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

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ABSTRACT

Proteolysis has a critical role in defining the typical organoleptic characteristics of Grana Padano, a well-known Italian cheese. During the ripening process, hydrolysis of β-casein produces different fragments, the most abundant and widely studied of which are γ-caseins, three polypeptides containing the HOOC-terminal portion of β-casein. By sodium dodecyl sulfate-PAGE and a specific anti-β-casein monoclonal antibody, two β-casein-derived bands were identified in Grana Padano cheese: β_a and β_b. Thanks to the identification of the amino acid sequences, it was shown that: a) β_a contains γ_1-casein [β-casein (29–209)] and the correlated peptide [β-casein (30–209)]; b) β_b contains γ_2-casein [β-casein (106–209)] and γ_3-casein [β-casein (108–209)].

The production of β_a and β_b by the three enzymes most involved in cheese proteolysis (pepsin, chymosin, and plasmin) was evaluated by performing in vitro digestions. A significant correlation between abundance of some polypeptides and ripening process was shown.
(**Key words:** β-casein, grana cheese, proteolysis, ripening)

Abbreviation key: R_f = relative mobility.

INTRODUCTION

Grana Padano is a well-known Italian cheese, appreciated internationally both for its organoleptic qualities and for its nutritional value. Grana Padano is included in the list of Italian cheeses having Denominazione di Origine Controllata, a definition that includes technological characteristics and geographical restrictions (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 main stages of the production of Grana Padano cheese are as follows. After partial skimming by creaming for 6 to 8 h, the milk is heated to 32°C in copper cauldrons. A natural whey culture is added as a starter and calves' rennet is used for coagulation. The coagulum is cut, the curds are heated to 54 to 55°C, and the warm "pasta" is transferred to special molds. After immersion in a saline solution for 20 to 25 d at 16 to 18°C, the cheeses are ripened for at least 12 mo at 16 to 18°C.

In different cheeses, proteolysis plays a critical role in determining the typical organoleptic characteristics and represents a significant indicator of quality, as shown for Cheddar (Farkye and Fox, 1991, 1992; Farkye and Landkammer, 1992), Emmental (Lawrence et al., 1987), and Mozzarella (Farkye et al., 1991). Proteolysis is caused by enzymes contained in milk (plasmin) and rennet (pepsin and chymosin) or released by microorganisms. Because of the enzyme activity, the casein content decreases during ripening and in commercial grana cheeses (Grana Padano and Parmesan) it corresponds to 10 to 15% of the total proteins (Addeo et al., 1992; 1994; Ferranti et al., 1997).

There are four major caseins in bovine milk: α_{s1}, α_{s2}, β, and κ caseins (Mepham et al., 1985). We previously described the proteolysis of α_s-casein during the ripening of Grana Padano cheese (Gaiaschi et al., 2000). Three $\alpha_s(\alpha_{s1} + \alpha_{s2})$-casein-deriving polypeptides were identified: α_a, α_b, and α_c. The α_a was released in cheese mainly by the chymosin attack, while α_b and α_c were caused by the action of plasmin. In another paper by our group (Restani et al., 1996a), γ-caseins were identified as markers of quality and ripening for the same cheese.

β-Casein is formed from 209 AA and contains five phosphate groups (Ribadeau-Dumas et al., 1972); several authors have reported how β-casein is susceptible to proteolysis both "in vitro" by purified enzymes and during cheese ripening (Fox, 1989; Grappin et al., 1985; Green and Foster, 1974; Mulvihill and McCarthy, 1993). To improve knowledge of the biochemical events

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that occur during the ripening of Grana Padano cheese and to identify further markers of quality and ripening, we evaluated the profile of β-casein proteolysis from 4 to 22 mo of ripening.

MATERIALS AND METHODS

Purified Proteins and Enzymes

Proteolytic enzymes and purified β-casein were purchased from Sigma Aldrich (Milan, Italy). They had the following characteristics: β-casein: lyophilized powder, minimum 90% by electrophoresis; 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, containing approximately 98% protein.

We prepared chemically enriched $\beta + \gamma$-caseins from a total casein extract (Resmini et al., 1980), following the fractionated precipitation method of Tripathi and Gehrke (1969).

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 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 mM acetate buffer (pH 5) at 37°C; b) chymosin: enzyme/protein ratio 1/100 (wt/wt) in 50 mM acetate buffer (pH 5) at 25°C; c) plasmin: enzyme/protein ratio 1/800 (wt/wt) in 50 mM phosphate buffer (pH 6.6) at 37°C. The final concentration of β-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 hydrolysis 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 (Gaiaschi et al., 2001).

SDS-PAGE

Cheese samples were separated with a polyacrylamide gradient running gel (9 to 19%) according to Gaiaschi et al. (2000). Purified β-casein (from Sigma Aldrich) 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). Quantitative analyses were performed using a gel scanner (Sharp JX-330, Pharmacia Biotech, Sweden) and the Image Master 1D Software. This allows 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. Every sample was analyzed at least five times; the coefficient of variation for the values of each sample was always below 5%. 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).

Immunoblotting

After SDS-PAGE, immunoblotting was performed as described previously (Gaiaschi et al., 2000). The only difference was the monoclonal antibody used; in this study, the membranes were immersed in 10 ml of 0.25% gelatin solution, containing 10 μl of anti-β casein monoclonal antibodies.

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 using a Perkin-Elmer Applied Biosystems 492 pulse liquid-phase sequencer; phenylthiohydantoin derivative AA were identified by reversed-phase HPLC (Perkin Elmer-Applied Biosystems, Monza, Italy).

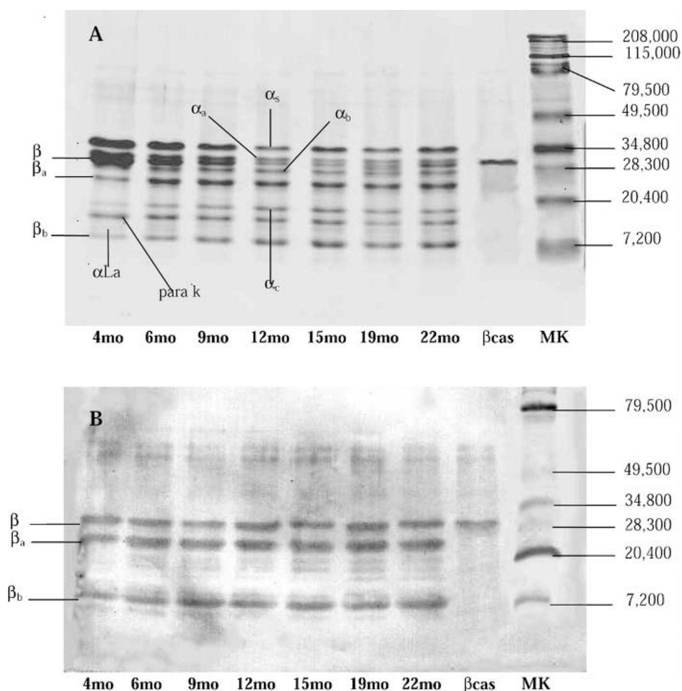

Figure 1. The SDS-PAGE (A) and immunoblotting (B) of cheese samples at different times of ripening. MK = molecular weight standard solution; mo = months of ripening; α_s = α_s-casein, β = β-casein, para κ = para κ casein; α-La = α-lactalbumin; β_a and β_b = β-casein derived-fragments; α_a = α_{s1} (24–199), α_b = α_{s1} (35–199)+α_{s2} (25–188) and α_c = α_{s1} (80–199).

Statistical Analysis

The amount of protein associated with β-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

As described for α_s-casein (Gaiaschi et al., 2000), we used the combination of SDS-PAGE and immunoblotting to detect and quantify the proteolytic fragments of β-casein.

In Figure 1, the electrophoretic (A) and immunoblotting (B) patterns of cheese samples at different times of ripening (from 4 to 22 mo) are shown. Several bands can be observed in the gel. Thanks to purified protein markers (not shown), some of them were recognized as milk proteins (α_s-casein, β-casein, para κ-casein, and traces of α-lactalbumin) or milk protein-derived fragments (α_a, α_b, α_c; Gaiaschi et al., 2000).

After incubation with a specific anti β-casein monoclonal antibody, three bands were clearly visible on the membrane (Figure 1B): β-casein (as expected) and two further bands. Because these two fragments were recognized by the specific monoclonal antibody, they derived from the proteolysis of β-casein. They were indicated as β_a and β_b.

The correspondence between bands in the SDS-PAGE and those in immunoblotting was based on relative mobilities ($\boldsymbol{R_f}$) of prestained molecular weight markers (see Figure 1A and B).

Profiles of β-casein, β_a, and β_b During Cheese Ripening

The amount of protein associated with each band was calculated by densitometry and referred to a calibration curve generated by plotting known values of purified protein loaded onto the gel versus the corresponding area. The profiles of β-casein and its derived bands are reported in Figure 2. β-Casein decreased during ripening; this was mainly evident from 4 to 15 mo of ripening (from 6.70 ± 0.40 to 2.08 ± 0.19 g/100 g of cheese, mean ± SE), when a plateau was reached. Statistically significant differences were observed between values of the first period of ripening and 12/15 mo (P < 0.01; from 4 to 7 mo vs. 12 mo, and from 4 to 9 mo vs. 15 mo).

The profile of the β_a showed an increase from 4 to 15 mo of ripening (from 3.21 ± 0.17 to 4.29 ± 0.21 g/100 g of cheese) and afterwards a slight decrease (at 22 mo, 3.5 ± 0.23 g/100 g of cheese).

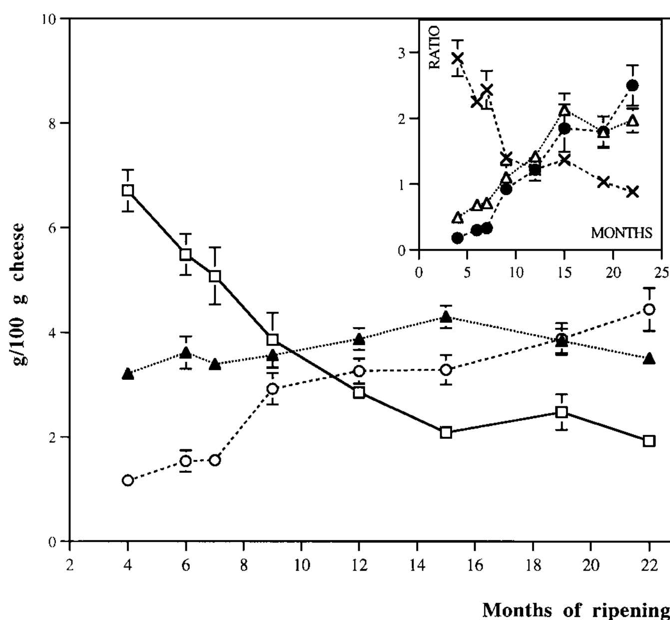

Figure 2. Ripening profiles of β-casein (-□-) and its derived proteolytic fragments: β_a (-▲-) and β_b (-○-). Values are mean ± SE (n ≥ 10). In the inset: β_a/β-casein (-△-), β_b/β-casein (-●-) and $\beta_a\beta_b$ (- X -).

Table 1. β-Casein-associated polypeptides identified by microsequencing.

Band	Polypeptide	Percentage
β_a	β (29–209)	65
	β (30–209)	35
β_b	β (106–209)	40
	β (108–209)	60

The β_b increased during the whole period considered, from 1.16 ± 0.1 g/100 g of cheese at 4 mo to 4.43 ± 0.41 at 22 mo (mean ± SE).

The amount of protein associated with β-casein and related fragments was lower at 22 mo (9.85 g/100 g of cheese) than that calculated at 4 mo of ripening (11.07 g/100 g of cheese); this means that 11% of initial protein was liberated by proteolysis as low molecular weight material (AA and short polypeptides), which cannot be quantified in SDS-PAGE.

Ratios Between Areas of β_a/β-Casein, β_b/β-Casein, and β_a/β_b

In the inset of Figure 2, the ratios between β_a/β-casein, β_b/β-casein, and β_a/β_b are shown. β_a/β-Casein and β_b/β-casein showed increasing trends, while β_a/β_b ratio decreased with the time of ripening. In the β_b/β-casein ratio, there was a statistically significant difference between the first months and 12 to 15 mo of ripening ($P < 0.01$; from 4 to 7 mo vs. 12 mo, and from 4 to 12 mo vs. 15 mo). Similarly, in β_a/β_b ratios, statistically significant differences were observed between values of 4 to 7 mo versus 9, 12, and 15 mo ($P < 0.01$).

Automatic Determination of the AA Sequence

Membranes containing the blotted bands were cut and β_a and β_b were sent for automatic microsequencing; the results are reported in Table 1. The β_a contained the well-known γ_1-casein [β (29–209)] and the correlated polypeptide free of the NH_2-terminal Lys29 [β (30–209)]. Their relative abundance was approximately 2:1.

The β_b contained two known polypeptides: γ_2-casein [β (106–209)] and γ_3-casein [β (108–209)]. Their relative abundance was approximately 1:1.5.

In Vitro Proteolysis of β-Casein

The in vitro proteolysis assays were performed at the optimal conditions (pH and temperature) for each selected enzyme. The in vitro digestions were blocked at different times of incubation and, after the addition of sample buffer, the samples were analyzed by SDS-PAGE and immunoblotting, as previously described for cheese. Some samples coming from the proteolysis of β-casein with the three selected enzymes are shown in Figure 3. Quantitative analyses were performed by densitometry, and the R_f of the polypeptidic bands obtained by in vitro digestion of purified β-casein were compared to those of bands present in cheese samples.

Proteolysis of β-casein by pepsin. β-Casein hydrolysis (performed at pH 2) was stopped at 5, 10, 20, 30, 40, 60, and 90 min. The hydrolysis produced three fragments called Pe1, Pe2, and Pe3 (Figure 3A). After 40 min of in vitro hydrolysis, the production of Pe1 and Pe2 was maximal. By this time, β casein had decreased to 19% of initial protein, Pe1 and Pe2 represented 48 and 29% of initial protein, respectively. Pe3 appeared only after 60 min of in vitro digestion. The digestion performed at pH 5 (nearer to the pH of milk and still suitable for pepsin) showed the production of the same pattern of fragments but with a drastic reduction of the pepsin activity.

Pe2 had the same R_f of β_a as shown in Figure 3A, but it did not react with the anti-β-casein monoclonal antibody (Figure 3B). For the absence of immunoreactivity, it was possible to conclude that this fragment was different from β_a, even though the R_f (and then molecular weight) was similar.

Proteolysis of β-casein by chymosin. The hydrolysis of β-casein by chymosin lasted 5, 10, 20, 40, and 60 min. It produced two fragments: C1 and C2 (Figure 3A).

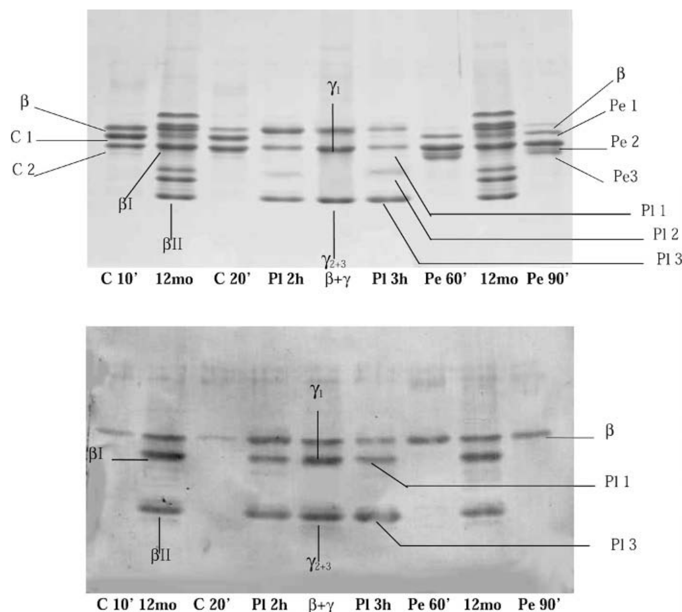

Figure 3. The SDS-PAGE (A) and immunoblotting (B) of the samples obtained from the in vitro digestion of β-casein by chymosin (C), plasmin (Pl), and pepsin (Pe). The times indicate the minutes or the hours of incubation. β + γ = chemically enriched β + γ -caseins (see Materials and Methods).

The maximal production of C1 was observed after 40 min, where it represented 29% of initial protein; for C2 the maximal value was reached at 60 min (31% of initial protein). After 60 min, only 51% of initial protein was still present, thus an important release of AA and short peptides by this enzyme must be hypothesized. As previously observed with pepsin, C1 has the same R_f of β_a but did not react with the anti-β-casein monoclonal antibody.

Proteolysis of β-casein by plasmin. As for chymosin, the hydrolysis of β casein by plasmin produced three fragments: Pl 1, Pl 2, and Pl 3 (Figure 3A). Two of them had the same R_f of β_a and β_b, and showed a positive reaction with the monoclonal antibody. The action of this enzyme is slow and the digestion was stopped at 1, 2, 3, 4, 5, and 6 h. After 1 h of in vitro hydrolysis, the three fragments were all present, but the maximal production was at 5 (Pl 1 and Pl 2) or 6 h (Pl 3). After 6 h, β casein had decreased to 9% of initial protein, while Pl 1, Pl 2, and Pl 3 represented 10, 18, and 36% of initial protein, respectively.

DISCUSSION

Our previous studies showed that the proteolysis of casein follows a time-dependent trend during the ripening period of Grana Padano cheese. In particular, it has been shown that γ-caseins, analyzed by isoelectric focusing, can be considered useful and reliable markers to evaluate the ripening and quality of Grana Padano cheese (Restani et al., 1996a). The pattern of α_s-casein proteolysis and the enzymes involved in the phenomenon have been also described (Gaiaschi et al., 2000). In particular, α_s-casein profile, α_b/α_s-casein, and α_c/α_s-casein ratios have been identified as markers of Grana Padano cheese ripening.

In this paper, we studied β-casein proteolysis, considering both its trend during cheese ripening and in in vitro digestion assays.

Proteolysis caused a progressive release of polypeptides in cheese; two fragments could be associated with β-casein because of their reactivity with the specific anti-β-casein monoclonal antibody: β_a and β_b.

The concentration of β-casein in cheese decreased rapidly during the first 15 mo, when a plateau was reached. An inverse trend was observed for β_a and β_b concentrations; the production of these two polypeptides was evident until the 15th mo of ripening for β_a and until the 22nd mo for β_b. The period required by β-casein to reach a constant trend was slightly longer than that observed for α_s-casein, which showed a slowing of proteolysis after 9 to 12 mo of ripening.

The production of β_a and β_b in cheese must be associated only with the action of plasmin; in fact, as shown by our in vitro hydrolysis, only plasmin was able to release peptides, recognized by the specific monoclonal antibodies, having the same R_f of β_a and β_b.

The results of the microsequencing and the data from the literature about the production of γ-caseins by plasmin (Gordon and Groves, 1975; Grappin et al., 1985; Fox, 1989) permitted us to associate the two immunoreactive bands present in cheese with γ_1- (β_a) and γ_2-+γ_3-caseins. (β_b). We showed in previous papers that purified γ-caseins (γ_1-, γ_2-, and γ_3-caseins) were all able to bind the anti-β-casein monoclonal antibody (Restani et al., 1996b). A further polypeptide was associated with βa: β [30–209]. Chymosin and pepsin were not involved in β-casein hydrolysis in Grana Padano cheese; in fact, the unreactive polypeptides released in in vitro digestion by chymosin (C1 and C2) and pepsin (Pe1, Pe2, and Pe3) were not found in β_a and β_b, as shown by microsequencing. The lack of activity of chymosin and pepsin in grana cheese ripening could be due to their thermosensitivity. In fact, these enzymes probably lose their proteolytic activity as a consequence of thermal (54 to 55°C) treatment of curd (Boudjellab et al., 1994). The unreactive polypeptides, liberated in in vitro assays by pepsin and chymosin, could be identified as the breakdown products described by Creamer (1976) and Visser and Slangen (1977) named βI or β-CN f(1-189/192) and βII or β-CN f(1-165/167). This correlation is supported by their localization in the NH_2-terminal portion of β-casein; in fact, since monoclonal antibody recognized γ-caseins, its epitopic area must include the COOH-terminal portion of β-casein.

The results obtained in this study permit us to define the profile of the main proteolytic products of β-casein in Grana Padano cheese. Their patterns, together with the parameters identified for α_s-casein, could be considered useful tools for the control of cheese ripening and quality.

Data presented in this and our previous papers (Gaiaschi et al., 2001; Restani et al., 1996a) confirm the necessity of a minimum ripening period of 12 mo to guarantee the important biochemical modifications that ensure the typical flavor and the high quality of Grana Padano cheese.

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