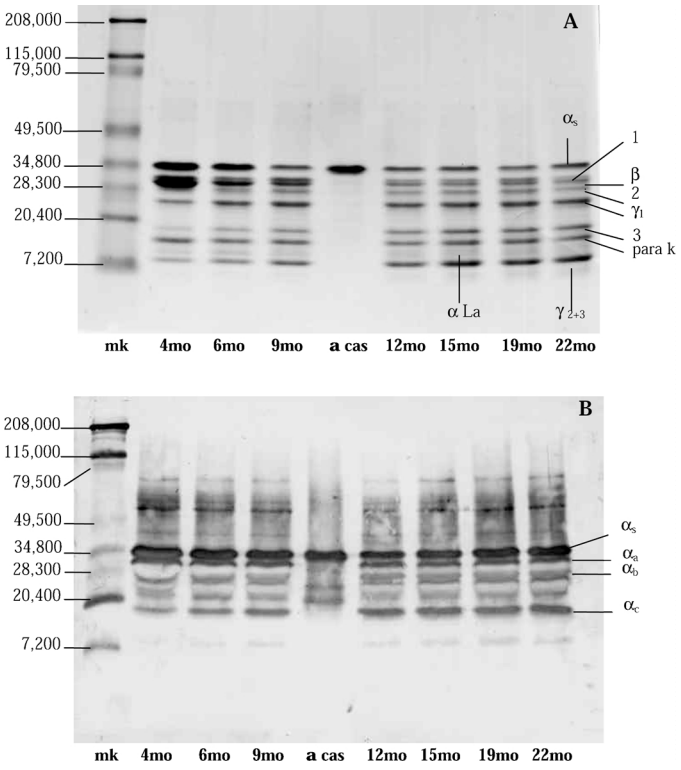

Figure 1. The SDS-PAGE (A) and immunoblotting (B) of cheese samples at different times of ripening. mk = molecular weight standard solution; mo = months of ripening; α, β, αs_1, γ_{2+3}, para k = α, β, αs_1, γ_{2+3}, para k caseins; α-La = α-Lactalbumin; 1, 2, and 3 unknown fragments, α_a, α_b and $\alpha_c = \alpha_a$, α_b, and α_c fragments.

Automatic Determination of the Amino Acid Sequence

Membranes, prepared for sequencing, were stained directly (0.1% Coomassie brilliant blue G-250 in 50% methanol and 10% acetic acid) and the bands were cut with a scalpel.

Amino acid sequences were determined with a Perkin-Elmer Applied Biosystems 492 pulse liquid-phase sequencer, phenylthiohydration-derivative AA were identified by reversed-phase HPLC (Perkin Elmer-Applied Biosystems, Monza, Italy).

Statistical Analysis

The amount of protein associated with α_s-casein (α_{s1} + α_{s2}-casein) and related fragments is expressed as mean ± SE. The significance of the differences between the mean values was calculated by analysis of variance (MANOVA) and then by the Fisher's multiple test.

RESULTS

Among the possible techniques at our disposal for the separation of α_s-casein and the identification of correlated proteolytic fragments, we found the association of SDS-PAGE and immunoblotting the most suitable. In fact, the use of specific monoclonal antibodies permitted easy detection of the products obtained by the enzymatic attack on α_s-casein (α_{s1} + α_{s2}-casein).

In Figure 1A, the electrophoretic patterns of cheese samples at different times of ripening (from 4 to 22 mo) are shown. Several bands can be observed using purified proteins (not shown); some of them are recognized as milk proteins (α_s-, β-, γ-casein and traces of α-lactalbumin) or milk-derived proteins (para k-casein). Because of their very close molecular weights, α_{s1} (22.9 kDa) and α_{s2} casein (24.3 kDa) are not separate in SDS-PAGE.

Other bands could not be identified and were defined as bands 1, 2, and 3.

Figure 1B illustrates the immunoblotting obtained after incubation of a gel, identical to that shown in Figure 1A, with a specific anti α_s-casein (α_{s1} + α_{s2}-casein) monoclonal antibody. The correspondence between bands in the SDS-PAGE and those in immunoblotting was based on relative mobility ($\mathbf{R}_f$) of prestained molecular weight markers.

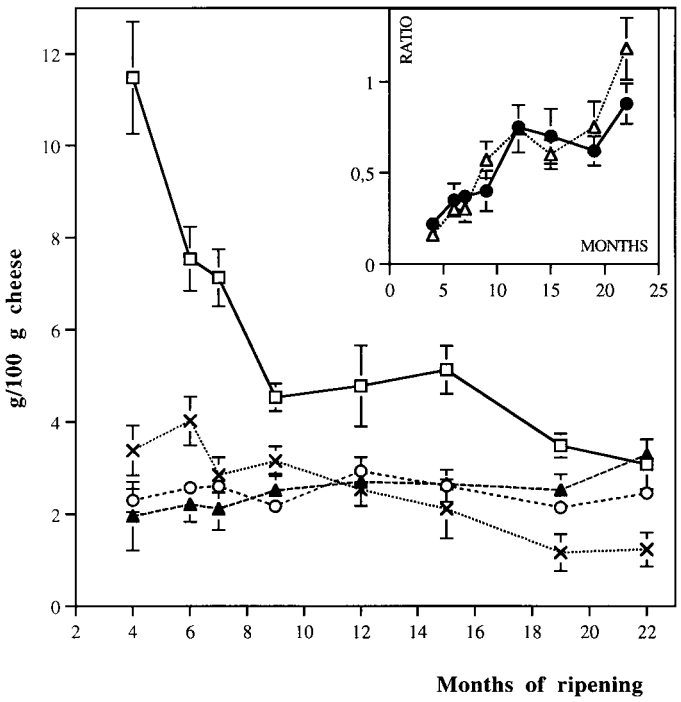

Figure 2. Ripening profiles of α_s-casein (-□-) and its derived proteolytic fragments: α_a (-x-), α_b (-○-) and α_c (-▲-). Values are mean ± SE (n ≥ 10). In the inset: α_b/α_s-casein (-●-) and α_c/α_s-casein (-△-).

Four bands are clearly visible: α_s-casein (as expected) and three fragments with R_f corresponding to bands 1, 2, and 3, previously described. Since the three fragments are recognized by the specific monoclonal antibody, they derive from the proteolysis of α_s- (α_{s1} and α_{s2}) casein, and for this reason they were indicated as α_a, α_b, and α_c (Figure 1B). They could each contain several proteolytic fragments with similar molecular weights.

The amount of protein associated with each band was calculated by densitometry and referring to a calibration curve generated by plotting known values of purified protein loaded onto the gel versus the corresponding area. The profiles of each α_s-casein derived band are reported in Figure 2.

Profiles of α_s-Casein and its Products During Cheese Ripening

α_s-Casein decreased during ripening, mainly from 4 to 9 mo of ripening (from 11.48 ± 1.22 to 4.53 ± 0.30 g/100 g of cheese, mean ± SE); after this period, the proteolysis was slower and at 22 mo of ripening the α_s-casein content was 3.08 ± 0.54. Significant differences were observed between values of the first period of ripening ($P < 0.01$; 4 vs. 6 mo, 6 vs. 9 mo, 7 vs. 9 mo). From 9 mo of ripening, no significant difference was found apart from that between the mo 15 and 22 ($P = 0.04$).

The α_a decreased throughout ripening (from 3.38 ± 0.54 g/100 g of cheese at 4 mo to 1.23 ± 0.37 at 22 mo, mean ± SE), while the profile of the α_b was approximately constant (mean value = 2.47 g/100 g of cheese).

The α_c was the only fragment that increased throughout ripening (from 1.95 ± 0.75 g/100 g of cheese at 4 mo to 3.28 ± 0.19 at 22 mo, mean ± SE).

The amount of protein associated with α_s-casein and related fragments was lower at 22 mo (10.04 g/100 g of cheese) than that calculated at 4 mo of ripening (19.11 g/100 g of cheese); this means that a high quantity of low molecular weight material (AA and short polypeptides), which cannot be analyzed by SDS-PAGE, was liberated by proteolysis.

Ratios Between Areas of α_b/α_s-Casein and α_c/α_s-Casein

In the inset of Figure 2, the ratios between α_b/α_s-casein (solid marks) and α_c/α_s-casein (open marks) are shown. In both cases, there was a statistically significant difference between the values of the first months and the 12th mo of ripening ($P < 0.01$, from 4 to 9 mo vs. 12 mo).

Automatic Determination of the Amino Acid Sequence

Membranes containing the blotted samples were cut with a scalpel, and α_a, α_b, and α_c were sent for automatic microsequencing. The results from sequencing are reported in Table 1; all identified bands apart from one (α_b fragment) derived from proteolysis of α_{s1}-casein.

In Vitro Proteolysis of α_s-Casein

The in vitro proteolysis assays were performed at different values of pH and temperature, taking into consideration both the range of activity of each selected enzyme and the cheese production conditions. The α_s-casein digestion was stopped at different times, and after the SDS buffer was added, the samples were analyzed by SDS-PAGE and immunoblotting, as previously

Table 1. The α_s associated-polypeptides identified by microsequencing.

Band	Polypeptide	Percentage
α_a	α_{s1} (24-199)	~100
α_b	α_{s1} (35-199)	70
	α_{s2} (25-188)	30
α_c	α_{s1} (80-199)	98

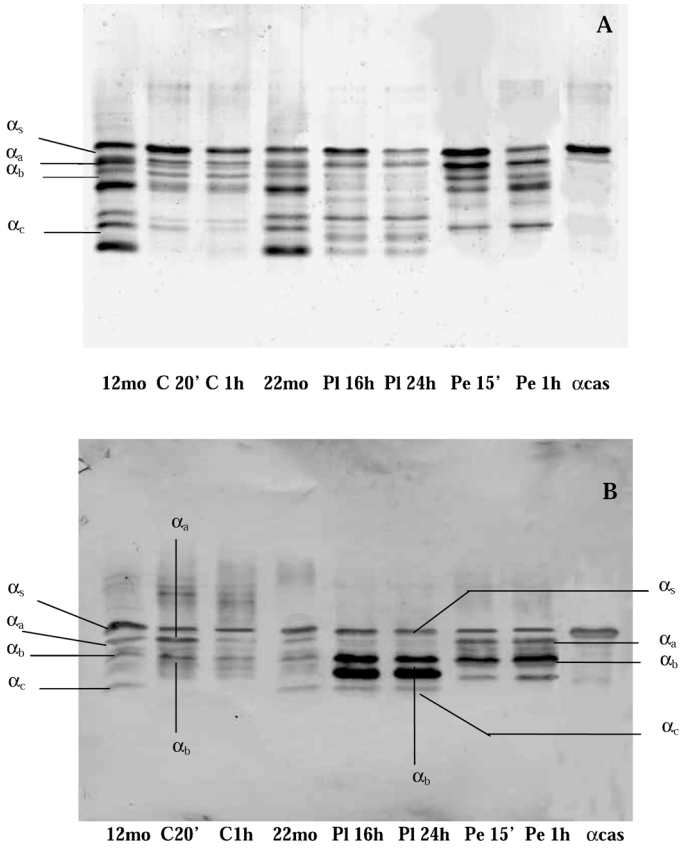

Figure 3. The SDS-PAGE (A) and immunoblotting (B) of the samples obtained from the in vitro digestion of α_s-casein by chymosin (C), plasmin (Pl), and pepsin (Pe). The times indicate the minutes or the hours of incubation.

described for cheese. An example of SDS-PAGE and immunoblotting profiles of digestion products obtained by proteolysis of α_s-casein with the three selected enzymes is reported in Figure 3A and B; two cheese samples were run in parallel to compare the R_f of the protein bands obtained in the in vitro digestion with those of cheese bands. Also, in this case, a quantitative analysis was performed by densitometry.

Proteolysis of α_s-casein by pepsin. The peptic hydrolysis, performed at pH 2, was stopped at 5, 15, 30, 40, 60, and 90 min. The hydrolysis produced α_a and α_b, and some other fragments, which are not present in cheese samples (Figure 3A). After 15 min of in vitro hydrolysis, the production of α_a and α_b was maximal. By this time, α_s casein had decreased to 48.5% of initial protein, α_a and α_b, represented 27.2 and 15.4% of initial protein, respectively. The remaining 8.9% was due to the other three unknown fragments. The digestion performed at pH 5 (nearer to the pH of cheese and still suitable for pepsin) showed the production of the same pattern of fragments but with a drastic reduction of the pepsin activity.

Proteolysis of α_s-casein by chymosin. The hydrolysis of α_s casein by chymosin lasted 5, 10, 20, 40, 60, and 90 min. It produced α_a and α_b and some other polypeptides, which are not present in cheese samples (Figure 3A). At 20 min, α_s casein had decreased to 26.5% of initial protein, α_a and α_b represented 29.7 and 12.4% of initial protein, respectively. The remaining 31.4% was only partially due to the five unknown fragments; thus an important release of AA and short peptides by this enzyme must be hypothesized.

Proteolysis of α_s-casein by plasmin. The hydrolysis of α_s casein by plasmin produced α_b and α_c and some other polypeptides, which are not present in cheese samples (Figure 3A). A fragment with an R_f similar to that of α_a is present in the gel, but it is not recognized by the antibody (Figure 3B); as a consequence, this polypeptide cannot be associated with α_a present in cheese.

The action of this enzyme is slow, and the digestion was stopped at 16, 20, and 24 h. After 16 h of in vitro hydrolysis, the three fragments were all present. By this time, α_s casein had decreased to 29.6% of initial protein; α_b and α_c represented 6.1 and 12.5% of initial protein, respectively. The remaining 51.8% is only partially due to the unknown fragments; plasmin also hydrolyzed the protein to AA and short peptides.

DISCUSSION

Grana cheese is one of the most famous Italian cheeses; its quality is known and appreciated all over the world. According to Italian law, Grana Padano cheese can be sold only after at least 12 mo of ripening. In fact, this period is necessary to obtain the important biochemical modifications that ensure the typical flavor and the high quality of this cheese. Our previous studies showed that the proteolysis of casein is an important parameter to evaluate the ripening period of a cheese both at the production site (dairies) and once the cheese is on the market. In particular, γ-caseins can be considered useful and reliable markers for the characterization of Grana Padano cheese (Restani et al., 1996).

The duration of ripening is one of the most important steps in reaching the standard quality of this cheese; it is also important for certifying the product, using objective parameters to protect it on the international market. The possibility of discriminating objectively between a cheese with only a few months of ripening and a fully ripened cheese could help to prevent fraud.

When the profiles of α_s-casein in cheese samples at different times of ripening are considered, the expected proteolysis, which caused a progressive release of polypeptides, can be confirmed. Among these, three fragments could be associated with $\alpha_s(\alpha_{s1}+\alpha_{s2})$-casein be-

cause of their reactivity with the specific anti-α_s-casein monoclonal antibody; they are called α_a, α_b, and α_c.

Because of AA sequence determinations we were able to identify the polypeptides associated with α_a, α_b, and α_c fragments. The α_a corresponded to the α_{s1}-I fragment (24-199 of α_{s1}-casein AA sequence), which is one of the proteolytic products obtained by the digestion of α_{s1}-casein with rennet (Mulvihill and Fox, 1979; McSweeney et al., 1993). Moreover, this polypeptide has been previously reported in Grana Padano cheese by Ferranti et al. (1997). The identification is supported by our in vitro digestion, in which α_a was produced by chymosin and pepsin. In cheese, the production of α_a could be mainly associated with chymosin; in fact, since it is heat-sensitive (Boudjellab et al., 1994), its action must be more significant in the earliest stages of cheese production, that is, before the heating of the curd. This could be demonstrated by the profile of the α_a concentration, which was highest at the beginning (4 mo) and then decreased gradually during ripening. Pepsin seems to be less involved, since its activity is very low at milk pH (pH 6.6). As shown in our assay, pepsin activity at pH 5 was approximately 20% of that measured at pH 2.

The α_b-associated band contained two polypetides identified by AA sequence: α_{s1} (35-199) and α_{s2} (25-188), as shown by AA sequences. These polypeptides have not been previously identified and are produced by plasmin as shown by in vitro hydrolysis and the presence of the AA Lys in position α_{s1} (24) and α_{s2} (34). As is well known, plasmin cleaves specifically the peptide bonds Lys-X and Arg-X (Humbert and Alais, 1979). Since the α_b concentration is similar during all the periods considered (22 mo), its presence must be the result of a balance between its formation by hydrolysis of α_s-casein (and α_a) and its breakdown to shorter peptides.

According to the AA sequence, α_c was identified as plasmin-deriving fragment α_{s1} (80-199); the same polypeptide has been found in Parmesan cheese by Addeo et al. (1995). Our in vitro hydrolysis assays confirmed the specific release of α_c by plasmin.

To identify a marker of cheese ripening, we performed statistical analyses of different proteolysis parameters; three of them were particularly interesting: α_s-casein profile as well as the ratios α_b/α_s-casein and α_c/α_s-casein. These parameters showed statistically significant differences when the values of "young" cheeses (from 4 to 9 mo) were compared to those of fully ripened cheese (at least 12 mo). No significant correlation was observed between the ratio α_a/α_s-casein and ripening period, although this correlation (α_{s1}-I/α_s-casein) has been found for other cheeses, having a shorter time of ripening (Gouda-type cheese, Izawa et al., 1997).

In conclusion, the results obtained in this study permit us to define the profile of the main proteolytic products of $\alpha_s(\alpha_{s1}+\alpha_{s2})$-casein in Grana Padano cheese. The pattern of α_s-casein and the ratios α_b/α_s-casein and α_c/α_s-casein have been identified as useful tools for the control of cheese ripening and quality; in fact, hydrolysis not only follows time-dependent trends, but is also based on the suitable mixture of proteolytic enzymes.

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