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Review

Advances in the role of a plant coagulant (*Cynara cardunculus*) in vitro and during ripening of cheeses from several milk species

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Abstract – Extracts from dried flowers of *Cynara cardunculus* have been employed successfully for many centuries in Portugal and some regions of Spain for the manufacture of traditional cheeses. Several studies have been performed in vitro and in actual cheeses focusing on the activity and specificity of proteinases from *C. cardunculus*, in attempts to further characterise this plant coagulant as a proteolytic agent in cheese ripening. In vitro studies encompassed extraction conditions, storage of extracts, proteolysis and specificity on caseins using experimental conditions that parallel milk and cheese during ripening. In vivo studies encompassed the effect of the type of milk (bovine, ovine and caprine) in terms of proteolysis, specificity of those plant proteinases during ripening, comparison of microbiological, chemical, proteolytic and lipolytic characteristics in ovine milk cheeses relative to animal rennet counterparts, and effect of native microflora, thermal processing and addition of starter cultures in ovine cheeses.

milk clotting enzyme / plant coagulant / cheese / ewe's milk / cow's milk / goat's milk / ripening

Resumé – **Rôle de l'extrait coagulant de chardon (*Cynara cardunculus*) in vitro et au cours de l'affinage de fromages fabriqués à partir de laits de plusieurs espèces.** L'extrait de fleurs séchées de *Cynara cardunculus* a été employé avec succès depuis plusieurs siècles au Portugal et dans quelques régions d'Espagne pour la fabrication de fromages traditionnels. De nombreuses études ont été réalisées in vitro et en fabrication fromagère sur l'activité et la spécificité des protéases de *C. cardunculus*, dans le but de mieux caractériser ce coagulant végétal comme agent protéolytique dans l'affinage du fromage. Les études in vitro ont concerné les conditions d'extraction, la conservation des extraits, la protéolyse et la spécificité sur les caséines en utilisant des conditions expérimentales qui

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permettent de faire la comparaison avec le lait et le fromage au cours de l'affinage. Les études in vivo ont concerné l'effet du type de lait (bovin, ovin ou caprin) en termes de protéolyse, la spécificité de ces protéases végétales au cours de l'affinage, la comparaison des caractéristiques microbiologiques, chimiques, protéolytiques et lipolytiques des fromages au lait de brebis par rapport à ceux obtenus à partir de présure animale, et les effets de la microflore d'origine, du traitement thermique et de l'addition de levains sur les fromages de brebis.

enzyme coagulante / coagulant végétal / fromage / lait de vache / lait de brebis / lait de chèvre / affinage

1. INTRODUCTION

Cynara cardunculus L., a plant from the *Asteraceae* family, is a prickly variety of thistle similar to the globe artichoke [44], which produces large heads and purple flowers throughout the summer. This variety of thistle grows wild and abundantly in the dry, stony and uncultivated areas of the southern and north-eastern parts of Portugal, although it has been identified in several regions of the Mediterranean basin, Canary islands, Madeira islands and Northern Africa. Standard conditions for the cutting and drying processes do not in practice exist. After collection from the mature plants, the flowers are usually dried in the shade in the open air, stored in a dry place and eventually sold in local markets. Consequently, the activity of the plant extract is extremely variable depending on the variety, the stage of maturity, the part of the flower used, the drying time and the final moisture content [1, 12, 33]. It was once reported that enzymatic activity exists in the top and middle parts of the flower head [10, 11]; however, it is now accepted that only the stylets and stigmae (violet part) possess a milk-clotting capacity [19, 40]. Traditionally, dried flowers are used throughout the cheesemaking season (October to May) to prepare crude, enzymatically active aqueous extracts, which are then used mainly for coagulating sheep's milk, and goat's and cow's milk to a lesser extent [49]. Unstandardised utilisation of this coagulant is claimed to cause poor organoleptic quality and contribute to day-to-day variation of cheeses; however, dried flowers of *C. cardunculus* have been successfully employed for many centuries in the Iberian Peninsula for the manufacture of traditional semi-hard cheeses, i.e. such traditional cheese varieties as Serra da Estrela [1, 30, 44, 65], La Serena (from ewe's milk) [23, 36, 42], Guía (from a mixture of ewe's and cow's milk) [25] and Los Pedroches (from ewe's milk) [7, 24, 66]. A few experiments have also been carried out pertaining to the manufacture of such French cheeses as Camembert and Gruyère [2], and such Italian cheeses as Bel Paese, Grana and Provolone [3]. However, a few problems were encountered; these included slightly more acid and bitter flavours and softer curd, with concomitant increased tendencies for loss of shape [2, 3].

Several studies have meanwhile been performed using extracts of *C. cardunculus* as coagulant in cheese, in attempts to further characterise this plant coagulant as a proteolytic [7, 24, 51–53, 56] and lipolytic [54, 57] agent during cheese ripening.

2. AQUEOUS EXTRACTION AND STORAGE OF EXTRACTS

One of the most popular methods of extraction involves soaking a handful of stylets and stigmae in a bowl of tap water for several hours, grinding with a mortar and pestle and filtering through a piece of cotton cloth; the brownish liquor thus obtained is added to raw milk to induce coagulation [30]. Salt may be added to the initial

aqueous infusion, although it has been shown that NaCl has no significant effect on the rate of enzyme extraction [50, 62]. An alternative method of extraction is grinding the dried flowers with crude kitchen salt, laying the paste on a cotton cloth (which acts as a strainer) and solubilising the enzymes by percolation with warm milk.

The effects of four processing parameters encompassing the liquid extraction of enzymes from *C. cardunculus* (viz. time of grinding, pH of buffer, NaCl concentration of buffer and homogenisation time) on their final proteolytic activity were studied by Sousa and Malcata [50]. More intense grinding and higher pH affected positively the proteolytic activity of the extracts of *C. cardunculus*, whereas longer homogenisation affected it negatively; the NaCl had no effect on the proteolytic activity [50]. Although in the traditional extraction process crude kitchen salt is often used for its abrasive features, in addition to its own contribution to the ionic strength and the osmotic pressure of the extraction solution, only the contributions of NaCl towards ionic strength have been considered. As mentioned before, NaCl (0–3%, w/w) had no effect on the proteolytic activity of *C. cardunculus* extracts [50], but NaCl concentrations above 5% decreased proteolysis of caseins [48, 55], and at 20% NaCl proteolysis of β-casein was completely inhibited [48]. The maximum (estimated) proteolytic activity was obtained after grinding stylets and stigmae for 36 s, using an extraction buffer with pH 5.9 in the absence of salt, and after having homogenised the ground stylets and stigmae in a buffer suspension for 15 min [50]. It should be emphasised that optimisation of the specific proteolytic activity of extracts of *C. cardunculus* meant maximisation of the product of the total amount of enzyme extracted by the catalytic activity. Hence, the aforementioned processing conditions were associated with maximum enzymatic activity of the extract, and not necessarily with maximum yield of enzyme or maximum specific activity of the enzyme extracted.

The effects of storage and lyophilisation of extracts of dried flowers of *C. cardunculus*, and reconstitution either in water or in citrate were also studied [49, 60]. Clotting and proteolytic activities are important parameters when evaluating coagulants for cheesemaking; hence, both these activities were assessed. Tavaria et al. [60] reported that storage at 4 ºC decreased significantly the clotting activity of the extracts to a greater extent in fresh than in lyophilised form. The rates of breakdown of β- and α_s-caseins by extracts of *C. cardunculus* were not significantly affected by storage time; however, lyophilised extracts reconstituted in citrate buffer were significantly less proteolytic [60]. These authors reported that the chelating effect of citrate ions (via taking up of colloidal calcium) associated with the lyophilisation effect (which provides a tentatively more stable structure) may account for the observed low proteolytic activity. The lyophilisation process seemed to retard loss of the clotting activity throughout storage, and to prevent proteolytic breakdown of the milk caseins when the extracts were reconstituted in citrate. Therefore, the use of lyophilised extracts reconstituted in citrate buffer is suggested as an apparently better alternative to the fresh extracts that have been employed for years in the manufacture of traditional cheeses in the Iberian Peninsula.

3. PURIFICATION OF PROTEINASES

Flowers of *C. cardunculus* were claimed to contain one acid proteinase with two glycosylated sub-units, with molecular masses of 31 and 16 $kg \cdot mol^{-1}$; that enzyme was found to induce milk coagulation through cleavage of the Phe_{105}-Met_{106} bond in bovine κ-casein [20]. This enzyme

preparation was further shown to be heterogeneous in that three active proteinases (initially referred to as cynarases or cyprosins 1, 2 and 3) could be isolated, purified and partly characterised in terms of activity [6, 13, 19, 27]. Those proteinases were reported to have a pH optimum of 5.1 and they were classified as aspartic proteinases [27]. Sousa [48] indicated that only two of the three original peaks (peaks 2 and 3) possessed clotting and proteolytic activities; these two proteinases were later named cardosin A (peak 2) and cardosin B (peak 3) [63]. These authors also reported that each cardosin occurs in dimeric form, with apparent molecular masses of 31 and 15 kg·mol^{-1} for the monomers of cardosin A, and 34 and 14 kg·mol^{-1} for those in cardosin B. The kinetic parameters associated with hydrolysis of the synthetic peptide Leu-Ser-Phe-(NO_2)-Nle-Ala-Leu-oMe were determined, and together with their specificity (see Section 4) they were compared to those of chymosin and pepsin [63]; it was thus shown that cardosin A is similar to chymosin, whereas cardosin B resembles pepsin. These proteinases share pH optima in the acid range, inhibition by pepstatin and preferential cleavage of peptide bonds between hydrophobic residues (as happens with other aspartic proteinases) [64]. The drying process decreases the proteolytic activity, especially that of cardosin B (which is the stronger enzyme in the crude extract), and thus has a considerable effect on the observed overall proteolytic activity [17]. More recent studies [21, 63, 64] have indicated that those two enzymes appear chiefly in the female part of the flowers of *C. cardunculus*, i.e. in the upper portion (where the ratio of concentrations of cardosin A to cardosin B is also higher); in the lower portion, only cardosin B is present [9]. Very few aspartic proteinases from plant origin have been isolated and partially characterised to date [59]; one of their unique features is an extra segment of ca. 100 amino acids, which bears no sequence similarity with aspartic proteinases of mammalian or microbial origin [21].

4. SPECIFICITY OF PROTEINASES IN VITRO

The specificity of proteinases from *C. cardunculus* was studied using insulin B-chain as well as caseins as substrates. Extracts of *C. cardunculus* were reported to hydrolyse the insulin B-chain at the carboxylic side of the peptide bond of Phe-X, Leu-X and Ile-X, where X was preferentially Val or Tyr; however, the specificity was not entirely determined by the nature of the residues since Leu_{11}-Val_{12} of the insulin B-chain was not cleaved [20]. Later, cardosin A was reported to cleave insulin B-chain at the bonds Leu_{15}-Tyr_{16}, Leu_{17}-Val_{18} and Phe_{25}-Try_{26}, whereas cardosin B also cleaved Glu_{13}-Ala_{14}, Ala_{14}-Leu_{15} and Phe_{24}-Phe_{25}, thus implying a broader specificity for the latter [63].

The major cleavage site of bovine [31] and ovine κ-casein is Phe_{105}-Met_{106}, whereas caprine κ-casein is preferentially cleaved at Lys_{116}-Thr_{117} [55] (Fig. 1a). The hypothesised distinct primary site of proteolysis on κ-casein may lead to more extensive losses of soluble peptides [14]. The most susceptible bond on bovine β-casein is Leu_{192}-Tyr_{193}, [32, 48], although proteinases from *C. cardunculus* were reported to cleave Leu_{127}-Thr_{128}, Leu_{165}-Ser_{166} and every single peptide bond of Ala_{189}-Tyr_{193} (i.e. Ala_{189}-Phe_{190}, Phe_{190}-Leu_{191}, Leu_{191}-Leu_{192} and Leu_{192}-Tyr_{193}) in bovine β-casein as well [32] (Fig. 1b). In ovine and caprine caseinates, β-caseins (i.e. β_1- and β_2-casein, which are two variants that differ in their level of phosphorylation, 6 and 5, respectively) are cleaved at both Leu_{127}-Thr_{128} and Leu_{190}-Tyr_{191}, but in caprine caseinate the bonds Glu_{100}-Thr_{101} and Leu_{136}-Pro_{137} are also broken down [55] (Fig. 1b). The most susceptible site in bovine α_{s1}-casein is Phe_{23}-Phe_{24} [32, 48] (Fig. 1c). Macedo et al. [32] reported additional cleavage sites in bovine α_{s1}-casein, viz. Phe_{145}-Tyr_{146}, Leu_{149}-Phe_{150}, Phe_{153}-Tyr_{154}, Leu_{156}-Asp_{157}, Ala_{163}-Trp_{164}, Trp_{164}-Tyr_{165}, Tyr_{165}-Tyr_{166}

Figure 1a. Comparison of primary structures of bovine, ovine and caprine κ-casein (a), β-casein (b), α_{s1}-casein (c) and α_{s2}-casein (d) (adapted from [4, 5, 34, 41, 43, 58, 61]). Identical sequences are indicated by (----); deletions of amino acid residues are indicated by (////); bonds cleaved by *C. cardunculus* (↓), cardosin A (A), cardosin B (B), and cardosins A and B (A+B) are explicitly indicated.

and Tyr_{166}-Val_{167}. In ovine (and caprine) caseinates, the primary bond cleaved in α_{s1}-casein is Phe_{23}-Val_{24}, followed by Trp_{156}-Tyr_{157} (Trp_{164}-Tyr_{165}) and Tyr_{165}-Thr_{166} (Tyr_{173}-Thr_{174}) [55] (Fig. 1c). Macedo et al. [32] reported that proteinases from *C. cardunculus* show a clear preference for bonds between hydrophobic regions of both α_{s1}-casein (f163-167) and β-casein (f189-193), which were less attacked by chymosin under various experimental conditions. When studying degradation of β- and α_s-caseins by proteinases of *C. cardunculus*, Tavaria [60] reported that, in the initial period of hydrolysis, the former appears to be more resistant to degradation than the latter casein (6.0 vs. 21.4% for ovine, and 9.1 vs. 21.6% for caprine caseinates, respectively); this was attributed to the effect of NaCl upon hydrolysis of β-casein via modification (folding or aggregation) of the substrate rather than of the enzyme. However, as hydrolysis time elapsed, those authors reported that two such caseins were degraded to virtually the same extent in ovine

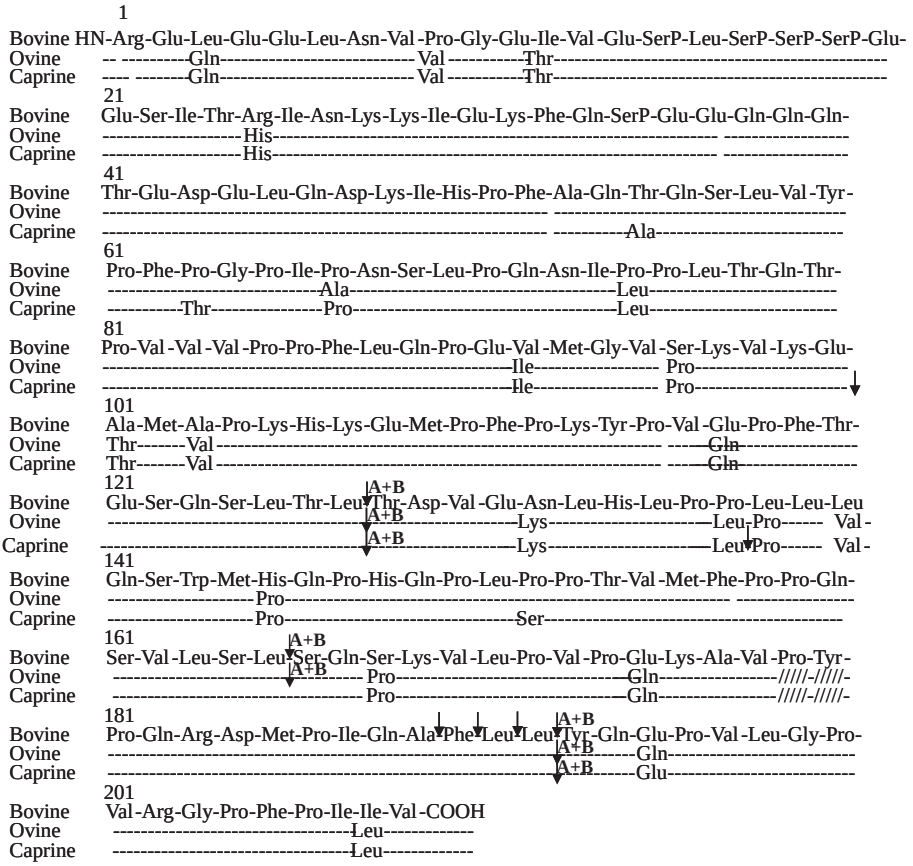

Figure 1b.

caseinate (51.8 vs. 49.3%) but not in caprine caseinate (68.9% of β-casein vs. 50.1% of α_s-casein). Finally, α_{s2}-casein was cleaved at Phe_{88}-Tyr_{89} in bovine [32], ovine and caprine caseinates [55] (Fig. 1d). The bond Tyr_{95}-Leu_{96} was also cleaved in bovine α_{s2}-casein [32], whereas the bonds Ser_9-Ser_{10} and Tyr_{179}-Leu_{180} were cleaved in caprine caseinate as well [55].

The specificity of previously isolated cardosins A and B was studied on bovine α_{s1}-casein [38], bovine β-casein [39], ovine caseinate and pure ovine α_{s1}- and β-caseins [45, 46], and caprine caseinate and pure caprine α_{s1}- and β-caseins [47].

Cardosin A and cardosin B both cleaved bovine α_{s1}-casein at the bonds Phe_{23}-Phe_{24}, Trp_{164}-Tyr_{165} and Phe_{153}-Tyr_{154}; cardosin A further cleaved the bond Tyr_{165}-Tyr_{166}, whereas cardosin B cleaved in addition the bond Phe_{150}-Arg_{151} [38]. The peptide bond Leu_{192}-Tyr_{193} in bovine β-casein was reported to be the most susceptible to hydrolysis by cardosin A or cardosin B, but the bonds Leu_{127}-Thr_{128} and Leu_{165}-Ser_{166} were also cleaved [39].

In ovine caseinate, α_s-caseins were reported to be more susceptible than β-casein to proteolysis by cardosin B, whereas the reverse was observed when the caseins

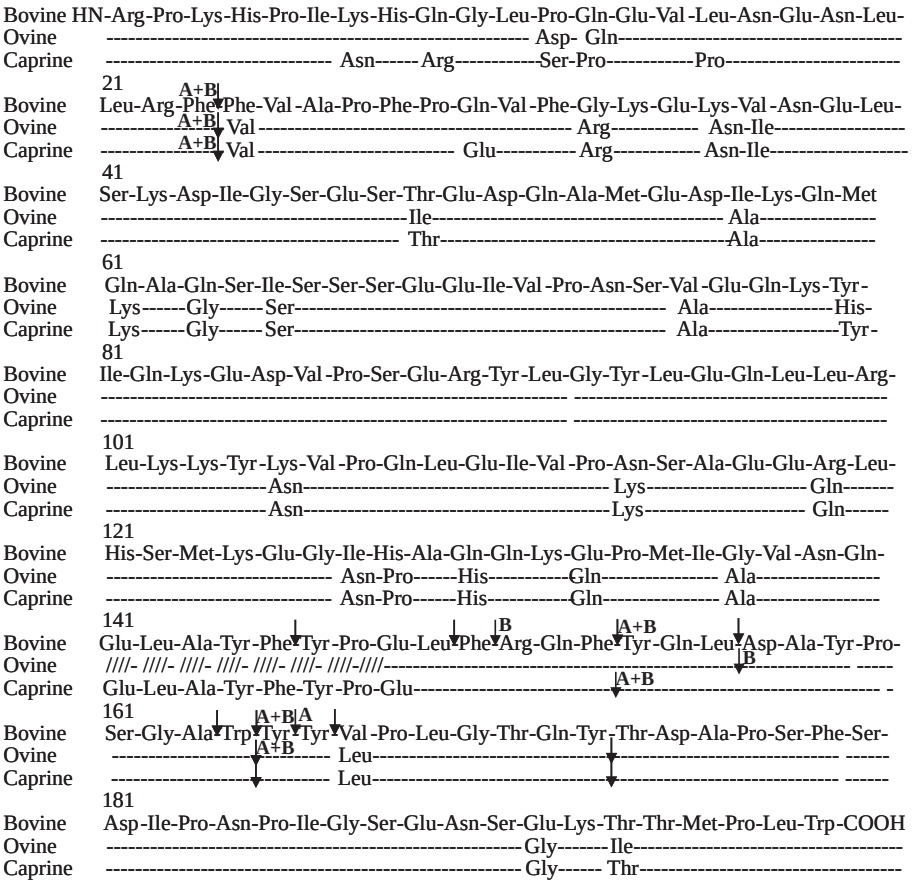

Figure 1c.

were in isolated form [46]. Cardosin A cleaved ovine caseinate at the bonds Phe_{105}-Met_{106} of ovine κ-casein, Leu_{127}-Thr_{128}, Leu_{165}-Ser_{166} and Leu_{190}-Tyr_{191} of ovine β-casein, and Trp_{164}-Tyr_{165} of ovine α_{s1}-casein [45]; cardosin B cleaved ovine β-casein at three primary sites, viz. Leu_{127}-Thr_{128}, Leu_{165}-Ser_{166} and Leu_{190}-Tyr_{191}, and ovine α_{s1}-casein at two primary sites, viz. Leu_{156}-Asp_{157} and Trp_{164}-Tyr_{165} [46]. The bonds Leu_{165}-Ser_{166} and Trp_{164}-Tyr_{165} were reported by Silva and Malcata [46] to be the most susceptible to cardosin B when this enzyme acted upon isolated ovine β- and α_{s1}-caseins, respectively. However, comparison of the results reported by Ramalho-Santos et al. [38, 39], by Sousa and Malcata [55], and by Silva and Malcata [46] suggests that the bonds in pure caseins (or in sodium caseinate, for that matter) most susceptible to cardosins A and B are Leu_{190}-Tyr_{191} and Leu_{127}-Thr_{128} in β-casein (Fig. 1b), and Phe_{23}-Val_{24} and Trp_{164}-Tyr_{165} in α_{s1}-casein (Fig. 1c).

In caprine caseinate, β-caseins were hydrolysed to a higher degree than α_s-caseins, and proteolysis by cardosin A was reported to be faster than by cardosin B [47]. These authors claimed that, in isolated form, β-caseins are broken down more rapidly and more extensively by cardosin A than by cardosin B, whereas α_s-casein is quickly

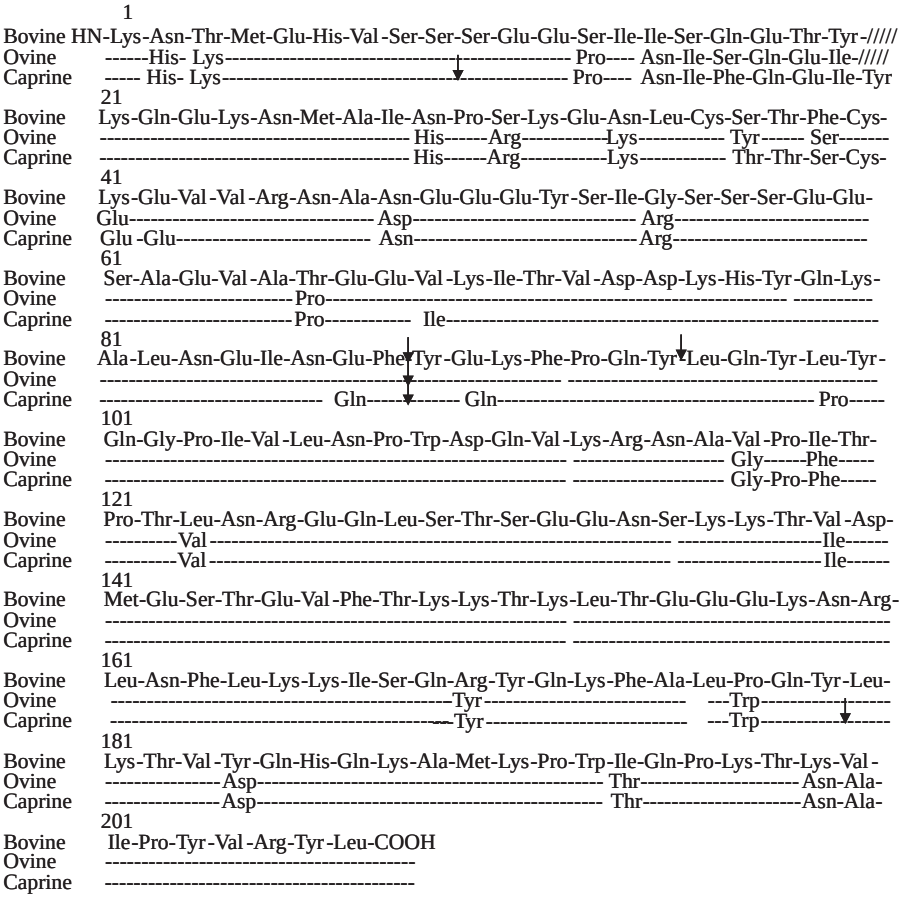

Figure 1d.

(and completely) hydrolysed by cardosin B but not by cardosin A. Regarding the specificity of cardosins A and B upon β-casein, either in caseinate or in pure forms, the major cleavage sites were found to be Leu_{127}-Thr_{128} and Leu_{190}-Tyr_{191}; conversely, in α_s-casein, either in caseinate or in pure forms, both cardosins were reported to cleave the peptide bond Phe_{153}-Tyr_{154} [47]. As mentioned above, and together with the results by Silva and Malcata [47], one concludes that the primary (and most susceptible) sites with regard to attack by both cardosins in caprine β-casein are Leu_{190}-Tyr_{191} and Leu_{127}-Thr_{128} (Fig. 1b), whereas in caprine α_{s1}-casein they are Phe_{23}-Val_{24} and Phe_{153}-Tyr_{154} (Fig. 1c).

Primary proteolysis of ovine and caprine caseinates by proteinases from extracts of *C. cardunculus* was studied using experimental conditions that mimic milk (pH 6.5), fresh cheese (pH 5.5) and cheese at the beginning of ripening (pH 5.2 with 5% NaCl) [55]. Caprine caseinate underwent more extensive proteolysis than the ovine one, under similar conditions (i.e. pH 6.5 and pH 5.5); proteolysis of β- and α_s-caseins in ovine and, to a lesser extent, in caprine caseinates was reduced in the presence of 5% NaCl [55].

5. *C. CARDUNCULUS* PROTEINASES AS COAGULANT

5.1. Bovine, ovine and caprine cheeses

The main biochemical characteristics of ovine, caprine and bovine cheeses manufactured with *C. cardunculus* proteinases as coagulant were studied throughout the ripening period, following an adaptation of the traditional technology [49, 52, 57], so as to shed further light on its mode of action. Interspecies comparison of primary proteolysis (measured by the amount of water-soluble nitrogen as percent of total nitrogen, WSN/TN) was reported to be highest for ovine milk cheese (11.9%) (Fig. 2) [52]. Statistically significant differences (on the 5% level) were found for WSN/TN between ovine cheese and either caprine (8.28%) ($P = 0.03$) or bovine (6.59%) ($P = 0.01$) cheeses, at early ripening, but no statistically significant differences were found between caprine and bovine cheeses ($P = 0.10$) [49]. These authors also showed that, by 68 d of ripening, WSN/TN was significantly higher for caprine (48.7%) ($P < 0.0001$) and ovine (46.4%) ($P < 0.0001$) than for bovine (32.9%) cheeses; however, caprine and ovine cheeses were significantly different from one another ($P < 0.0001$). Those three types of cheese display similar levels of trichloroacetic acid-soluble nitrogen (TCASN) and phosphotungstic acid-soluble nitrogen (PTASN) [52]. Water-soluble nitrogen (WSN) compounds are produced mainly via the action of coagulant enzymes [16, 37, 53, 67] and, to a lesser extent, milk proteinase [28, 67]; conversely, TCASN and PTASN result from the action of peptidases contributed by the starter and non-starter microflora.

Primary proteolysis was also assessed by urea-polyacrylamide gel electrophoresis (urea-PAGE) of the water-insoluble fraction (WISF) from bovine, ovine and caprine cheeses, and degradation of caseins was determined by densitometry. Sousa and Malcata [52] found that the percent degradation of bovine α_{s1}-casein is 86% by 68 d of ripening, whereas the bands with highest

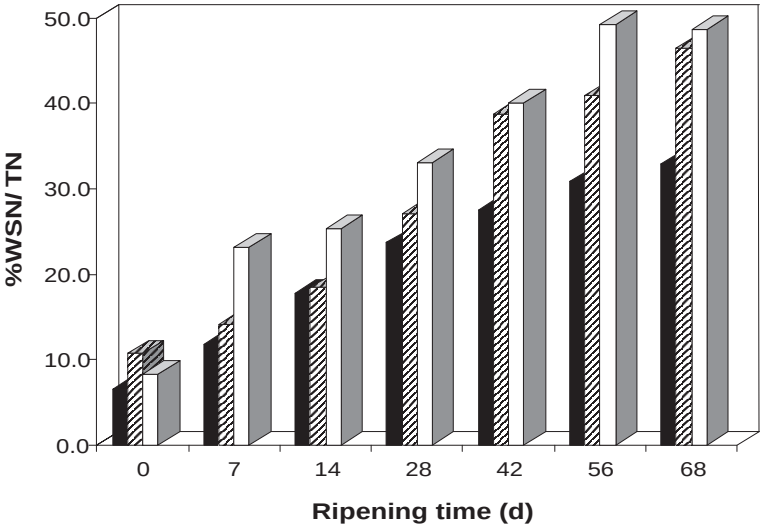

Figure 2. Mean values for the water-soluble nitrogen as percentage of total nitrogen (WSN/TN,%) for bovine (black) ovine (stripes) and caprine (white) milk cheeses, manufactured with extracts of *C. cardunculus,* throughout ripening.

mobility (i.e. those designated as the α_s-casein region) exhibit degradation levels of 47% and 86% in ovine and caprine milk cheeses, respectively (Fig. 3). The degradation of α_s-caseins (again assessed by densitometry) decreased as the NaCl content increased (to yield 86% for bovine and caprine cheeses, and 46% for ovine cheeses, containing ca. 2.1% or 3.0% NaCl, respectively) [49], in agreement with results by Noomen [35] for Meshanger-type cheese, and Delacroix and Trossat [15] for Gruyère-type cheeses. Degradation of bovine β-casein was 33%, and that of combined β_1- and β_2-caseins was 33% and 43% in ovine and caprine milk cheeses, respectively (Fig. 3) [52]. NaCl affects degradation of β-casein via modification (folding or aggregation) of the substrate, thus making it more resistant to enzymatic attack; however, the hydrophobic nature of β-casein might putatively also allow it to bind to fat globules, and hence protect it from enzymatic attack. This fact should be more noticeable in ovine than in caprine or bovine milk cheeses, as ovine milk has a higher fat content than those of the latter (even though ovine milk has about twice the amount of casein as bovine milk). This is in agreement with Tavaria et al. [60], who reported that ovine β-casein is degraded by 48.2%, whereas caprine β-casein is degraded by 68.9%, both in caseinate form. In Cheddar cheese manufactured with milk standardised to different fat contents, reduction in the fat level resulted in greater degradation of β-casein; the concentration of intact β-casein in the full fat and low fat Cheddar cheeses after 180 d of ripening were ca. 90% and 58%, respectively [22]. The γ-caseins (i.e. γ_1-, γ_2- and γ_3-caseins) arising from degradation of the β-casein by plasmin [68] increased in concentration throughout ripening of bovine cheeses, but could not be identified in the WISF of either ovine or caprine cheeses [49].

Sousa [49] reported that the peptide profiles of the water-soluble fraction (WSF) of cheeses (performed by reversed-phase high performance liquid chromatography, RP-HPLC) exhibited different patterns depending on the source of milk (Fig. 4). The concentration of hydrophobic peptides (Tab. I) increased up to 68 d of ripening in caprine cheese, and up to 14 d in bovine and ovine cheeses, but apparently decreased thereafter; the concentration of hydrophilic peptides (Tab. I) increased up to 68 d of

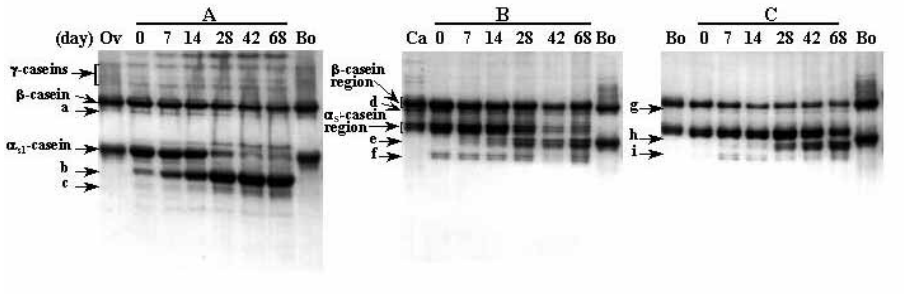

Figure 3. Urea-PAGE electrophoretograms of the water-insoluble fraction after 0, 7, 14, 28, 42 and 68 d of ripening, for bovine (a), ovine (b) and caprine (c) milk cheeses manufactured with extracts of *C. cardunculus.* Bovine Na-caseinate (Bo), ovine Na-caseinate (Ov) and caprine Na-caseinate (Ca) were included as standards. Peptide fragments labelled were duly isolated and identified (Tab. II).

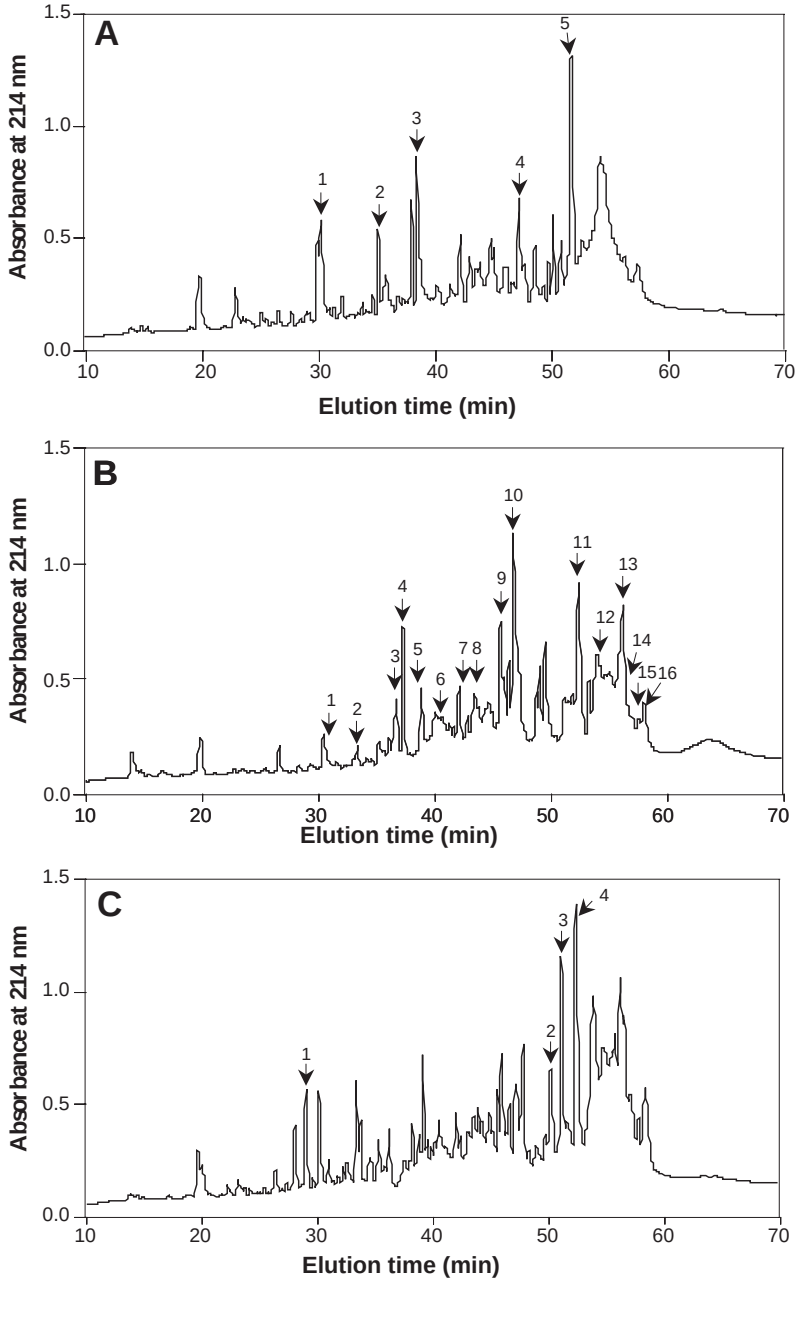

Figure 4. Reverse-phase high performance liquid chromatography (RP-HPLC) profiles of the water soluble fraction of bovine (a), ovine (b) and caprine (c) milk cheeses, manufactured with extracts of *C. cardunculus* and ripened for 68 d. Peptide fragments labelled were duly isolated and identified (Tab. III).

Table I. Evolution throughout ripening of areas associated with hydrophobic and hydrophilic peptides, as determined by RP-HPLC[a] of the WSF of bovine, ovine and caprine raw milk cheeses manufactured with extracts of *C. cardunculus.*

Ripening time (d)	Hydrophobic peptides (1)	Hydrophilic peptides (2)	Ratio (1) / (2)
Bovine			
0	2607.8	14.9	175.1
7	3226.7	50.4	64.0
14	5615.1	80.1	70.1
28	4521.9	132.8	34.1
42	4977.7	222.1	22.1
56	6331.0	470.5	13.5
68	5453.8	363.0	15.0
Ovine			
0	778.4	60.8	12.8
7	3877.2	67.8	57.2
14	6077.2	64.7	93.9
28	4854.7	114.4	42.4
42	5182.0	134.1	38.7
56	5710.7	177.6	32.2
68	5647.7	189.0	29.9
Caprine			
0	2332.8	19.0	133.4
7	2791.0	62.5	44.7
14	3746.1	111.7	33.5
28	5604.3	387.4	14.5
42	6109.8	593.4	10.3
56	6696.5	798.4	8.4
68	6985.9	962.1	7.3

[a] See Figure 4.

ripening in ovine and caprine cheeses, and up to 56 d in bovine cheese [49]. The ratio of hydrophobic to hydrophilic peptides (Tab. I) decreased consistently during ripening in bovine and caprine cheeses, hence attaining 15.0 and 7.3, respectively, by the end of ripening; however, this ratio went through a maximum by 14 d in ovine cheese and decreased thereafter to reach 29.9 by 68 d of ripening [49]. These results suggest that ovine milk caseins are apparently a less appropriate substrate than bovine milk caseins for the release of hydrophobic peptides brought about by *C. cardunculus* extracts, in agreement with findings by Carrera et al. [8]. Gaya et al. [26] reported that the formation of hydrophobic peptides in cheese manufactured with plant coagulant proceeded for a longer time than in cheese manufactured with chymosin; these authors also pointed out the highest level of hydrophobic peptides by 24 h of ripening when plant coagulant was employed, which was ca. two-fold that found in cheese manufactured with chymosin. Proteinases from *C. cardunculus* (and also chymosin) have been implicated in the formation of bitter peptides in cheese, so factors that affect retention (and also activity) of coagulant in the curd will surely influence development of bitterness. Visser et al. [69, 70] concluded that the relatively slow hydrolysis of β-casein by coagulant and by starter proteinases leads to the gradual appearance of the bitter peptide β-casein (f193-209) as the first degradation product,

which in turn resists further degradation well. The proteinases from *C. cardunculus* (like those of most coagulants) and, probably to a lesser extent, plasmin hydrolyse caseins down to large- and intermediate-size peptides, which are then broken down by residual coagulant, as well as by proteinases and peptidases from starter and non-starter bacteria. Proteolysis in cheese coagulated with *C. cardunculus* does not occur much further, as ascertained by the relatively low TCASN/TN and PTASN/TN indices [52]; therefore, the role of coagulant in the development of bitterness may be an indirect one, via production of long peptides that are (or should be) subsequently degraded to small, bitter peptides by microbial proteinases.

Electrophoretic bands corresponding to degradation products in WISF (Fig. 3) were isolated by electroblotting from urea-PAGE gels using a mini Trans-Blot™ electrophoretic transfer cell; the peptide fragments of the WSF from bovine, ovine and caprine milk cheeses (Figs. 4a-c) were isolated [49] for the purpose of identification. Sequencing (five to ten cycles) was performed via Edman degradation on an automated pulsed, liquid-phase protein/peptide sequencer; the free amino acids released were detected as their phenylthiohydantoin derivatives.

Caseins from the three types of milk share relatively similar amino acid sequences (Fig. 1a-d), thus implying that they should be similarly hydrolysed in the (same) susceptible regions. Upon sequencing, the bands (a), (d) and (g) (Fig. 3) of the WISF yielded peptides of the type β-casein(f1-) (Tab. II) [49]. Those bands have electrophoretic mobilities similar to that of the primary product produced by proteinases from *C. cardunculus* on β-casein (i.e. β-I-casein) in bovine [48], ovine and caprine caseinates [56], via cleavage of the most susceptible bond Leu_{192}-Tyr_{193} in bovine β-casein or Leu_{190}-Tyr_{191} in ovine and caprine β-caseins (Fig. 1b). The sequencing of a peptide containing the N-terminus of β-casein (f193-) in bovine cheeses, or

Table II. Electrophoretic bands, as determined by urea-PAGE, of the WISF of bovine, ovine and caprine raw milk cheeses manufactured with *C. cardunculus* and ripened for 68 d.

Electrophoretic band[a]	N-terminal sequence	Cleavage site in casein sequence
Bovine		
a	H_2N-Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-?	β-f1-?
b	Phe-Val-Ala-Pro-Phe-Pro-Gln-Val-Phe-?	α_{s1}-f24-?
c	Phe-Val-Ala-Pro-Phe-Pro-Gln-Val-Phe-?	α_{s1}-f24-?
Ovine		
d	H_2N –Arg-Glu-Gln-Glu-Glu-Leu-?	β-f1-?
e	Val-Val-Ala-Pro-Phe-Pro-Gln-?	α_{s1}-f24-?
f	Val-Val-Ala-Pro-Phe-Pro-?	α_{s1}-f24-?
Caprine		
g	H_2N –Arg-Glu-Gln-Glu-Glu-Leu-?	β-f1-?
h	Val-Val-Ala-Pro-Phe-Pro-Gln-?	α_{s1}-f24-?
i	Val-Val-Ala-Pro-Phe-Pro-?	α_{s1}-f24-?

[a] See Figure 3.

Table III. Peptides accounting for RP-HPLC peaks of the WSF of bovine, ovine and caprine raw milk cheeses manufactured with *C. cardunculus* and ripened for 68 d.

HPLC peak[a]	N-terminal sequence	Cleavage site in casein sequence
Bovine		
1	H_2N-Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-Gly-Leu-Pro-Gln-Glu-?	α_{s1}-f1-?
2	H_2N-Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-Gly-Leu-Pro-Gln-Glu-? + Ser-Gln-Ser-Lys-Val-Leu-Pro-Val-Pro-Glu-Lys-Ala-Val-Pro-?	α_{s1}-f1-? + β-f166-?
3	H_2N-Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-Gly-Leu-?	α_{s1}-f1-?
4	H_2N-Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-Gly-Leu-Pro-Gln-Glu-? + Thr-Asp-Val-Glu-Asn-? + Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-?	α_{s1}-f1-? β-f128-? β-f193-?
5	Thr-Asp-Val-Glu-Asn-Leu-His-Leu-? + Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro?	β-f128-? β-f193-?
Ovine		
1	H_2N-Arg-Pro-Lys-His-Pro-? + Gln-Ser-Trp-Met-Pro-?	α_{s1}-f1-? + β-f141-?
2	Ser-Gln-Pro-Lys-Val-Leu-?	β-f166-?
3	H_2N-Arg-Pro-Lys-His-Pro-Ile-Lys-? + Ser-Gln-Pro-Lys-Val-Leu-?	α_{s1}-f1-?+ β-f166-?
4	H_2N-Arg-Pro-Lys-His-Pro-Ile-Lys-? + Ser-Gln-Pro-Lys-Val-Leu-?	α_{s1}-f1-?+ β-f166-?
5	H_2N-Arg-Pro-Lys-His-Pro-? + Arg-Asp-Met-Pro-Ile-?	α_{s1}-f1-?+ β-f183-?
6	Ser-Gln-Pro-Lys-Val-Leu-? + Arg-Asp-Met-Pro-Ile-?	β-f166-?+ β-f183-?
7	Val-Val-Pro-Pro-Phe-Leu-? + Asp-Val-Glu-Lys-Leu-?	β-f83-?+ β-f129-?
8	Asp-Val-Glu-Lys-Leu-?	β-f129-?
9	Lys-Ile-Glu-Lys-Phe--? + Thr-Asp-Val-Glu-Lys-Leu-His-? + Asp-Val-Glu-Lys-Leu-?	β-f29-?+ β-f128-?+ β-f129-?
10	H_2N –Arg-Pro-Lys-His-Pro- ? + Thr-Asp-Ala-Pro-Ser-? Asp-Asp-Lys-His-Tyr-? + Asn-Glu-Phe-Tyr-Glu-? Thr-Gln-Pro-Lys-Thr-Lys-? + Val-Val-Pro-Pro-Phe-Leu-? Gly-Val-Pro-Lys-Val-? + Ser-Gln-Pro-Lys-Val-Leu-? Gly-Pro-Phe-Pro-Ile-Leu--?	α_{s1}-f1-?+ α_{s1}-f166-?+ α_{s2}-f74-?+ α_{s2}-f86-?+ α_{s2}-f194-?+ β-f83-?+ β-f94-?+ β-f166-?+ β-f201-?
11	Val-Val-Ala-Pro-Phe-Pro-? Tyr-Gln-Glu-Pro-Val-Leu-?	α_{s1}-f24-?+ β-f143-?+ β-f191-?
12	Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-?	β-f191-?
13	H_2N-Arg-Glu-Gln-Glu-Glu-? +Asn-Val-Val-Gly-Glu-?+ Leu-Gln-Asp-Lys-Ile-? + Thr-Asp-Val-Lys-Asn-? + Ala-Tyr-Pro-Gln-Arg-Met-?	β-f1-?+ β-f7-?+ β-f45-?+ β-f128-?+ β-f177-?
14	Thr-Asp-Val-Lys-Asn-?	β-f128-?
15	Asn-Val-Val-Gly--?	β-f7-?
16	Asn-Val-Val-Gly-Glu-?	β-f7-?

Table III. (continued).

Caprine		
1	Lys-His-Pro-Ile-Asn-His-Arg-Gly-Leu-? + Thr-Asp-Val-Lys-Asn-Leu-? +	α_{s1}-f3-? + β-f128-?
2	Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-?	β-f191-?
3	Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-?	β-f191-?
4	Leu-Leu-Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-?	β-f189-?

[a] See Figure 4.

β-casein (f191-) in ovine and caprine cheeses (Tab. III), confirmed that the most susceptible bond of β-casein has been cleaved, so the complementary fragment should be the peptide β-casein (f1-192) (a) in bovine β-casein, or β-casein (f1-190) (d and g) in ovine and caprine β-casein, respectively. Sousa [49] sequenced the peptides β-casein (f128-), β-casein (f166-) and β-casein (f193-) from bovine β-casein (Tab. III), and β-casein (f128-) and β-casein (f191-) from ovine and caprine β-casein (Tab. III), which were probably produced by the plant proteinases of interest. The corresponding cleavage sites were identified previously in solutions of bovine β-casein [32, 48], and of ovine and caprine β-caseins subjected to hydrolysis by proteinases of *C. cardunculus* [55]. The main hydrolysis sites identified in association with said coagulant action were Leu_{127}-Thr_{128}, Leu_{165}-Ser_{166} and Leu_{192}-Tyr_{193} in bovine β-casein, Leu_{127}-Thr_{128} and Leu_{190}-Tyr_{191} in ovine β-casein [56], and Leu_{127}-Thr_{128} and Leu_{190}-Tyr_{191} in caprine β-casein. Several peptide fragments were reported in bovine, ovine and caprine β-casein (Tab. III) [49], but only a few could be traced as having been released by coagulant proteinases. The existence of the other fragments is probably due to proteinases or peptidases from the milk, coupled with enzymes from the microorganisms prevailing in cheese. The bands (b), (c), (e), (f), (h) and (i) in Figure 3 were sequenced as the peptides α_{s1}-casein (f24-) (Tab. II), produced following cleavage of bond Phe_{23}-Phe_{24} in bovine α_{s1}-casein, or Phe_{23}-Val_{24} in ovine and caprine α_{s1}-caseins [49, 52]. In solution, the fragment bovine α_{s1}-casein (f24-199) was identified as the major peptide produced by the action of proteinases from *C. cardunculus* [48], a result confirmed independently by Macedo et al. [32]. The peptide α_{s1}-casein (f24-199) was claimed to be present, at least in early stages of ripening, in all types of cheese; increasing the salt-in-moisture concentration of cheese does not apparently influence the rate of initial hydrolysis of α_{s1}-casein, although it inhibits subsequent hydrolysis of α_{s1}-casein (f24-199) [18]. The bond in ovine and caprine α_{s1}-casein most labile to the action of proteinases from *C. cardunculus* is Phe_{23}-Val_{24}, which produces a set of bands of higher electrophoretic mobility than α_{s1}-casein, thus corresponding to bovine α_{s1}-I-casein (i.e. f24-199) [55]. These bands were produced in ovine milk cheeses from the very beginning of ripening, in caprine milk cheeses after 7 d and in bovine milk cheeses after 28 d, and all became more intense as ripening progressed. In ovine cheese manufactured with *C. cardunculus,* Phe_{23}-Val_{24} is already hydrolysed by 7 d, but not before 28 d of ripening when animal coagulant is employed instead. Other bands with higher mobility (Fig. 3) in bovine cheese (c), in ovine cheese (f) and in caprine cheese (i) were reported to share the same N-terminus, viz. α_{s1}-casein (f24-) (Tab. II); these bands were associated with the peptide α_{s1}-casein

(f24-165), which is present in ovine cheese manufactured with *C. cardunculus* but not with animal rennet [56]. Hydrolysis of the peptide bond Phe_{23}-Val_{24} in α_{s1}-casein has been associated with a rapid change in the rubbery texture of young curd into a smoother, more homogeneous product [29]; it may indeed account for the fact that ovine and caprine cheeses manufactured with *C. cardunculus* typically possess smoother textures.

5.2. Plant coagulant versus animal rennet

Cheeses manufactured with ovine milk using extracts from *C. cardunculus* as coagulant were compared with cheeses manufactured with commercial animal rennet [24, 53], or with extracts of *Cynara humilis* [66]. Comparing averages and 95% confidence intervals, it has been stated [53] that the type of coagulant has no significant effect upon cheese bulk chemical composition throughout ripening, and that no significant microbiological differences were detected between cheeses manufactured with *C. cardunculus* or animal rennet. Vioque et al. [66] reported that extracts of *C. cardunculus* or *C. humilis* have no appreciable effect on bulk composition, but extracts of the latter resulted in higher lactic acid content and higher pH values than in those cheeses produced with *C. cardunculus*. Those authors reported that microbiological viable counts (total, coliforms and lactobacilli) were similar in cheeses produced with both types of plant coagulant, although enterobacteria, yeasts and moulds were found at higher viable numbers in cheese produced with *C. humilis* than with *C. cardunculus*. Cheeses manufactured with plant coagulant exhibited significantly higher levels of WSN/TN; that coagulant had significantly less proteolytic activity upon α_s- and β-caseins than animal rennet [53]. In contrast, Fernández-Salguero and Sanjuán [24] showed that residual α_s- and β-caseins were more concentrated in cheese produced with animal rennet than in cheese produced using plant coagulant (*C. cardunculus*), but no statistically significant differences were found between these two types of coagulant. Vioque et al. [66] showed that WSN was higher in cheeses manufactured with extracts of *C. cardunculus* than with *C. humilis,* but WSN in cheeses with *C. humilis* was much higher than in Los Pedroches cheese manufactured with chymosin [24]. The electrophoretic patterns of the WISF of both cheeses were claimed to unfold a higher susceptibility of α_s- than β-casein to proteolysis [24, 36, 53]. The peptides β-casein (f1-190) and α_s-casein (f24-191) were reported to be the primary products originated by both coagulants from the corresponding caseins [56]; however, the bond Phe_{23}-Val_{24} was cleaved by 7 d but not before 28 d of ripening when animal coagulant was employed (note that identical conditions, including standardised amounts of plant and animal coagulants, were employed, so such differences were attributable to different enzyme activities; the fact that in Cheddar and Camembert the bond Phe_{23}-Val_{24} is rapidly hydrolysed is probably due to the larger amounts of animal rennet added). These bands were identified as the peptide α_{s1}-casein (f24-165), which is present only in ovine cheese manufactured with *C. cardunculus*, whereas the peptide α_{s1}-casein (f120-191) was only produced by animal rennet [56]. The peptides β-casein (f128-), β-casein (f166-) and β-casein (f191-) were produced by plant rennet only, whereas β-casein (f164-) and β-casein (f191-) were produced by animal rennet only [56]. Proteolysis in cheese manufactured with these extracts is associated mainly with the action of this rennet rather than with the prevailing microflora or otherwise adventitious enzymes, and proceeds to a high extent but a low degree.

5.3. Raw milk versus pasteurisation versus addition of starter cultures

Ovine cheeses manufactured from raw milk exhibited higher microbial counts than those from pasteurised milk without starter addition [51]; however, the lowest microbial counts were recorded in cheese with starter added [7, 51]. Pasteurisation [51] and addition of starter cultures [7, 51] were found to have no detectable effects on the characteristics of the cheeses throughout ripening; nevertheless, differences were found between the cheeses in terms of WSN/TN, as well as between cheeses manufactured from pasteurised milk without starter addition and those manufactured from pasteurised milk with starter addition in terms of TCASN and PTASN [51]. Proteolysis was generally more intense and rapid in cheeses containing starter cultures than in starter-free cheeses [7, 49]. Sousa and Malcata [51] reported that cheeses manufactured with raw milk, pasteurised milk without starter or pasteurised milk with starter addition could not be distinguished by urea-PAGE; this means that caseins were apparently degraded into products that originated similar electrophoretic patterns. However, their WSF showed differences by long ripening times for pasteurised milk cheeses with starter addition relative to raw milk cheeses and pasteurised milk cheeses without starter addition; furthermore, a higher number of peptides was present in pasteurised milk cheeses with starter addition [49].

6. CONCLUSIONS

In the last decade, extensive work has been carried out on the properties of the proteolytic action of various species of *Cynara*, including isolation and independent characterisation of their proteinases (i.e. cardosins A and B).

In vitro studies were performed to investigate the clotting and proteolytic activities, as well as the specificity of the aqueous extracts from *C. cardunculus* towards caseinate and isolated caseins. The maximum activity of extracts of *C. cardunculus* was obtained via grinding the stylets for 36 s, using an extraction buffer with pH 5.9 without salt, and after homogenisation of the suspension for 15 min. Lyophilised extracts of *C. cardunculus* (reconstituted in citrate buffer) are recommended for use rather then fresh extracts in the manufacture of cheeses owing to their higher clotting/proteolytic activity ratio. *Cynara cardunculus* proteinases acting upon caseins show preference for bonds between bulky regions; cardosin A was claimed to be similar to chymosin, whereas cardosin B was claimed to be similar to chymosin, in terms of their specificity and kinetic parameters.

In vivo studies encompassed comparison of the breed source of milk (bovine, ovine and caprine milk), comparison of plant and animal coagulants, as well as the effects of microflora, pasteurisation of milk and addition of starter cultures on the biochemical characteristics of the cheeses during ripening that were prepared with *C. cardunculus*. Significant effects of the breed source of milk were observed with respect to the ripening index (WSN/TN); higher proteolysis was recorded in ovine and caprine than in bovine cheeses. Proteinases of *C. cardunculus* produced higher levels of the ripening index than commercial animal rennet, even though the former led to lower levels of the ripening depth index (TCASN/TN) and the free amino acid index (PTASN/TN). Pasteurisation and addition of starter cultures have no appreciable effect on the characteristics of the cheeses throughout ripening; nevertheless, differences were found between the cheeses in terms of WSN/TN, as well as between cheeses manufactured from pasteurised milk with or without starter addition, in terms of TCASN and PTASN.

Nevertheless, further experimental work is still necessary to more consistently and deeply describe the role of these proteinases in such a complex matrix as cheese.

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