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Influence of vegetable and animal rennet on proteolysis during ripening in ewes' milk cheese

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Abstract

The renewed interest in using enzymes from thistles of the genus *Cynara* in the making of traditional ewes' milk cheese prompted us to investigate the effect of vegetable and animal rennet on proteolysis during ripening of Los Pedroches cheese. Casein hydrolysis was found to be much more extensive and faster in cheese made by using vegetable rennet (the amount of soluble nitrogen at 60, 80 and 100 days of ripening was more than 28% greater than that in cheese produced using animal rennet). The levels of insoluble Tyr and Trp were higher in cheese produced with vegetable rennet. PAGE, using gels containing 7 M urea, revealed decreased contents in residual α_s-CN and β-CN, as well as markedly increased levels of the more mobile components in cheese produced from vegetable rennet at the end of ripening. On the other hand, the degree of proteolysis in terms of NPN or its main components (peptides, amino acids and ammonia) was similar in cheese produced using animal or vegetable rennet.

Keywords: Los Pedroches cheese; Vegetable rennet; Proteolysis

1. Introduction

Some Spanish, Portuguese, French and Italian varieties of ewes' milk cheese (Barbosa, Valles, Vassal, & Mocquot, 1976) are made with aqueous extracts of dried wild thistle flowers from various species of the genus *Cynara* L. The species *C. cardunculus* L. grows spontaneously in stony and waste places, as well as in dry grassland, mainly on clay soil in southern and western Mediterranean regions and southern Portugal, the Canary Islands and Madeira; it is usually used in the making of Portuguese Serra and Serpa cheeses (Macedo, Malcata, & Oliveira, 1993; Vieira de Sá & Barbosa, 1972) and Spanish Los Pedroches, La Serena and Torta del Casar cheeses (from ewes' milk) as well as Los Ibores cheese (from goats' milk) and Flor de Guía cheese (from a mixture of ewes' and cows' milk) (Fernández-Salguero, Sanjuán, & Montero, 1991). The other *Cynara* species such as *C. humilis* L., grow in dry, waste places of the central and southern regions of the Iberian peninsula; it is more abundant and also used in the making of various traditional cheeses as a replacement for or, allegedly, mixed with *C. cardunculus* when this is scanty. The enzymes in these two thistle species vary in activity (Pires, Faro, Macedo, Esteves, Morgado, Veríssimo, Pareira, & Gomez, 1994; Fernández-Salguero & Gómez, 1997).

The proteinases of *C. cardunculus* L. have been purified and characterized (Heimgartner, Pietrzak, Geertsen, Brodelius, da Silva Figueiredo, & Pais, 1990). This species contains three proteinases, the proteolytic activities of which are maximal at pH 5.1, 5.7 and 6.0, at variable temperatures under ionic strengths equivalent to 0.1–0.6 M in NaCl (Macedo et al., 1993). They are thus acidic proteinases belonging to the aspartic proteinase group called 'cyprosins' (Cordeiro, Jacob, Puhan, Pais, & Brodelius, 1992) or 'cardosins' (Pires et al., 1994). It has been claimed

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that two forms of cyprosin are similar to each other as well as to chymosin, while the other is similar to pepsin (Pires et al., 1994). Isoelectric focusing showed that the apparently pure cyprosins can be resolved into three isoenzymes with close isoelectric points (Cordeiro, Pais, & Brodelius, 1994). Procedures for the isolation and partial characterization of the three isoenzymes of cyprosin 3 have been developed (Brodelius, Cordeiro, & Pais, 1995). Just as recombinant chymosin has been obtained by biotechnological means in various microorganisms, the expression for cyprosin in both microbial and plant cells is being optimized with a view to producing cyprosin on a large scale in transgenic organisms.

One of the most representative artisanal ewes' milk cheeses manufactured in Spain is Los Pedroches (Carr, 1981). It is named after the valley where it is typically made, on farms and at small dairies in the province of Córdoba (southern Spain) from December to May. It is a fatty cheese made from uncooked, hard paste, made from raw Merino ewes' milk that is not inoculated with a lactic culture and is usually coagulated with vegetable rennet. Los Pedroches cheese is ripened for about 2 months. During that period, various physical, chemical, microbiological and organoleptic changes endow it with a peculiar aroma and a slightly piquant, creamy flavour (particularly when made with vegetable rennet) that is highly appreciated in the region. This has fostered its production as an outlet for the milk produced in the area, as well as to increase the profitability of ewe breeding and boost rural development in the framework of sustainable agriculture. Los Pedroches cheese is included in the list of 'traditional foods' presented by Spain to the European Union.

The ripening of Los Pedroches cheese made with animal rennet has been the subject of biochemical studies (Fernández-Salguero, Barreto, & Marsilla, 1981; Fernández-Salguero, 1987a, b). Also, our laboratory has characterized various commercially available samples of this cheese. In order to assess the effect of animal rennet (chymosin) and vegetable rennet (cyprosins) on physico-chemical and biochemical changes during ripening of Los Pedroches cheese, a comprehensive study (Sanjuán, 1992) has been undertaken of the influence of various factors (Sanjuán & Fernández-Salguero, 1994) on the time of clotting with vegetable rennet (*C. cardunculus* L.), as well as the fatty acid composition of Los Pedroches cheese produced using animal and vegetable rennet (Sanjuán, Millán, Gomez, & Fernández-Salguero, 1995), and the effect of the type of rennet used on the raw chemical composition of the cheese. This paper reports information on changes in the principal nitrogenous components during ripening of Los Pedroches cheese made with animal and vegetable rennet.

2. Materials and methods

2.1. Cheesemaking procedure

Cheese samples were made at a traditional factory in the production area from unpasteurized Merino ewes' milk to which no starter culture was added. The amount obtained during the milking day was split into two batches, one of which was coagulated with animal rennet (Ha-bo commercial powder from Chr. Hansen), according to the instructions of the manufacturer, and the other with vegetable rennet. The milk obtained on another day was split equally into two batches and clotted with each type of rennet. Therefore, two experimental batches were clotted with animal rennet and another two with vegetable rennet. Each of the four experimental batches consisted of 10 cheeses. The aqueous extract from *C. cardunculus* L. was obtained from 70 g of dried flowers, which was macerated in 1 litre of water for 24 h. The extract was added at a level of about 8 ml per litre of milk so that coagulation took 80–90 min at 29 ± 1°C. After pressing, the cheese was salted by rubbing its surface with dry salt and ripened under the conditions prevailing in the farmhouse cellar, viz. 10–15°C and a relative humidity of 80–90%. The temperature and relative humidity were measured by means of a thermohydrograph. One cheese from each experimental batch was transferred to the laboratory at 2, 4, 7, 14, 22, 30, 43, 60, 80 and 100 days of ripening for analysis.

2.2. Analytical methods

Moisture content and total nitrogen (TN) were determined by AOAC (1980) methods. The nitrogen fractions, including soluble nitrogen (SN) at pH 4.6, non-protein nitrogen (NPN) in 12% TCA, ammonia nitrogen (NH_3-N) and soluble tyrosine and tryptophan were prepared and analysed as described by Fernández-Salguero, Marcos, Alcalá, & Esteban (1989), and Fernández-Salguero, Sanjuán, & Montero (1991). Amino acid nitrogen (AAN) was determined by the procedure of Reiter, Sorokin, Pickering, & Hall (1969).

Samples for electrophoretic separation of caseins were prepared (El-Shibiny & El Salam, 1976) and analyzed by PAGE in gels containing 7 M urea; gels were scanned and bands quantified densitometrically at 600 nm, as described by Marcos, Esteban, León, & Fernández-Salguero (1979) and Fernández-Salguero et al. (1989).

The pH was measured by using a Beckman 3500 digital pH-meter, as described by Fernández-Salguero et al. (1991).

All samples were analysed in duplicate.

3. Results and discussion

3.1. Proteolysis characteristics

Table 1 shows the mean analytical results for moisture, total nitrogen (TN), different soluble fractions (SN, NPN, AAN and NH_3-N, all as percentages of TN) and pH obtained during ripening for the two cheese batches produced using animal rennet (A) and the two made using vegetable rennet (V).

As can be seen, SN values differed markedly between the cheeses coagulated with the two types of rennet. After 2 days of ripening, the SN values for the cheeses made using vegetable rennet were more than double those of samples produced with animal rennet. The amount of SN increased gradually until 60 days, after which it levelled off in both types of cheese. The mean SN values for the last three samples analyzed (at 60, 80 and 100 days after manufacture) showed that cheese made with vegetable rennet contained about 31% more soluble nitrogen (33.4% of TN) than that made with animal rennet (22.9% TN), with $p = 0.003$ ($0.001 < p < 0.01$). On the other hand, NPN values and their changes during ripening were very similar for both types of rennet. A previous study (Fernández-Salguero, 1978a) on Los Pedroches cheeses, which were ripened for 67 days at a higher ambient temperature, showed higher SN levels (39–42% of TN) and also higher levels of NPN (21–22% of TN).

The SN fraction in La Serena cheese, which is very similar to Los Pedroches cheese and is produced using *C. cardunculus* L. proteinases, after at 60 days of ripening, was found to be 38.8% of TN (Fernández del Pozo, Gaya, Medina, Rodríguez-Marín, & Núñez, 1988); also, NPN was found to account for 14.5% of TN, a value similar to ours. A different study of La Serena cheese during ripening (Núñez, Fernández del Pozo, Rodríguez-Marín, Gaya, & Medina, 1991) showed higher levels of SN and NPN than those obtained in this work. Consistent with the present results, however, Núñez et al. (1991) showed that cheeses made with vegetable rennet contained more SN after 60 days of ripening than cheese produced with animal rennet (52.5% vs 34.5%). Also, NPN levels were similar for the two types of rennet. A study on traditional Andalusian ewes' milk cheese (Fernández-Salguero & Gómez, 1997) showed levels of SN and NPN similar to those now obtained for Los Pedroches cheese made from animal rennet. Finally, cheese obtained from ewes' milk from the Serra de la Estrela region (Portugal) using animal rennet and *C. cardunculus* (Sousa & Malcata, 1997) showed water-soluble N (WSN) levels similar to those in Table 1; however, previous authors found NPN values lower than those in Table 1 and also those lower than reported by Núñez et al. (1991) for La Serena cheese. Also Sousa and Malcata (1997) obtained NPN values significantly higher in cheeses manufactured with animal rennet compared to those made with *C. cardunculus* at 70 days of ripening. These results are not in agreement with those for Los Pedroches (Table 1) or for La Serena cheeses (Núñez et al., 1991) which had similar NPN levels by the end of the ripening period in each of the cheese varieties manufactured with both types of rennet.

The low initial levels of SN in the cheese made using animal rennet are the result of the curdling mass being under low microbial activity at the early stages of ripening; also, the protein solubilization observed can be ascribed to the action of proteolytic enzymes in the rennet, which give rise to the formation of high-molecular weight products. On the other hand, the high levels of SN found in our cheese batches produced using vegetable rennet suggest an increased proteolytic activity of cyprosins rather than the involvement of microbial endo- and exo-proteases, since the difference was observed throughout the ripening process. Also, the

Table 1
Changes in moisture, TN, pH and the different soluble nitrogen fractions (SN, NPN, AAN and NH_3-N) in the cheese batches obtained with animal rennet[a] (A) and vegetable rennet[a] (V) during ripening

Ripening time (days)	% Cheese						% TN							
	Moisture		TN		pH		SN		NPN		AAN		NH_3-N	
	A	V	A	V	A	V	A	V	A	V	A	V	A	V
2	50.5	51.6	3.0	2.9	5.6	5.6	6.5	13.8	2.6	3.1	0.7	0.9	0.2	0.2
4	49.3	48.2	3.2	3.0	5.4	5.4	6.6	17.5	3.0	3.4	0.8	1.2	0.2	0.3
7	48.4	48.4	3.1	3.0	5.4	5.4	7.7	19.9	3.5	3.6	0.9	1.4	0.3	0.3
14	47.9	46.8	3.3	3.0	5.3	5.3	12.9	22.0	6.2	5.2	1.2	1.9	0.3	0.3
22	46.0	45.0	3.3	3.2	5.4	5.5	14.3	25.5	6.0	8.5	1.6	2.5	0.4	0.6
30	43.7	42.5	3.4	3.4	5.4	5.4	19.9	25.6	8.1	7.1	2.7	2.9	0.9	1.0
43	43.9	40.3	3.6	3.5	5.3	5.5	19.6	27.4	9.8	9.5	3.7	3.2	1.3	1.5
60	40.5	40.1	3.7	3.5	5.2	5.2	25.3	35.5	11.1	11.8	4.4	3.8	1.7	2.1
80	38.3	36.6	3.8	3.7	5.1	5.2	20.7	33.2	15.3	13.8	6.4	6.6	1.9	2.2
100	35.3	34.1	4.0	3.8	5.1	5.1	22.6	31.6	14.9	15.0	8.7	8.0	2.8	3.2

[a] Mean values for two experiments.

similarity between NPN contents between the two types of cheese suggests that the production of nitrogenous compounds of low molecular weight is independent of the type of rennet used; these compounds arise largely from the activity of microbial enzymes present in the cheese.

The proportion of total soluble nitrogen (SN) has traditionally been regarded as a 'ripening index' for cheese as it reflects the extent of proteolysis. The strong proteolytic activity shown by the enzymes (cyprosins) in the flowers of *Cynara cardunculus* L. thistles relative to chymosin in the formation of soluble nitrogen could be used as a proteinase system in the accelerated ripening in some ewes' milk cheese varieties involving the addition of enzymes (Law & Wigmore, 1982). An alternative could be cyprosin + peptidase combinations or mixtures of cyprosin and attenuated cultures. Cyprosins show more specific hydrolysis of proteins in ewes' milk (Cordeiro et al., 1992). That is the reason why some typical cheeses made in Spain and Portugal from ewes' and goats' milk with cardoon possess no markedly bitter flavour whereas cheeses produced from cows' milk and flower extracts of *C. cardunculus* tend to have a bitter taste (Barbosa, Corradini, & Battistoni, 1981). Some studies on the specificity of cyprosins suggest that these proteinases may act as both endopeptidases and as exopeptidases (Faro, 1991; Heimgartner et al., 1990) but tend to prefer peptide bonds containing polar amino acids in their hydrolytic attack.

The data in Table 1 were used to calculate the levels of protein nitrogen (TN–NPN) in the two fractions, viz. casein nitrogen (TN–SN) and proteose–peptone nitrogen (SN–NPN or protein N–casein N), as well as peptide nitrogen [NPN–(AAN + NH_3-N)]. The results are given in Table 2. Of the 97% of TN consisting of protein nitrogen at the beginning of ripening in both cheese batches, casein nitrogen was found to account for about 93.5% in samples produced using animal rennet and only for 86.2% in those made with vegetable rennet, resulting from the rapid increase in SN. This difference in the extent of casein solubilization remained throughout ripening; thus, at 100 days, the amount of protein N in both batches was still similar (85% TN), but so was the difference between the amount of unhydrolysed casein (77.4% of TN for cheese produced using animal rennet vs 68.4% for that made with vegetable rennet; $p = 0.003$ ($0.001 < p < 0.01$). These differences in casein nitrogen are reflected in the other protein subfraction of proteoses–peptones [with some oscillations, the levels of proteose–peptone in the cheese made with vegetable rennet were much higher ($p < 0.001$) than those produced using animal rennet]. The levels of peptide N (Table 2) generally increased during ripening up to about 2 months; at the end of the study, they accounted for about 5% of TN for both types of rennet ($p > 0.05$), which suggests that peptides account for 37% of NPN.

Unlike levels of SN, the AAN values, with both types of rennet (Table 1), initially increased more markedly in the cheese made with vegetable rennet but then levelled off in both types of cheese. The levels of AAN continued to rise throughout the study (to over 8% of TN) in such a way that amino acids were the most quantitatively significant compound group in the NPN fraction after 80 days of ripening. La Serena has reported contents (expressed as nitrogen soluble in phosphotungstic acid, PTA-N) above those in Table 1 (Fernández del Pozo et al., 1988; Núñez et al., 1991).

NH_3-N values also increased more markedly from the beginning of ripening in cheese made with vegetable rennet; however, the differences remained throughout. The low proportion of ammonia nitrogen at the beginning of ripening suggests that deaminase activity was quite low, which can be ascribed to the pH of these cheeses (Table 1) being well below the optimum value for the enzyme (Sanjuán, 1992).

Table 3 shows the mean soluble tyrosine and tryptophan contents in the batches produced using animal and vegetable rennet. As can be seen, the levels of Tyr

Table 2
Changes in different groups of nitrogen components in the cheese batches obtained with animal rennet[a] (A) and vegetable rennet[a] (V) during ripening (all values in g/100 g TN)

Ripening time (days)	Protein N		Casein N		Proteose–peptone		Peptide N	
	A	V	A	V	A	V	A	V
2	97.4	96.9	93.5	86.2	3.9	10.7	1.7	2.1
4	97.0	96.6	93.4	82.5	3.6	14.1	1.9	2.0
7	96.5	96.4	92.3	80.2	4.2	16.3	2.3	1.8
14	93.8	94.9	87.1	78.0	6.8	16.8	4.7	2.9
22	94.0	91.5	85.7	74.5	8.3	17.0	4.0	5.4
30	91.9	92.9	80.2	74.4	11.8	18.5	4.5	3.2
43	90.2	90.5	80.4	72.7	9.8	17.8	4.9	4.8
60	88.9	88.2	74.7	64.5	14.2	23.7	5.0	5.9
80	84.7	86.2	79.3	66.8	5.4	19.4	7.1	5.0
100	85.1	85.0	77.4	68.4	7.7	16.6	3.4	3.8

[a] Mean values for two experiments.

Table 3
Changes in the mean soluble tyrosine (Tyr) and tryptophan (Trp) contents (in mg/100 g) in the cheese batches obtained with animal[a] and vegetable rennet[a] during ripening

Ripening time (days)	Animal		Vegetable	
	Tyr	Trp	Tyr	Trp
2	113	76	120	76
4	111	51	133	80
7	122	49	143	79
14	168	47	188	86
22	156	87	199	92
30	188	87	222	96
43	232	51	234	98
60	216	68	267	127
90	215	70	264	125
100	217	83	285	142

[a] Mean values for two experiments.

exceeded the levels of Trp throughout ripening. The levels of both soluble amino acids increased gradually with ripening, with some oscillations, in the batches coagulated with animal rennet. The lower tryptophan values shown throughout ripening may be the result, not only of the lower original casein contents, but also of the fact that this amino acid is more readily hydrolysed by microorganisms. A comparison of the results for the two types of rennet studied reveals higher contents in both soluble amino acids in the cheese samples made with vegetable rennet (Tyr $p > 0.05$, Trp $p < 0.001$). The values in these amino acids are known to reflect the extent of proteolysis since, at least that of tyrosine, is significantly related to SN ($p < 0.001$) in both types of rennet.

3.2. Electrophoretic patterns of the caseins

The extents of degradation of major caseins and their hydrolysis products were determined by using the urea-PAGE technique. The bands making up the four groups of casein components were quantified photometrically. The results are shown in Table 4, together with the proportion of para-κ-CN and the α_s/β-CN ratio.

As can be seen, the relative proportion of α_s-CN decreased throughout ripening in both types of cheese. The initial proportion of this casein was higher in the samples made with vegetable rennet (42.3%) and also decreased more rapidly than in the samples produced using animal rennet (initial content 38.2%). Based on the mean values for the last three samples analyzed (at 60, 80 and 100 days of ripening; Table 4), the amount of residual α_s-CN in the samples made with animal rennet was higher relative to the samples produced using vegetable rennet (18.6 vs 16.0%); however, the difference was not statistically significant ($p > 0.05$).

The β-CN region revealed a slight decrease in the proportion of these bands throughout ripening; however, the amount of residual protein was higher in the cheese samples produced using animal rennet. La Serena cheese was also found to undergo more rapid hydrolysis of α_s-CN and β-CN in samples made with vegetable rennet (Núñez et al., 1991). The level of residual β-CN showed with both types of rennet was much greater than that of α_s-CN, which confirms the higher resistance to enzyme hydrolysis of the former.

The α_s-CN/β-CN ratio (Table 4) decreased from nearly unity to about 0.5 at the end of ripening; the ratio was slightly lower for the samples made with vegetable rennet. Cheese from ewes' milk has been reported to exhibit α_s-CN/β-CN ratios from 0.78 to 0.98 (Storry, Grandison, Millard, Owen, & Ford, 1983).

The components with the higher electrophoretic mobilities located in the λ-CN region resulted from the hydrolysis of α_s-CN under the action of milk plasmin, microbial enzymes and enzymes in the rennet used as coagulant agent. The contents of these components increased with ripening in such a way that their mean values in the last three samples analysed (cheeses with more than 60 days of ripening) were almost twice as high in the cheese samples produced using vegetable rennet as in those made with animal rennet; this is

Table 4
Per cent relative contributions of major casein regions in cheese samples obtained with animal rennet (A) or vegetable rennet[a] (V) during ripening

Ripening time (days)	λ-CN		α_s-CN		β-CN		κ-γ-CN		Origin		α_s-CN/β-CN	
	A	V	A	V	A	V	A	V	A	V	A	V
2	0.0	0.0	38.2	42.3	47.3	46.0	14.6	14.7	3.4	2.3	0.9	1.0
4	0.0	0.0	37.8	41.0	43.6	41.6	16.4	14.9	2.2	2.3	0.9	1.0
7	4.1	0.0	36.1	43.0	39.3	37.4	14.3	14.3	3.1	5.3	0.9	1.1
14	5.7	8.5	31.7	33.8	38.9	33.8	18.3	18.3	4.6	5.4	0.6	1.0
22	5.0	9.6	30.0	30.1	36.5	33.8	22.2	22.2	4.1	4.2	0.8	0.9
30	10.5	12.4	27.0	26.1	37.9	35.4	21.9	21.9	3.9	4.0	0.7	0.7
43	8.3	12.8	23.7	24.3	39.0	36.8	19.8	19.8	5.4	6.3	0.6	0.7
60	8.4	17.6	21.8	16.6	43.3	37.4	23.3	23.3	4.2	4.8	0.5	0.4
80	10.2	17.2	18.9	15.7	41.3	38.6	23.5	23.5	4.0	4.8	0.5	0.4
100	9.2	15.0	15.2	15.6	41.3	39.6	25.0	25.0	5.8	4.7	0.5	0.4

[a] Mean values for two experiments.

consistent with the higher SN values previously shown in the spectrophotometric analyses (Table 1). In any case, the proportions for the components of α_s-CN and those located in the λ-CN region were inversely correlated ($r = -0.8742$ for cheese made with animal rennet and -0.9771 for samples produced using vegetable rennet) to a high degree for the two cheese batches ($p < 0.001$).

The contents in the components located in the κ-γ-CN region changed similarly with the two types of rennet; they increased from 14.6% at the beginning of ripening to about 24–25% at the end. These components are known to result from the hydrolytic action of alkaline protease or microbial enzymes on ovine β-caseins (Gordon, Groves, Greenberg, Jones, Kalan, Peterson, & Townsend, 1972; Eigel, Butler, Ernstrom, Farrel, Harwalker, Jenness, & Whitney, 1984), or from proteolysis by the animal or vegetable rennet added. The decreased anodic mobility of these components was the result of the absence of the *N*-terminal sequence in β-CN, which is where most of the negative charges of the molecule lie. The contents of the components located in the κ-γ-CN and β-CN regions shown by both types of rennet were inversely—though not significantly—related ($p > 0.05$). However, other authors (Marcos et al., 1979; Fernández-Salguero et al., 1981) reported a highly significant correlation between the two casein fractions in different varieties of Spanish and European cheeses. γ-CN fractions remain undegraded in the ripened cheese (Creamer, 1975).

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