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Hydrolysis of α_s- and β-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 α_s- and β-caseins were degraded up to 82 and 76%, respectively, by 35 days of ripening. The α_s-casein variants (α_{s2}- and α_{s3}-) displayed similar degradation patterns to one another, but different from those of β-casein variants (β_1- and β_2-). Although the α_s-caseins were broken down more slowly than β-caseins at early stages of ripening (97, 95, 80, and 60% of α_{s2}-, α_{s3}-, β_1-, and β_2-caseins, respectively, were still intact by 7 days), this observation was reversed for later stages of ripening (18, 18, 30, and 20% of α_{s2}-, α_{s3}-, β_1-, and β_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 β_2- and α_{s3}-caseins. Degradation of α_{s3}-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 β_2-casein when manufactured in May than in November or February. The magnitude of the correlation coefficients pertaining to concentrations of intact α_s- and β-caseins indicated that the products of proteolytic breakdown with higher mobility than α_s-caseins (tentatively termed α_1-I, α_2-I, and α_3-I) were preferentially correlated with α_s-caseins, the products of proteolytic breakdown with mobility between β-caseins and α_s-caseins (tentatively termed β_1-I and β_2-I) were preferentially correlated with β_1- and β_2-caseins rather than with α_s-caseins, and the products of proteolytic breakdown with the highest mobility (tentatively termed α/β_1-II and α/β_2-II) were preferentially correlated with β-caseins. Copyright © 1996 Elsevier Science Ltd

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 adition 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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curd composition) and the environmental conditions prevailing during ripening. This communication reports work on the evolution of hydrolysis of α_s- and β-caseins in Serra cheese as assayed by polyacrylamide gel electrophoresis at different times in the ripening period and in the cheesemaking season. In view of the unique characteristics of Serra cheese encompassing use of raw ovine milk, addition of a plant rennet, essentially manual cheesemaking and ripening conditions determined chiefly by the outer weather, unique proteolysis profiles are anticipated.

MATERIALS AND METHODS

Cheese manufacture and sampling

Three batches of twelve 0.5 kg cheeses were prepared in a small, certified dairy farm in the *Appéllation Serra Controllée* region in three sequential periods within the cheesemaking season (namely, November, February and May). The three periods within the cheesemaking season were chosen sufficiently appart from each other to be representative of different milk compositions (arising from different physiological states of the sheep and different composition of the feed) and different ripening conditions (arising from different temperatures and relative humidities of the local weather).

The traditional method was used to manufacture the cheeses: crude kitchen salt was added to the milk (at a level of 12 g of salt per litre of milk) and stirred to accelerate solubilization; ground dry thistle flower (at a level of 0.4 g per litre of milk) was mixed with tap water until a brown suspension was obtained, this suspension was filtered through a fine, clean cloth and the clear filtrate was added to the milk and gently stirred; the milk was then allowed to rest at about 28 °C until complete coagulation had occurred (for about 1 h); the curd was manually cut in order to obtain small, irregularly shaped pieces which were then poured into a fine cloth bag and lightly pressed by hand for about 10 min; the curd paste was then divided into several plastic perforated moulds and drainage of whey was completed via pressing of the fresh cheese while in the mould via a 10 kg metal block for about 12 h (cheeses were turned upside down 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 Creamer (1991). Standard ovine casein solution was prepared by isoelectric precipitation of sheeps' 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 α_s- 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.

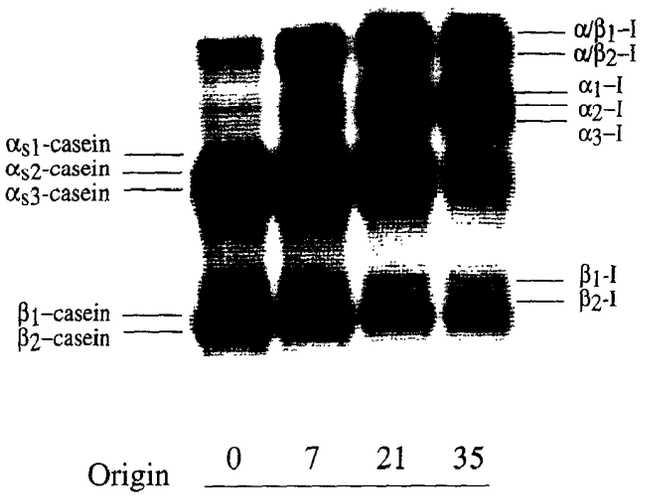

Fig. 1. Typical urea-PAGE electrophoretogram of samples of Serra cheese obtained during ripening; the numbers below the lanes refer to the age of cheese in days.

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 Bordaleira sheep contain two major groups of electrophoretic bands. According to Richardson & Creamer (1976), the group with the lower mobility consists of β-caseins and is subdivided into two variants, β_1- and β_2-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_s-caseins and is subdivided into three variants, α_{s1}-, α_{s2}- and α_{s3}-casein; the clearly dominant α_{s2}- and α_{s3}-casein have similar molecular weights and calcium sensitivities but different behaviour in Mg^{2+}-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_s-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, these two bands were therefore tentatively labelled as β_x-I. Figure 1 also shows the appearance and increase in intensity of bands with greater electrophoretic mobility than those of α_s-caseins. These bands had electrophoretic mobilities similar to the band produced when bovine α_{s1}- casein was incubated with extracts from flowers of *C. cardunculus* in solution (Sousa, 1993) and were tentatively labelled as α_x-I. Two other bands with greater electrophoretic mobility than α_x-I were produced from the very beginning of ripening and became thicker as ripening elapsed. These two bands were tentatively termed α/β_x-II.

Figure 2 shows the ratio of concentrations of intact α_s- and β-caseins to their initial concentrations during ripening and during the cheesemaking season. The coefficients of correlation between intact caseins and first breakdown products are listed in Table 1.

Table 1. Correlation coefficients ($P < 0.0001$) between intensities of bands accounted for by intact caseins and their primary degradation products

		Casein substrate			
		α_{s2}	α_{s3}	β_1	β_2
P	α/β_1-II	−0.849	−0.835	−0.906	−0.931
r	α/β_2-II	−0.665	−0.653	−0.792	−0.884
o	α_1-I	−0.934	−0.931	−0.919	−0.847
d	α_2-I	−0.956	−0.941	−0.938	−0.868
u	α_3-I	−0.967	−0.953	−0.936	−0.857
c	β_1-I	+0.883	+0.876	+0.928	+0.912
t	β_2-I	+0.881	+0.867	+0.935	+0.947

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 α_s- 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 α_s-caseins occur to similar extents in Serra cheese) seems to be related with the type of rennet (plant instead of animal) used since Dinakar *et al.* (1989) have reported that a plant rennet obtained from *Withania coagulans* 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 (α_{s2}-, α_{s3}-, β_1-, and β_2-) was affected significantly by the ripening time ($P < 0.0001$ for all such caseins, see Fig. 2). The results

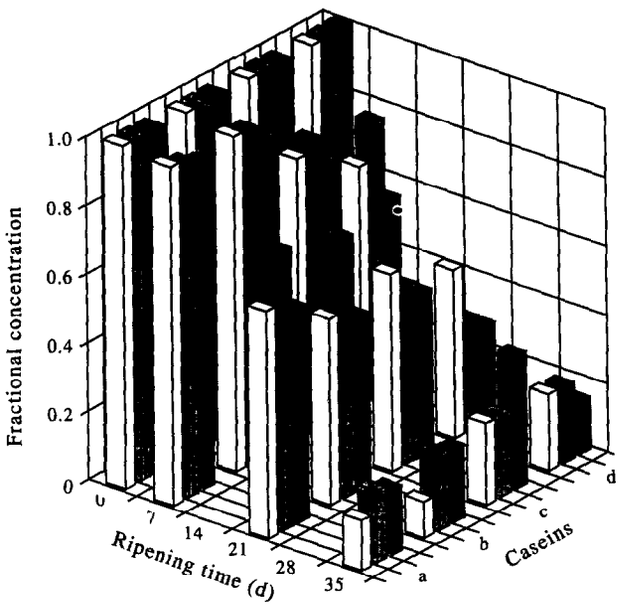

Fig. 2. Fractional disappearance of caseins (a: α_{s2}; b: α_{s3}; c: β_1; d: β_2) by hydrolysis throughout the ripening period and throughout the cheesemaking season.

of Fisher's PLSD analyses for α_s-caseins indicate that these proteins were significantly degraded only after 7 days of ripening; ca. 97% of α_{s2}-casein was still intact at 7 days, 70% at 21 days and 18% at 35 days, whereas in the case of α_{s3}-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 β_1-casein was still intact at 7 days, 50% at 21 days and 30% at 35 days; on the other hand, β_2-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_s-caseins were degraded more slowly than β-caseins during the initial stages of ripening, the degree of degradation of α_s-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 α_{s1}-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 α_{s1}-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 α_s-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 α_s-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 10^6 cfu/g of cheese only after 7 days (Macedo *et al.*, 1995; Macedo *et al.*, 1996).

Hydrolysis of β_1- and α_{s2}-caseins were not affected significantly by the period within the cheesemaking season ($P=0.921$ and $P=0.127$, respectively). Hydrolysis of α_{s3}-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 β_2-casein at 7 days 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 α_s-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 α_{s3}- and β_2-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 β_2-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 α_{s3}-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

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 α_s- 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, α_1-I, α_2-I, and α_3-I correlate better with α_s-caseins than with intact β_1-casein or, to a lesser extent, with intact β_2-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 α_{s1}-casein incubated with extracts of flowers of *C. cardunculus* in solution (Sousa, 1993). Conversely, the breakdown products β_1-I and β_2-I correlated better with β_1- and β_2-caseins ($r > 0.91$) than with intact α_s-caseins ($r < 0.88$). This result agrees with Sousa (1993) in that proteinases from *C. cardunculus* and chymosin exhibit comparable specificities on bovine β-casein. The breakdown products labelled as α/β_1-II and α/β_2-II correlate better with β-caseins than with α_s-caseins (see Table 1). These products could result from the action of the plant rennet on the β-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 α_s- and β-caseins.

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