γ-Casein as a Marker of Ripening and/or Quality of Grana Padano Cheese

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Several biochemical reactions occur in cheese during ripening. Among these, proteolysis of β-casein to produce γ fractions is one of the most evident. In search of new quality markers, an isoelectric focusing technique in a suitable pH range has been used to evaluate γ-casein profiles in Grana Padano cheese samples at different times of ripening (from 4 to 36 months). The following ratios between densitometric values were considered: γ_2-casein/γ_1-casein, γ_3-casein/γ_1-casein, and γ_3-casein/γ_2-casein. Another parameter investigated was the presence of modified γ_2-casein (with pI = 6.7), which derives from the interaction between γ_2-casein and aldehydes coming from microbial metabolism. The appearance of this protein band correlates well with the cheese ripening period. Data shown in this paper indicate that γ fractions could be considered as new markers of ripening and quality and contribute to the certification of Grana Padano cheese on the international market.

Keywords: *Cheese; ripening; quality certification; γ-casein; modified γ-casein; isoelectric focusing*

INTRODUCTION

Grana cheese is one of the most famous Italian cheeses; its quality is known and appreciated all over the world. Grana Padano is produced in a restricted area of Italy, namely, certain provinces of Piedmont and Lombardy (Italian President's Decree, 1955). The cheeses are cylindrical and weigh from 24 to 40 kg. The principal stages of the standard industrial procedure for the production of Grana cheese are as follows: after partial skimming in stainless steel vats and lasting 6−7 h, the milk is transferred to copper cauldrons and heated at 32 °C. A natural whey culture is added as a starter. Calves' rennet is used for coagulation, and the resulting curd is heated at 54−55 °C. The warm "pasta" is transferred to special molds, then immersed in salted solution for 20−22 days, and finally ripened for 1−2 years.

The casein fraction represents 80% of the cow's milk proteins; cow's milk contains an average of 25 g/L of caseins (Jennes, 1970). Caseins have an important role in cheese making, since this protein fraction precipitates during milk clotting to produce curd. Several biochemical modifications and interactions, involving caseins, occur in Grana cheese during ripening. The proteolytic processes produce a decrease in casein content; in commercial Grana cheese, caseins as such correspond to 10−15% of the total proteins (Addeo et al., 1988). Endo- and exopeptidases that cause proteolysis have different natures: milk deriving (plasmin), presamic (pepsin and chimosine), and microbial enzymes. Their presence in a balanced ratio is important for the final quality of the cheese.

Among the proteolytic products, some different polypeptides are produced; in this paper γ-casein subfractions (γ_1, γ_2, and γ_3) are considered; they derive from β-casein hydrolysis at the NH_2 terminal (fragments 29−209, 106−209, and 108−209, respectively). γ-Casein formation begins during milking as a consequence of the proteolytic action of plasmin on β-casein (Eigel et al., 1979) and lasts throughout the ripening period.

Proteolysis due to the plasmin action is important for defining cheese characteristics, and it can also influence cheese quality; this has been shown for different cheeses such as Cheddar (Farkye and Fox, 1991, 1992; Farkye and Landkammer, 1992), Emmental (Lawrence et al., 1987), and Mozzarella (Farkye et al., 1991).

Another important group of substances produced by microorganisms during ripening is the volatile fraction; in a suitable quantity, it contributes to the typical flavor of Grana cheese. The volatile fraction includes esters, aldehydes, acids, ketones, hydrocarbons, lactones, etc. Our previous studies showed that the γ_2-casein is able to react with aldehydes (Restani et al., 1988), producing a cyclic derivative at the amino-terminal histidine. As a consequence of histidine cyclization, γ_2-casein changes its pI, producing a new molecule called "modified γ_2 casein" with pI = 6.7 (Restani et al., 1989).

Starting from these considerations, the aim of this study was the identification of new parameters that could be useful as ripening markers or for certifying the quality of Grana Padano cheese.

MATERIALS AND METHODS

Chemicals. Chemicals were of the highest purity grade available commercially, and when unspecified, they were from Bio Rad (Richmond, CA).

Cheese Samples. In this study, 132 Grana Padano cheese samples (without formaldehyde addition as a food additive) 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.

Isoelectric Focusing. Gels for isoelectric focusing (IEF) had the following final concentration: 7% acrylamide (*CAUTION*: Acrylamide is a neurotoxic substance!), 0.19% *N*,*N*′-methylenebis(acrylamide), 2.5% carrier ampholytes pH 5−8 and 2.5% carrier ampholytes pH 6−8 (Pharmacia Biotech

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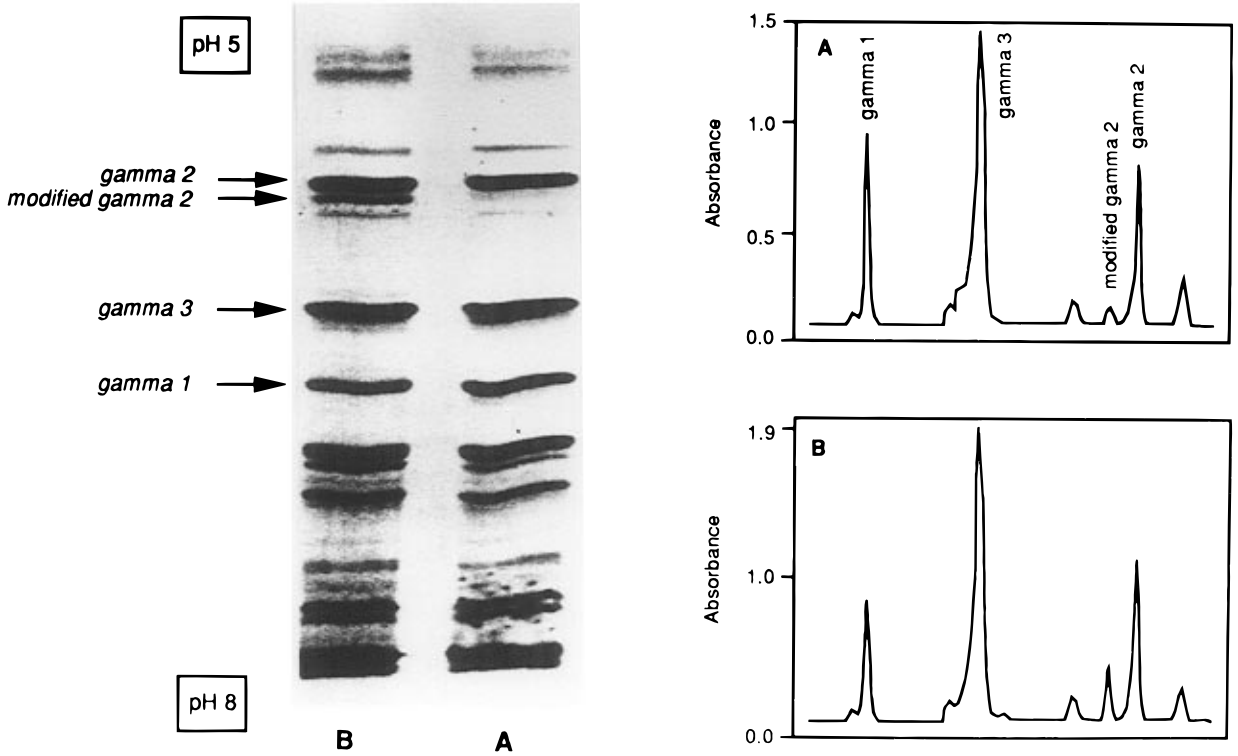

Figure 1. Electrophoretic (IEF) patterns and densitometries of two Grana Padano cheeses at 4 (A) and 34 (B) months of ripening.

AB, Uppsala, Sweden), 6 M urea, 0.02% ammonium persulfate, and 0.03% *N,N,N′,N′*-tetramethylendiamine (TEMED). Samples (30 μL corresponding to 1.2 mg of cheese in freshly prepared 8 M urea) were loaded onto the gel, using cellulose application pieces, in the anodic area. IEF analyses were performed at 8 °C (Multitemp II, Pharmacia Biotech) and at constant power (15 W, Microdrive 5, Pharmacia Biotech) for 4 h. Gels were stained with Fast Green (Gorovsky et al., 1970), overnight at room temperature (22 ± 2.0 °C).

The quantity of protein associated with each band was evaluated by analyzing IEF gels with a laser densitometer (Ultroscan XL, Pharmacia Biotech) and expressed as a percent of the total area (associated with all γ-caseins).

Statistical Analysis. Results are expressed as mean ± SEM. The significance of the differences between the mean values was calculated by analysis of variance (MANOVA) and then by the Fisher's multiple range test.

RESULTS

In this paper, only γ-casein is considered. This choice comes from our previous studies where this group of proteins showed interesting changes during ripening (Restani et al., 1988, 1989). The best technique for separating γ-casein subfractions is IEF, and since pI of γ-caseins is close to 7, the pH range between 5 and 8 was chosen. In Figure 1, the electrophoretic patterns and corresponding densitometries of two cheese samples at different months of ripening (4 and 34) are shown. Main protein bands are indicated and considered: γ_1, γ_2, and γ_3 are easily identifiable in both samples. They are present in different amounts, as is evident from the relative abundance. The subfraction called modified γ_2 is negligible in most samples at the beginning of cheese making, while it is clearly visible at 34 months of ripening.

After densitometric analysis, data on the specific ripening profiles of γ-casein subfractions can be processed.

Ripening Profiles of γ_1, γ_2, and γ_3. Since in the chosen pH range only a small part of the cheese proteins is included, to describe the protein changes during ripening, we could not use absolute values, so it was necessary to resort to relative quantities. In Figure 2 the profiles of γ_1-, γ_2-, and γ_3-caseins are expressed as the relative abundance with reference to the total protein associated with the γ-casein. γ_1-Casein, the fraction corresponding to the sequence 29−209 of β-casein, decreases during ripening because of the enzymatic hydrolysis that releases γ_2 (106−209 of β-casein) and γ_3 (108−209 of β casein) (Farkye and Fox, 1991, 1992; Farkye and Landkammer, 1992; Farkye et al., 1991; Gordon et al., 1972; Ribadeau Dumas et al., 1972). Significant differences are observed between values of the first period of ripening ($p < 0.01$; 4 mo vs 7 mo, 6 vs 7, 7 vs 12, 9 vs 15, 10 vs 15, 12 vs 15, and 15 vs 34). From 19 months of ripening no significant difference is found.

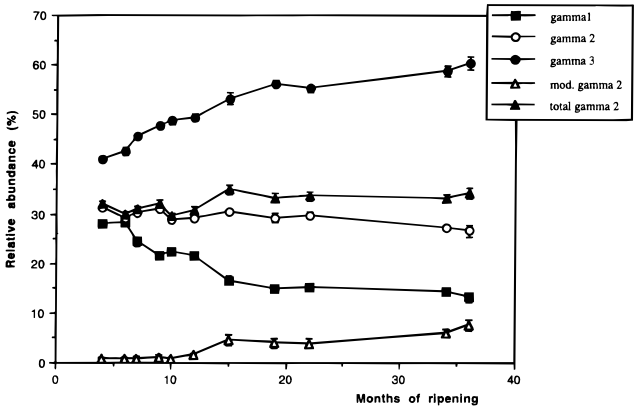

Figure 2. Relative abundance (%) of γ_1-, γ_2- (unmodified, modified and total), and γ_3-caseins during ripening. Data are expressed as the area of each protein subfraction in relation to the total area associated to γ-casein. The number of samples considered at each time is ≥ 8, and values are average ± SE.

γ_3-Casein increases continuously with significant differences throughout ripening ($p < 0.01$; 4 mo vs 7

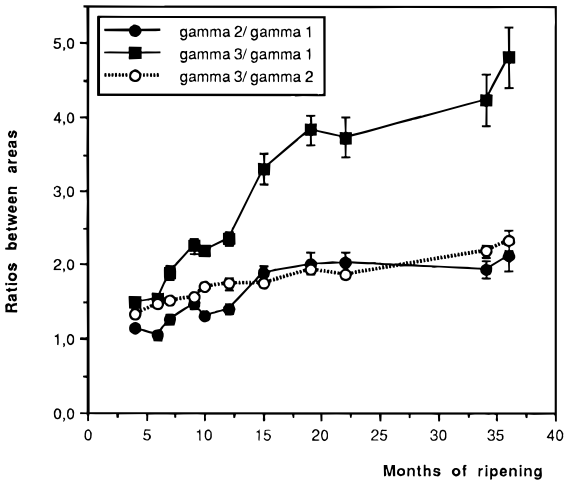

Figure 3. Ripening profiles of the ratios between areas of γ_2/γ_1; γ_3/γ_1, and γ_3/γ_2. The number of samples considered at each time is ≥8, and values are average ± SE.

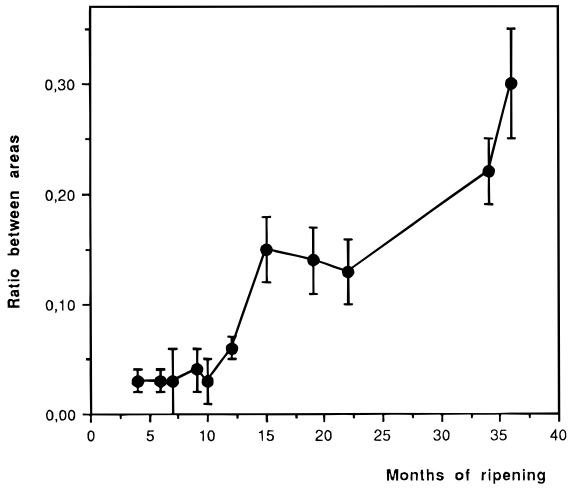

Figure 4. Ripening profiles of the ratios between areas of modified γ_2- and γ_2-caseins. The number of samples considered at each time is ≥8, and values are average ± SE.

mo, 6 vs 7, 7 vs 10, 9 vs 15, 10 vs 15, 12 vs 15, 15 vs 19, 19 vs 36, and 22 vs 34).

Unmodified γ_2 is approximately constant, and significant differences are observed only between values at the beginning and end of the ripening period ($p < 0.01$; 4−22 and 36 mo).

Modified γ_2 shows an important increase between 10 and 15 months of ripening; the significant differences are limited to the values before and after that period ($p < 0.01$; 4−12 mo vs 15 mo). The profile of total γ_2 is similar to that of modified γ_2 and the significant differences also correspond ($p < 0.01$; 4−12 mo vs 15 mo).

Ratios between Areas of γ_2/γ_1, γ_3/γ_1, and γ_3/γ_2. As clearly visible in densitometries reported in Figure 1, γ_3-casein has the biggest area in both samples (at 4 and 34 months of ripening), but its abundance in relation to γ_1- and γ_2-caseins is different. In the later months of ripening, the area of γ_3 increases as shown by the value of absorbance reported in the *Y* axis, while γ_1 and γ_2 present similar areas, although their relative abundance is inverted. In Figure 3, the ratios between γ_2/γ_1, γ_3/γ_1, and γ_3/γ_2 are shown. The most important change is observed in the curve representing the ratio between the areas of γ_3- and γ_1-caseins; in fact, its increase during ripening presents the best statistically significant differences ($p < 0.01$; 4 mo vs 9 mo, 6 vs 9, 7 vs 12, 9 vs 15, 10 vs 15, 12 vs 15, and 15 vs 34).

Ratios between Areas of Modified γ_2-Casein and γ_2-Casein. In Figure 4 the ratios between modified γ_2-casein (pI = 6.7) and γ_2-casein are illustrated: also in this case, the increase of modified γ_2-casein strictly parallels the cheese ripening. For this parameter the variability is high, as shown by the standard error values. Significant differences are shown between the values obtained before and after 15 months ($p < 0.01$; 4−12 mo vs 15 mo) and before and after 22 months ($p < 0.01$; 15−19 mo vs 34−36 mo).

DISCUSSION

Grana cheese is one of the most famous Italian cheeses; its quality is known and appreciated all over the world. To present the required flavor and fragrance, Grana cheese needs at least 12 months of ripening, which is the shortest period permitted before commercialization. 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 by using objective parameters to protect it in the international market.

The possibility of discriminating objectively a cheese having only a few months of ripening from a fully ripened one could help in preventing fraud, both in terms of commercialization of young cheese having a lower added value and from the hygienic point of view. In fact, a milk rich in undesired microorganisms (CO_2-producing) cannot reach the 12 months of ripening, because of the high incidence of breaking of the cheese (Bottazzi and Corradini, 1987).

The research here reported was mainly devoted to the study of the γ-casein fraction, which was the object of our previous paper (Restani et al., 1988). This fraction is released by hydrolysis of β-casein during ripening, starting from the milking process; moreover, it is a very reactive fraction as shown by the high capability of γ_2-casein to bind free aldehydes (Restani et al., 1988). γ-Casein subfraction profiles confirmed the proceeding of proteolysis during ripening with a continuous increase of the components having lower molecular weights. In particular γ_3 casein (the smallest one) rises throughout ripening; γ_1-casein (with higher MW) decreases in parallel, while unmodified γ_2-casein seems to remain constant. This unchangeability is really due to the balance between its release from γ_1 and its further hydrolysis to produce γ_3. Modified γ_2-casein appears only after 12 months and increases during the following period of ripening. The total γ_2-casein shows a profile similar to that of unmodified γ_2-casein, with a slight increase after 12 months of ripening in relation to the modified γ_2 appearance.

Considering the profiles obtained by dividing the areas of different γ-casein subfractions, three parameters are considered here. The most interesting of these is the ratio between γ_3 and γ_1, which shows an important and continuous increase during ripening. This increase is significantly faster after 12 months of ripening, that is, the time after which the cheese can be commercialized.

Another remarkable parameter is the ratio between modified γ_2-casein (pI = 6.7) and γ_2-casein. The profile shows an initial step where the abundance of modified γ_2 is negligible and then from 10 months of ripening a sharp increase. These data confirm our hypothesis that modified γ_2-casein derives from the interaction of NH_2-

terminal histidine of γ_2-casein with aldehydes produced by microbial metabolism of lactose and fats (Contarini and Zucchetti, 1992). In fact, it has been shown that aldehydes more reactive with γ_2-casein (such as butyraldehyde and 3-methylbutyraldehyde) are present in significant amounts after 13 months of ripening, where modified γ_2-casein appears in higher quantity (Contarini and Zucchetti, 1992). A higher variability was observed for this parameter as shown by the standard error values. This variability seems to be in relation to the province of production, and it could be associated with the amount of microorganisms present in milk at the beginning of cheese production and/or to the rennet used.

In conclusion, γ-caseins can be considered useful and reliable markers of cheese ripening; in particular, the evaluation of the ratios between γ_3-casein/γ_1-casein and modified γ_2-casein/γ_2-casein permits easy and acceptably precise identification of the month of ripening. These parameters could be used for the certification of Grana Padano cheese in the international market, and moreover they could help to keep frauds in check.

Finally, the IEF technique described here permits the loading of cheese as such, without time-consuming procedures. It is accurate and reproducible; it is rapid (16 samples in 36 h, including the time for the preparation of the gel and for elaboration of data), simple, relatively inexpensive, and easy to use in routine controls. The use of relative values, such as the ratio between γ-casein areas, makes this method particularly reliable since the most usual experimental variables (errors in loading or problems of solubility, etc.) cannot change it.

ACKNOWLEDGMENT

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