Hydrolysis of $\alpha_S$- and $\beta$-caseins during ripening of Serra cheese

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Hydrolysis of the major caseins in Serra cheese manufactured from raw sheep’s milk coagulated with a plant rennet (*Cynara cardunculus, L.*) was monitored by urea-PAGE electrophoresis throughout a 35 day ripening period (with sampling at 0, 7, 21 and 35 days) and throughout the cheesemaking season (with sampling at November, February and May). The $\alpha_\text{s}$ and $\beta$ caseins were degraded up to 82 and 76%, respectively, by 35 days of ripening. The $\alpha_\text{s}$-casein variants ($\alpha_{2\text{s}}$ and $\alpha_{3\text{s}}$) displayed similar degradation patterns to one another, but different from those of $\beta$-casein variants ($\beta_1$ and $\beta_2$). Although the $\alpha_\text{s}$-caseins were broken down more slowly than $\beta$-caseins at early stages of ripening (97, 95, 80, and 60% of $\alpha_{2\text{s}}$, $\alpha_{3\text{s}}$, $\beta_1$, and $\beta_2$-caseins, respectively, were still intact by 7 days), this observation was reversed for later stages of ripening (18, 18, 30, and 20% of $\alpha_{2\text{s}}$, $\alpha_{3\text{s}}$, $\beta_1$, and $\beta_2$-caseins, respectively, were still intact by 35 days of ripening). The position along the cheese-making season significantly affected the hydrolysis of only the $\beta_2$- and $\alpha_{3\text{s}}$-caseins. Degradation of $\alpha_{2\text{s}}$-casein was slower in February than in November or May for 21-day old cheeses; cheeses ripened for 7 days or 21 days showed more intact $\beta_2$-casein when manufactured in May than in November or February. The magnitude of the correlation coefficients pertaining to concentrations of intact $\alpha_\text{s}$- and $\beta$-caseins indicated that the products of proteolytic breakdown with higher mobility than $\alpha_\text{s}$-caseins (tentatively termed $\alpha_1$-I, $\alpha_2$-I, and $\alpha_3$-I) were preferentially correlated with $\alpha_\text{s}$-caseins, the products of proteolytic breakdown with mobility between $\beta$-caseins and $\alpha_\text{s}$-caseins (tentatively termed $\beta_1$-I and $\beta_2$-I) were preferentially correlated with $\beta_1$- and $\beta_2$-caseins rather than with $\alpha_\text{s}$-caseins, and the products of proteolytic breakdown with the highest mobility (tentatively termed $\alpha/\beta_1$-II and $\alpha/\beta_2$-II) were preferentially correlated with $\beta$-caseins.

INTRODUCTION

The most important and famous variety of traditional Portuguese cheese is manufactured in the inner regions of Portugal geographically confined to the Serra mountains; this type of cheese is made from raw sheep’s milk on the farm level only with the dried flowers of the plant *Cynara cardunculus* (thistle) as rennet and without any deliberate addition of a starter. In addition to a clotting activity similar to that of chymosin (Vieira de Sá & Barbosa, 1970, 1972; Barbosa, 1983), such vegetable rennet also displays a strong proteolytic action *in vitro* (Morgado, 1990; Sousa, 1993) which eventually leads to extensive breakdown of the caseins in the cheese matrix. Protein breakdown has an obvious role in determining the texture (which in the case of Serra is rather soft and buttery) and background flavour intensity (which in the case of Serra is rather clean, smooth and slightly acid) and thus in making flavour precursors available (Adda et al., 1982). Although primary proteolysis has been extensively studied for several cheese varieties manufactured with bovine or ovine milks coagulated with animal or microbial rennets, little information is available on the primary proteolysis of cheeses manufactured from ovine milk using a plant rennet, and essentially no information has been generated on the primary proteolysis of Serra cheese (as emphasized in the comprehensive review by Macedo et al., 1993).

The kinetic characteristics (i.e. rate), the thermodynamic characteristics (i.e. extent) and the selectivity characteristics (i.e. the relative rate of enzymatic action towards the various caseins and fragments thereof, available in the cheese matrix) of proteolysis are known to depend on the source of enzyme(s) in question, the type of cheesemaking technology (and thus the

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milk was then allowed to rest at about 28 °C until coagulation had occurred (for about 1 h); the curd was filtered through a fine, clean cloth and the clear filtrate was added to the milk and gently stirred; the curd paste was then divided into several plastic perforated moulds and drainage of whey was completed via rubberized rubber bottoms. The moulds were then inverted after about 6 h; salting was completed by rubbing the whole outer surface of the cheese with kitchen salt (at a level of about 15 g of salt per cheese); cheeses were ripened in chambers at 9 °C and relative humidity at 95% for the first week and thereafter in chambers without temperature or humidity control; the cheeses were inverted daily.

Three cheeses from each batch were taken randomly during the ripening period after 0, 7, 21 and 35 days and transported under refrigerated conditions (about 4 °C) for analysis. After having removed the rind, samples were taken from each cheese, homogenized and frozen at about -30°C in Whirl-pak® vacuum packages (Cole-Parmer, Chicago, IL), until analysed.

Chemical analyses

The total protein content of cheese was determined on 0.3 g samples by the micro-Kjeldahl method (Anon., 1993) using a Kjeltec system with a 2012 digestor and a 1002 distilling unit (Tecator, Hoganas, Sweden).

Biochemical analyses

Cheese samples (0.9 g) were thoroughly mixed with 20 ml of protein solvent as described by Cramer (1991). Standard ovine casein solution was prepared by isoelectric precipitation of sheep's milk at pH 4.3 using HCl; the precipitate was washed several times with distilled water, dissolved in distilled water at pH 6.6 by repeated addition of small volumes of 0.1 N NaOH and freeze-dried (FTS Systems, Stone Ridge, NY). Proteolysis in the aliquots of cheese samples was monitored by electrophoresis on 6 M urea-containing polyacrylamide gels (PAGE) (12.5% T, 4% C, pH 8.9) with stacking gel (4.2% T, 5% C, pH 7.6) as described by Andrews (1983) and carried out on a Protean II xi cell vertical slab-gel unit (Bio-Rad Laboratories, Watford, UK). The 1000/500 power supply (Bio-Rad Laboratories) was set at 280 mV for the stacking gel and at 300 mV for the separating gel. Gels were stained with Coomassie Blue G250 (Bio-Rad, Richmond, CA) using the method of Blakesley & Boezi (1977). Quantitation of each band in the electrophoretogram was done at 550 nm using a CD60 Desaga densitometer (Desaga Sarstedt-Gruppe, Heidelberg, Germany); the extent of hydrolysis of αs- and β-caseins was assessed via the ratio of intensities of the corresponding bands at the ripening time to those at the initial time. Each cheese sample was run in two gels prepared on different days. Staining and destaining were done for the same time always using similar amounts of freshly prepared solutions.

Statistical analyses

The Statview® 4.0 statistical package (Haycock et al., 1992) was used for statistical treatment of the results via analysis of variance (ANOVA table) and Fisher's protected least significance difference test (Fisher's PLSD). This methodology was acceptable from a statistical point of view because the experimental errors were independent and normally distributed (diagnostics not shown). The two types of tests were employed to determine overall and pairwise, respectively, statistical differences between the concentrations of each casein at the 5% level of significance throughout the ripening period and the cheesemaking season. The same software was employed to calculate correlation coefficients between intact caseins and primary breakdown products.
Hydrolysis of caseins in Serra cheese was studied to understand the changes in casein composition during ripening. Caseins were classified into two major groups based on electrophoretic mobility: one group with lower mobility consists of α-caseins and is subdivided into two variants, β1- and β2-casein, which have a common polypeptide chain and appear to differ only in the degree of phosphorylation (6 and 5 phosphate residues, respectively). The other group with higher mobility consists of β-caseins and is subdivided into three variants, β1-, β2- and γ-casein; the clearly dominant β1- and β2-casein have similar molecular weights and calcium sensitivities but different behavior in Mg2+-containing PAGE gels (Richardson & Creamer, 1976). The 0 d-sample in Fig. 1 shows bands with greater electrophoretic mobility than β-caseins but lower than α-caseins which decrease in intensity with ripening time. Following the claim by Sousa (1993) that *Cynara cardunculus* and chymosin possess comparable specificity on bovine α-casein and αs-casein, these two bands were therefore tentatively labelled as β1- and β2-caseins. Figure 1 also shows the appearance and increase in intensity of bands with greater electrophoretic mobility than those of αs-caseins. These bands had electrophoretic mobilities similar to the band produced when bovine αs-casein was incubated with extracts from flowers of *C. cardunculus* in solution (Sousa, 1993) and were tentatively labelled as αs-casein. Two other bands with greater electrophoretic mobility than αs-casein were produced from the very beginning of ripening and became thicker as ripening elapsed. These two bands were tentatively termed αβ1- and αβ2-caseins.

Figure 2 shows the ratio of concentrations of intact αs-casein and β-caseins to their initial concentrations during ripening and during the cheesemaking season. The results show significant changes in the ratio of intact caseins to their primary degradation products over time. The correlation coefficients between intact caseins and their primary degradation products are listed in Table 1.

### RESULTS

The urea-polyacrylamide gel electrophoretograms (urea-PAGE) of Serra cheese during ripening (a typical example is available as Fig. 1) show that the caseins from the Bordeira sheep contain two major groups of electrophoretic bands. According to Richardson & Creamer (1976), the group with lower mobility consists of β-caseins and is subdivided into two variants, β1- and β2-casein, which have a common polypeptide chain and appear to differ only in the degree of phosphorylation (6 and 5 phosphate residues, respectively). The group with higher mobility consists of α-caseins and is subdivided into three variants, αs1-, αs2- and αs3-casein; the clearly dominant αs2- and αs3-casein have similar molecular weights and calcium sensitivities but different behavior in Mg2+-containing PAGE gels (Richardson & Creamer, 1976). The 0 d-sample in Fig. 1 shows bands with greater electrophoretic mobility than β-caseins but lower than α-caseins which decrease in intensity with ripening time. Following the claim by Sousa (1993) that *Cynara cardunculus* and chymosin possess comparable specificity on bovine β-casein and α-casein, these two bands were therefore tentatively labelled as β1- and β2-caseins. Figure 1 also shows the appearance and increase in intensity of bands with greater electrophoretic mobility than those of αs-caseins. These bands had electrophoretic mobilities similar to the band produced when bovine αs-casein was incubated with extracts from flowers of *C. cardunculus* in solution (Sousa, 1993) and were tentatively labelled as αs-casein. Two other bands with greater electrophoretic mobility than αs-casein were produced from the very beginning of ripening and became thicker as ripening elapsed. These two bands were tentatively termed αβ1- and αβ2-caseins.

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### DISCUSSION

Proteolytic activity in cheese is determined chiefly by the levels and type of residual rennet and indigenous milk proteinases present, salt to moisture ratio, temperature of ripening, and changes in pH during ripening (Lawrence et al., 1987). By 35 days of ripening, the αs- and β-caseins had undergone extensive degradation, up to 82 and 76%, respectively. For long times of incubation, Sousa (1993) has reported that aqueous extracts of dried flowers of *C. cardunculus* had higher proteolytic activities in vitro than chymosin and Vieira de Sá & Barbosa (1972) have observed high proteolytic activity (measured as the increase in water-soluble nitrogen and 4% TCA soluble nitrogen contents) of this plant rennet using cow's and sheep's milk as substrates. The unusually extensive hydrolysis of β-caseins (degradation of β- and αs-caseins occur to similar extents in Serra cheese) seems to be related with the type of rennet (plant instead of animal) used since Dinshar et al. (1989) have reported that a plant rennet obtained from *Withania somnifera* also extensively breaks down β-caseins in Cheddar cheese.

In cow's milk, β-casein is the most susceptible substrate to be hydrolyzed by plasmin leading to the formation of γ-caseins and some of the proteose peptones (Fox et al., 1993). Plasmin activity in milk is increased by pasteurization, possibly by inactivation of plasmin inhibitors or by increasing the rate of activation of plasminogen (Grufferty & Fox, 1988). It is proposed that a similar situation occurs in cheeses when higher cooking temperatures are used during manufacture (Farkye & Fox, 1990). No bands were detected in the region of γ-caseins for Serra cheese (see Fig. 1), which is thus a clue to low plasmin activity; since raw milk without any type of thermal treatment is utilized and cooking is not used in Serra cheesemaking, no enhancement of that enzyme is expected either.

As expected, hydrolysis of caseins (αs2-, αs3-, β1-, and β2-) was affected significantly by the ripening time ($P < 0.0001$ for all such caseins, see Fig. 2). The results...
of Fisher’s PLSD analyses for α-caseins indicate that these proteins were significantly degraded only after 7 days of ripening; ca. 97% of α₂-casein was still intact at 7 days, 70% at 21 days and 18% at 35 days, whereas in the case of α₁-casein 95, 60, and 18% of protein was still intact at 7, 21 and 35 days of ripening, respectively. The results of Fisher’s PLSD analyses for β-caseins suggest that these proteins were significantly degraded at all stages of ripening; about 80% of β₁-casein was still intact at 7 days, 50% at 21 days and 30% at 35 days; on the other hand, β₂-casein was degraded faster and to a higher degree with about 60% still intact at 7 days, 40% at 21 days and 20% at 35 days. Although the α-caseins were degraded more slowly than β-caseins during the initial stages of ripening, the degree of degradation of α-caseins at the end of ripening (say 35 days) was higher than that of β-caseins (see Fig. 2). The overall proteolytic pattern during ripening is determined largely by the action of residual rennet enzyme(s) combined with that of enzymes produced by viable (or released by lysed) microorganisms. Although starter proteinases contribute little to the formation of large peptides (i.e. pH 4.6- or water-soluble peptides), proteinases from mesophilic streptococci are capable of hydrolyzing intact caseins in solution, especially β-casein; apparently, only few strains are capable of hydrolyzing α₃₁-casein, although this is hardly noticed in most cheeses since this protein is easily hydrolyzed by chymosin, the major constituent of animal rennets (Fox & Law, 1991). Furthermore, O’Keefe et al. (1975) found that the rate of proteolysis of α₃₁-casein in cheese was accelerated during manufacture and the early stages of cheese ripening when high levels of starters were used. Therefore, the significant rate of degradation of α₃-caseins only after 7 days may indicate that microflora play an important role in hydrolysing this protein, especially if it is assumed that the enzymes of C. cardunculus do not hydrolyse α₃-caseins as rapidly as β-casein (as discussed before) and that the microflora in Serra cheese are composed mostly of mesophilic lactic acid bacteria and coliforms, the numbers of which reach values above 10⁶ cfu/g of cheese only after 7 days (Macedo et al., 1995; Macedo et al., 1996).

Hydrolysis of β₁- and α₂-caseins were not affected significantly by the period within the cheesemaking season (P = 0.921 and P = 0.127, respectively). Hydrolysis of α₃₃-casein after 21 days of ripening was lower in February (75% of protein still intact) than in May (P = 0.042) or in November (P = 0.039), but these latter periods were statistically similar to one another (52%, P = 0.664); for the remaining ripening times, no statistical difference was detected at the 5% level of significance. Fisher’s PLSD analyses showed that hydrolysis of β₂-casein at 7 and 21 days of ripening is lower in May (69 and 49%, respectively, of protein still intact) than in the other 2 months (57 and 30%, respectively), but was statistically similar in all months tested in the case of cheeses ripened for 35 days (P = 0.184). As mentioned before, proteolysis in cheese is affected by such factors as salt to moisture ratio, pH and temperature of ripening. In Pedroches and Serena cheeses (both made from sheep’s raw milk and Cynara sp.), the extent of hydrolysis of α₁-casein decreases with increasing ash concentration while that of β-casein is unaffected (Marcos et al., 1976, 1979). In our study, one verified that both α₂- and β₂-caseins were positively correlated with moisture content (r = 0.724 and r = 0.652, respectively) and negatively correlated with ash to moisture ratio (r = -0.702 and r = -0.823, respectively) at the 5% level of significance. One also verified that 7 and 21 day-old cheeses manufactured in May possessed a significantly lower moisture content (51.2 and 48.21%, respectively) and higher ash to moisture ratio (7.44 and 7.86%, respectively) than those manufactured in November (7 and 21 day-old cheeses possessed 54.3 and 50.9% moisture content, respectively, and 6.65 and 7.11% ash to moisture ratio, respectively) and in February (7 and 21 day-old cheeses possessed 56.11 and 53.27% moisture content, respectively, and 6.91 and 7.21% ash to moisture ratio, respectively). The pH in 7 and 21 day-old cheeses did not show significant variations throughout the cheesemaking season (Macedo & Malcata, 1996). Therefore, the higher extent of hydrolysis of β₂-casein can probably be explained by differences in the moisture content and ash to moisture ratio in cheeses. However, the variations in the concentration of α₃₃-casein during the cheesemaking season for 21 day-old cheeses are not consistent with the variations in the aforementioned compositional factors; therefore, it seems that the ripening temperature may affect hydrolysis of this protein, especially knowing that after 7 days the ripening temperature is no longer constant but becomes mainly

Fig. 2. Fractional disappearance of caseins (a, α₂; b, α₃; c, β₁; d, β₂) by hydrolysis throughout the ripening period and throughout the cheesemaking season.
determined by the outside weather (which tends to be cooler in February, about 5°C on average, than in November, about 10°C, or May, about 14°C).

The results in Table 1 indicate that concentrations of all primary degradation products were statistically correlated with the concentrations of αs- and β-caseins at a significance level below 0.01%. However, based on the value of the correlation coefficients, it seems that the breakdown products, αs-1, αs-1, and δs-1 correlate better with αs-caseins than with intact β-casein or, to a lesser extent, with intact δs-casein. This observation is expected because, as mentioned before, the mobility of the electrophoretic bands associated with these proteins were similar to those produced by bovine αs-casein incubated with extracts of flowers of C. cardunculus in solution (Sousa, 1993). Conversely, the breakdown products βs-1 and βs-1 correlated better with βs-caseins and βδs-caseins (r > 0.91) than with intact αs-caseins (r < 0.88). This result agrees with Sousa (1993) in that proteases from C. cardunculus and chymosin exhibit comparable specificities on bovine β-casein. The breakdown products labelled as αs/βs-II and αs/βs-II correlate better with βs-caseins than with αs-caseins (see Table 1). These products could result from the action of the plant rennet on the βs-caseins because, as Sousa & Malcata (1996) have emphasized, cheeses manufactured with calf rennet do not show similar bands. These observations require, however, more fundamental work in order to clearly identify the degradation products via sequencing and to ascertain whether other low molecular weight products (not detectable by electrophoresis) result from hydrolysis of αs- and β-caseins.

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REFERENCES


