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Hydrolysis of caprine and ovine milk proteins, brought about by aspartic peptidases from *Silybum marianum* flowers

Sandra Vairo Cavalli [a,*], Sofia V. Silva [b], Cecilia Cimino [a], F. Xavier Malcata [b], Nora Priolo [a]

[a] *LIPROVE, Departamento de Ciencias Biológicas, Faculated de Ciencias Exactas, UNLP, CC 711, 1900 La Plata, Argentina*
[b] *Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, P-4200–072 Porto, Portugal*

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Abstract

The flowers of cardoon (*Asteraceae*) are a rich source of aspartic peptidases which possess milk clotting activity – and are thus used in traditional cheesemaking in the Iberian Peninsula. This study was aimed at characterizing the enzymatic action of the aspartic peptidases present in flowers of *Silybum marianum* (L.) Gaertn. (*Asteraceae*), specifically upon degradation of caseins. The proteolytic activities toward Na-caseinates previously prepared from caprine and ovine milks were studied, in a comparative fashion, using urea-PAGE, tricine-SDS-PAGE, densitometry, electroblotting and sequencing. Caprine α_{s1}- and β-caseins were degraded up to 68% and 40%, respectively, during 24 h of incubation. Only one important and well-defined band corresponding to a molecular weight of 14.4 kDa – i.e. a fragment of β-casein, was observed by 12 h of hydrolysis. By 24 h of incubation, ovine α_s- and β-caseins were degraded up to 76% and 19%, respectively. In what concerns specificity, the major cleavage site in ovine caseinate was Leu99-Arg100 in α_{s1}-casein.
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1. Introduction

Argentina is the second milk producer of Latin America, and it ranks eleventh place on a world-wide basis. Sales of goat's and ewe's milk cheeses appears to be an attractive alternative to reconvert and diversify primary production in that country, since possesses a relation of favorable price of 3:1 when compared with cheeses of bovine origin (Dayenoff, Ochoa, & Domínguez, 2002; Estación Experimental Agropecuaria Chubut, 2006). Furthermore, goats and ewes can grow in more unfavorable environmental conditions, from a feeding point of view, than cows (Dayenoff et al., 2002). On the other hand, cheeses manufactured from milk of ewes or goats possess a much higher added value as gourmet products are in stake.

The unique nutritional composition relative to cow's milk should also be emphasized. This reflects especially in cheese yield: ca. 5.5 l of ewe's milk is required to produce 1 kg of cheese, whereas ca. 11 l of cow's milk is required for the same amount of cheese. This greater cheese yield is explained by the quantitatively higher content of fat (7.5%) and caseins (4.6%) of ewe's milk than cow's counterpart (3.9% of fat and 2.6% of caseins); a similar realization holds with goat's milk (4.5% of fat and 3.0% of caseins). In addition to the aforementioned higher cheese yield, goat's and ewe's milk have beneficial health properties, because of their mineral and vitamin levels, and their different protein profile (Walstra, Geurts, Noomen, Jellema, & van Boekel, 1999; Dulce, 2006).

Aqueous extracts from flowers of *Cynara carduncul*us (Veríssimo, Esteves, Faro, & Pires, 1995; Veríssimo et al., 1996), *Cynara humilis*, and/or *Cynara scolymus* contain proteinases, so thus have accordingly been used in the Iberian Peninsula since Roman times to manufacture ovine and/or caprine milk cheeses (Reis et al., 2000). Milk clotting

* Corresponding author. Tel.: +54 221 4230121x57; fax: +54 221 422 6947.
E-mail address: svairo@biol.unlp.edu.ar (S. Vairo Cavalli).

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activity was also found in flowers of *Centaurea calcitrapa*, *Onopordum turcicum* and *Silybum marianum* (Domingos et al., 1998; Tamer, 1993; Vairo Cavalli, Claver, Priolo, & Natalucci, 2005). All these species belong to the *Asteraceae* family, and furthermore lie in the same tribe: *Cardueae* Cass. = *Cynareae* Less. (Ariza-Espinar & Delucchi, 1998). Flowers specifically of *S. marianum* were claimed to contain aspartic proteinases that were able to entertain milk clotting; such clotting activity is partially inhibited at NaCl concentrations above 50 mM, whereas the effect of $CaCl_2$ is biphasic – with an initial decrease, followed by an increase in rennet clotting times (Vairo Cavalli et al., 2005).

Primary proteolysis is caused chiefly by residual rennet, and produces large to medium-sized peptides from the starting caseins; these can be further degraded into small peptides, and eventually free amino acids – in a process generally known in whole as secondary proteolysis. Primary proteolysis plays an essential role upon development of proper cheese texture, whereas secondary proteolysis is often implicated with cheese flavour; it is thus of great importance to assure a well-balanced breakdown of caseins, in order to prevent development of such undesired attributes in cheese as low viscosity and high bitterness (Visser, 1993). Proteolysis is indeed the most important set of biochemical transformations during cheese ripening, in which the residual rennet plays a relevant role; hence, it is of the utmost importance to evaluate the degradation patterns of caseins in model systems that mimic actual cheesemaking (Irigoyen, Izco, Ibáñez, & Torre, 2000; Silva & Malcata, 1999).

Analysis of the degradation pattern of bovine Na-caseinate by aspartic proteinases from *S. marianum* was studied previously – and was even considered as a new source of plant rennet, with distinctive, useful characteristics for the dairy industry (Vairo Cavalli et al., 2005). The aim of this work was to complement that knowledge, via evaluating the action of those aspartic peptidases upon the hydrolysis of caprine and ovine Na-caseinates – using experimental conditions that parallel milk (pH 6.5), in order to address an increasing worldwide demand for alternative dairy products with improve organoleptic nutritional and health properties. Study of casein breakdown in model systems will likely generate important information to help elucidate the complex processes involved in ripening of cheeses from small ruminants, and eventually contribute toward development of better, non conventional final cheeses (Silva & Malcata, 2000).

2. Materials and methods

2.1. Enzyme extract preparation

The method described by Vairo Cavalli et al. (2005) was followed to prepare the enzyme extract. Fresh flowers of *S. marianum* (L.) Gaertn. were ground with a mortar and pestle under liquid nitrogen, homogenized at a ratio of 1 g per 3 ml of 0.1 M citric acid–sodium citrate buffer (pH 3.0) containing 1.0 mM EDTA, and stirred for 30 min. The homogenate was centrifuged at 5000*g* for 20 min at 4 °C, and a 10-ml aliquot of the supernatant was applied to a Pharmacia K 15/30 column packed with a size exclusion chromatographic medium (Sephadex G-25 Fine, from GE Healthcare, Uppsala, Sweden) – which had been previously equilibrated with 50 mM citric acid–sodium citrate buffer (pH 3.0). Elution was performed with the same buffer, at a 0.45 ml/min flow rate, so as to obtain a partially purified enzyme extract (EE).

2.2. Protein quantification

Protein concentration was determined by the method of Bradford (1976). Bovine serum albumin (Sigma, St. Louis MO, USA) was used as reference in the preparation of the calibration curve.

2.3. Milk clotting activity

A 100 μl-aliquot of EE was added to 1 ml of skim milk (San Regim, SanCor, Argentina) and 12% (w/v) in 10 mM $CaCl_2$, at 30 °C (Vairo Cavalli et al., 2005). The milk clotting activity (MCA) was measured following the procedure described by the International Dairy Federation (1992). One rennet unit (RU) was defined as the amount of enzyme that coagulates 10 ml of milk at 30 °C in 100 s (Barros, Ferreira, Silva, & Malcata, 2001).

2.4. Endopeptidasic activity

The endopeptidasic activity of the EE was determined by hydrolysis of the synthetic fluorogenic peptide MCA–Lys–Pro–Ala–Glu–Phe–Phe–Ala–Leu–DNP (Genosphere Biotechnologies, Paris, France), according to Veríssimo et al. (1996) with modifications. Enzyme preparations were incubated at 37 °C with 0.54 μg/ml of substrate in 50 mM Na-acetate buffer (pH 5.6), containing 140 mM NaCl. The rate of hydrolysis of the aforementioned Phe–Phe bond was monitored for 5 min, in a thermostated spectrofluorometer Luminiscence Spectrometer LS 50 B (Perkin Elmer, Wellesley MA, USA). The fluorofore was excited at 328 nm, and the light emitted was detected at 393 nm.

2.5. Sodium caseinate preparation

Whole ovine and caprine caseins were obtained from raw milk, via isoelectric precipitation following acidification to pH 4.25 with 6 M HCl – according to the method of Sousa and Malcata (1998) with slight modifications. The mixture of caseins and whey was warmed to 37 °C, and held at that temperature for 30 min. The caseins were recovered by filtration through a clean cloth, and washed several times with deionized water. The caseins were then resuspended in deionized water (to the initial volume), and pH was adjusted to 7.0 with 1 mM NaOH. The suspension was allowed to equilibrate at 4 °C for at least 2 h, freeze-dried and stored until use.

2.6. Enzymatic hydrolysis performance

Whole caseinates were dissolved to a final concentration of 1% (w/v) in 100 mM phosphate buffer (pH 6.5), containing 0.1% (w/v) NaN_3 to prevent protein degradation by adventitious microflora, and allowed to stabilize at 30 °C. The reactions were started by addition of 450 μl of each caseinate to 45 μl of EE (which corresponds to 44 ± 4 μg of protein/ml). At different times, the reactions were quenched by addition of 500 μl of 5% (w/v) trichloroacetic acid (TCA). The samples were left to precipitate overnight at 4 °C, and then centrifuged at 10,000*g*. The precipitates were redissolved in 450 μl of sample buffers: (a) for urea polyacrylamide gel electrophoresis (urea-PAGE), the buffer was 62 mM Tris–HCl (pH 7.6), containing 8 M urea, 2.0% (v/v) 2-mercaptoethanol and 0.002% (w/v) bromophenol blue, with pH adjusted with 1 M NaOH, and with samples vortexed four times for 30 s; (b) for tricine-sodium dodecylsulphate-PAGE (tricine-SDS-PAGE), the buffer was 62.5 mM Tris–HCl (pH 6.8), containing 2% (w/v) SDS, 0.5% (v/v) 2-mercaptoethanol, 0.002% (w/v) bromophenol blue and 10% (w/v) glycerol, with pH adjusted with 1 M NaOH, and with samples heated at 100 °C for 5 min.

Controls containing Na-caseinate and Na-azide, at the same concentrations but without addition of enzyme, were also sampled. Digestion of ewe's and goat's caseinates were also carried out with commercial animal rennet (Chris Hansen's, Copenhagen, Denmark), diluted so as to obtain the same coagulation time with EE.

In all cases, samples were dissolved in urea buffer.

2.7. Electrophoretic analysis

Electrophoresis were performed using a Mini Protean III cell (Bio-Rad Laboratories, Hercule, CA, USA). Urea-PAGE was according to Shalabi and Fox (1987); gels were pre-run at 50 mA for 10 min, and after sample loading, the power supply was set at 100 V at the stacking gel, and then increased to 200 V at the resolution gel.

Tricine-SDS-PAGE was according to Shägger and von Jagow (1987): after sample loading, the power supply was set at 30 V at the stacking gel, then increased at 15 V per min for four times, and finally maintained between 90 and 100 V.

Gels were stained with Coomassie Blue G-250 (Bio-Rad Bulletin, 2003). Quantification of intact caseins and polypeptides was done by gel scanning followed densitometry, using the software Scion Image Beta v. 4.02 for Windows (Scion Corporation, Frederick, MA, USA).

2.8. Electroblotting preparation

After Tricine-SDS-PAGE, the peptides were transferred onto a polyvinylidene difluoride (PVDF) membrane by electroblotting in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) containing 10% (v/v) methanol (pH 11), at 500 mA for 1 h.

2.9. Chemical sequencing

N-terminal amino-acid sequences of the protein bands recovered via electroblotting were determined by Edman degradation, using an automated pulsed liquid-phase-peptide sequencer (Applied Biosystem 477 A sequencer, Foster City CA, USA). Partial sequences obtained were checked against known sequences of caseins, in order to identify the cleaved peptide bonds. Sequence homology searches were performed using the BLAST network services (Altschul et al., 1997).

3. Results and discussion

3.1. Enzyme extracts – protein quantification, milk clotting activity and endopeptidasic activity

The EE obtained from fresh flowers of *S. marianum*, and partially purified by gel filtration (pH 3.0) displayed a protein content of 264 ± 4 mg/l, a clotting activity of 0.083 ± 0.003 RU/ml and an endopeptidasic activity of 1.50×10^{-5} IU.

As reported elsewhere (Vairo Cavalli et al., 2005) the nature of proteolytic activity associated with MCA is due to aspartyl endopeptidases. On the other hand, when endopeptidase inhibitors (PMSF – a serine protease inhibitor, E-64 – a cysteine protease inhibitor, and pepstatin A – an aspartic protease inhibitor) were preincubated with the EE, only the latter promoted inhibition of milk clotting activity. Recall that pepstatin is one of the most specific inhibitors known in enzymology, and is highly selective for aspartic peptidases (Dunn, 2001).

3.2. Caprine caseinate

3.2.1. Urea-PAGE analysis

It is known that only four genotypes of caseins exist, so the heterogeneity apparent in electrophoresis is due to effects of post-translational processing, alternative splicing of gene product or genetic polymorphisms (Recio, Perez-Rodriguez, Amigo, & Ramos, 1997). Recall that the American Dairy Science Association Committee on Nomenclature and Classification, back in 1984, proposed that the nomenclature developed for bovine caseins, viz. α_{s1}-, α_{s2}-, β- and κ-caseins, be adopted for milk proteins of other species (Ginger & Grigor, 1999).

A typical urea-PAGE electrophoregram of caprine caseinate, and its fractional degradation by EE is depicted in Fig. 1A. The group of bands with the lowest electrophoretic mobility therein is accounted for by β-casein. Conversely, the group of bands with the highest electrophoretic mobility is associated with α_s-casein (Silva & Malcata, 2000).

Sodium caseinate was hydrolyzed by EE to yield band (a) – which exhibits an intermediate mobility between β- and α_{s2}-casein, and which can be seen after 1 h of digestion and becomes more intense as reaction time elapsed (lanes

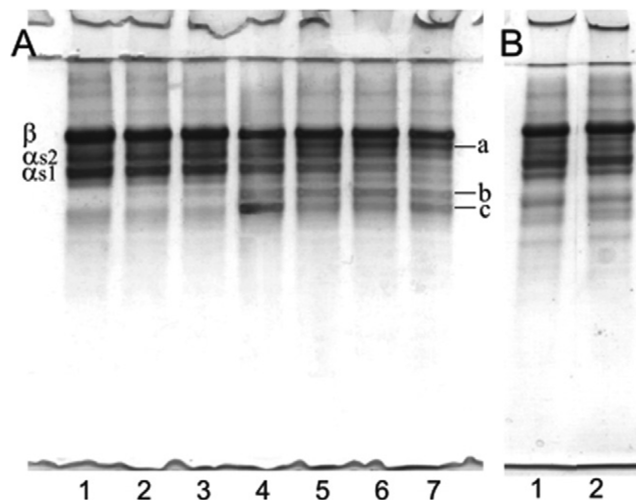

Fig. 1. Urea-PAGE electrophoretogram (resolution gel of $T = 12.5\%$ and $C = 4\%$, pH 8.9, overlaid by stacking gel of $T = 4\%$, $C = 3\%$, pH 7.6). A – degradation patterns of goat's caseins by EE; lanes 1–7: caseins after incubation for 0, 30 min, 1 h, 3 h, 6 h, 12 h, and 24 h, respectively. B – degradation patterns of caprine caseinate by commercial bovine curdle; lanes 1–2: caseins after incubation for 0 min and 24 h.

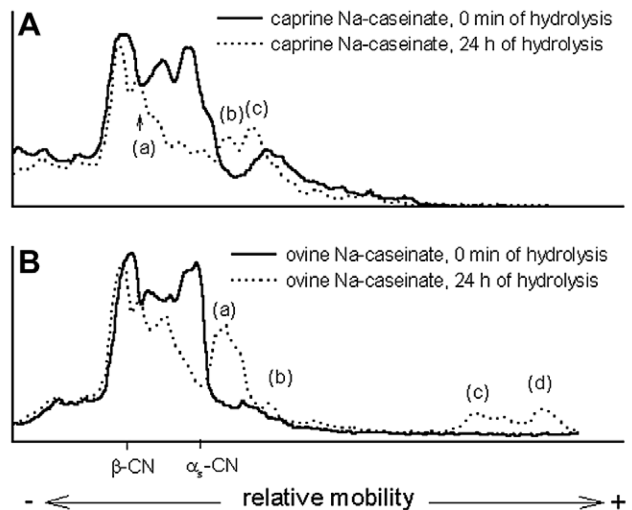

Fig. 2. Densitogram analysis of bands obtained by urea-PAGE of (A) caprine Na-caseinate and of (B) ovine Na-caseinate.

3–7 in Fig. 1A). A pair of bands of higher electrophoretic mobility – denoted as bands (b) and (c), in lanes 2–7 of Fig. 1A, were also produced; band (b) was first visible by 30 min, became much more intense until 3 h, and remained as such until 24 h of reaction. On the other hand, band (c) looked very intense by 3 h of hydrolysis, but vanished as time elapsed. The densitograms corresponding to the bands obtained by urea-PAGE are shown in Fig. 2A. Caprine β-casein was degraded up to 40% (Fig. 2A), whereas caprine α_{s1}-casein was hydrolyzed up to 68%, by 24 h of incubation. These results do not agree with those by Sousa and Malcata (1998) for caprine Na-caseinate hydrolyzed at pH 6.5 by enzymatic extracts of flowers of *C. cardunculus* (*Asteraceae*), and those by Silva and Malcata (2000) pertaining to degradation of caprine Na-caseinate at pH 6.8 with purified cardosins A and B. The breakdown patterns of bovine Na-caseinate by EE of *S. marianum* (Vairo Cavalli et al., 2005) showed a greater hydrolysis degree of β-casein, as well as very different profile relative to obtained with caprine Na-caseinate.

The kinetics of hydrolysis, measured as the decrease in area of densitogram peaks are apparent in Fig. 3. The rate of breakdown was similar for both caseinates during the first 3 h of reaction. These results are consistent with those reported by Sousa and Malcata (1998), who promoted reaction catalyzed by aqueous extracts of dry flowers of *C. cardunculus*, at pH 5.9. However, as hydrolysis proceeded, α_{s1}-casein was hydrolyzed to a greater extent than β-casein.

Hydrolysis of goat's caseinate was also carried out separately with commercial bovine curdle and with EE of flowers of *S. marianum* under identical reaction conditions. The results produced by urea-PAGE are also shown in Fig. 1A and B. It can be observed that the commercial

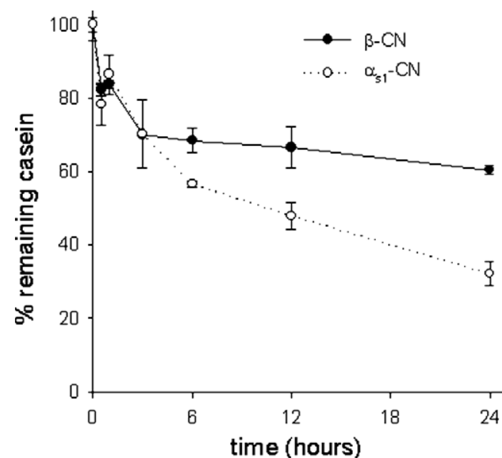

Fig. 3. Kinetics of hydrolysis of caprine α_{s1}- and β-caseins.

bovine curdle did not produce any observable effect on the two more important fractions of casein, but it partially hydrolyzed the proteins/peptides associated with bands with mobility between those of α_{s1}- and β-caseins – which corresponds to α_{s2}-casein. On the other hand – and as already observed in Fig. 1A, EE produced extensive degradation of α_s-caseins but only a slighter degradation of β-casein.

3.2.2. Tricine SDS-PAGE analysis

The results obtained by tricine SDS-PAGE of hydrolyzed goat's caseinate is shown in Fig. 4. Only one important and defined band (a), characterized by 15.0 kDa, appeared by 12 h of digestion – and became more intense by 24 h. The N-terminal sequence of the peptide with the molecular weight of 14.4 kDa was REQEELNV, which corresponds to the N-terminus of β-casein. Studies including casein breakdown in caprine cheeses by animal rennet have also reported the presence of bands accounted for by β-casein fragments (Silva & Malcata, 2000).

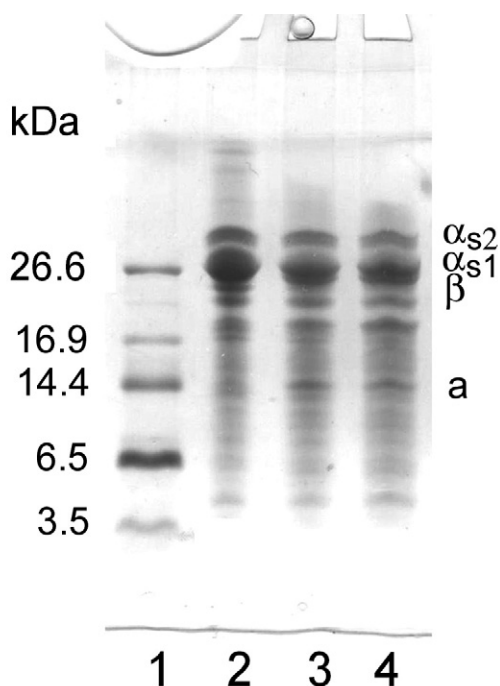

Fig. 4. Tricine SDS-PAGE electrophoretogram (resolution gel of $T = 14.5\%$, $C = 3\%$, overlaid by separating gel of $T = 10\%$, $C = 3\%$, and then by stacking gel of $T = 4\%$, $C = 3\%$). Degradation patterns of caprine caseins by EE; lane 1: polypeptide molecular weight markers (Bio-Rad): bacitracin (1.4 kDa), insulin b chain, oxidized (3.5 kDa), aprotinin (6.5 kDa), α-lactalbumin (14.4 kDa), myoglobin (17.0 kDa) and triose-phosphate isomerase (26.6 kDa); lane 2: intact casein; lanes 3–5: whole casein after 6 h, 12 h and 24 h of digestion, respectively. Proteins were stained with Coomasie Blue G.

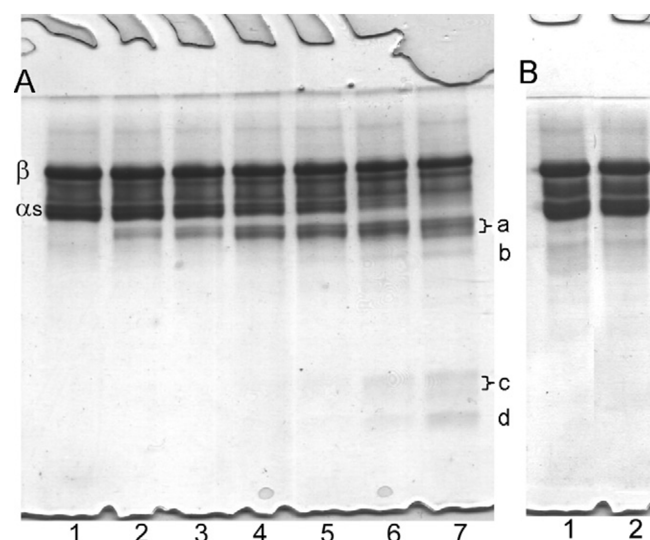

Fig. 5. Urea-PAGE electrophoretogram (resolution gel was $T = 12.5\%$ and $C = 4\%$, pH 8.9, overlaid by stacking gel of $T = 4\%$, $C = 3\%$, pH 7.6). A – degradation patterns of ewe's caseins by EE; lanes 1–7: caseins after incubation for 0, 30 min, 1 h, 3 h, 6 h, 12 h and 24 h, respectively. B – degradation patterns of ovine caseinate by commercial bovine curdle; lanes 1–2: caseins after incubation for 0 min and 24 h.

3.3. Ovine caseinate

3.3.1. Urea-PAGE analysis

The results of hydrolysis of ovine caseinate analysed by urea-PAGE are depicted in Fig. 5A. Two main groups of caseins can be identified – regions α_s- and β-caseins; this observation is consistent with those previously reported by Richardson and Creamer (1976). The group with greater mobility corresponds to α_s-caseins, and may include several variants; such microheterogeneity comes, as previously discussed, from the degree of glycosylation and/or the degree of phosphorylation, in addition to actual genetic polymorphism (Chianese et al., 1996). The group with lower mobility corresponds to the region of β-casein – formed by two variants, β_1 and β_2, with differences in phosphorylation level (Sousa & Malcata, 1998).

Although breakdown of ovine caseinate took place much slowlier than that of bovine counterpart (Vairo Cavalli et al., 2005), a region with bands of greater mobility than α_s-casein is visible as early as by 30 min (a). On the other hand, other bands appeared after 6 h of incubation and became thicker towards 24 h. In the urea-PAGE densitogram (Fig. 2B), peaks (a–d) can be seen by 24 h of hydrolysis. A noticeable decrease of α_s-casein from 0 to 24 h of degradation can also be observed. After 24 h of incubation, α_s-casein was degraded by EE to $76 \pm 4\%$, whereas β-casein was only degraded to $19 \pm 4\%$. Electrophoresis analysis of water insoluble fractions from La Serena cheese – semi-hard Spanish ewe's milk cheese manufactured with extracts of *C. cardunculus*, showed that α_s-casein was less susceptible to proteolysis than β-casein (Roa, López, & Mendiola, 1999). After ripening Serra da Estrela cheeses produced in Portugal from ovine milk curdled with extracts of flowers of *C. cardunculus*; were characterized by extensive hydrolysis of β- and α_s-caseins, and essentially similar viz. 75% and 82%, respectively (Macedo & Malcata, 1997). Silva and Malcata (1999) reported that cardosin B degraded both ovine caseins, but not to the same extent; however, when whole ovine caseinate was degraded by cardosin B, α_s-casein was more susceptible to proteolysis than β-casein, whereas the opposite behavior was observed when the isolated fractions were exposed to hydrolysis. In cheese-like systems, none of the highly susceptible peptide bonds of α_{s1}-casein were found to be cleaved during the initial 24 h of ripening – neither by crude aqueous extracts of *C. cardunculus* or by purified cardosin A (Silva & Malcata, 2005). Irigoyen et al. (2000) found that α_s-casein are more acutely hydrolyzed than β-caseins throughout ripening of ovine cheeses, that had been curdled with lamb artisan rennet, calf industrial rennet or a mixture of both. The clotting activity of the rennet used is one of the factors that has a major influence upon the degradation extent of caseins; similarly, the origin of the clotting enzyme used (from animal, microbial or plant sources) will constrain the proteolysis degree; plant and microbial clotting enzymes breakdown β-caseins faster than animal ones (Irigoyen et al., 2000).

In attempts to compare degradation patterns, ovine caseinate was hydrolyzed with commercial bovine rennet and EE, under identical conditions of reaction; the resulting electrophoregram is shown in Fig. 5B. By 24 h of hydrolysis, a region (a) and a band (b) close to the α_s-caseins can be visualized, and another region of dimmed band (c) and a band (d) but with much greater mobility can also be shown (Fig 5A). Electrophoresis of hydrolyzed caseinate (lane 2 in Fig. 5B), carried out for 24 h with commercial bovine rennet, displayed little difference with respect to the control (lane 1 in Fig. 5B); only a low intensity new band (e), of smaller mobility than β-casein, a band (f) and a region of bands (g) with greater mobility – yet close to fraction α_s-caseins can be observed.

The kinetic of hydrolysis of ovine α_s- and β-caseins carried out by the partially purified extract of flowers of *S. marianum*, are displayed in Fig. 6 As discussed above, α_s-casein was faster and more extensively degraded than β-casein – unlike the data reported by Sousa and Malcata (1998), who found similar rates of hydrolysis (until 3 h of reaction) for both ovine fractions when the reaction was effected by aqueous extracts (pH 5.9) of dry flowers of *C. carduncul**us*.

3.3.2. Tricine SDS-PAGE analysis

The results of tricine SDS-PAGE of hydrolyzed ewe's caseinate is shown in Fig. 7. After 12 h, bands characterized by molecular weights of 18.9, 16.5, 11.2 and 8.6 kDa became more intense, while smaller peptide bands (5.8 and 4.3 kDa) appeared.

In what concerns specificity, the major cleavage site in α_{s1}-casein was Leu99-Arg100 – as derived from N-terminal sequencing of the band featuring 14.4 kDa [i.e. α_{s1}-(f100-*)]. According to Sousa and Malcata (1998) and Silva and Malcata (2005), this peptide bound was not reported to be cleaved by aspartic peptidases of *C. cardunculus*; however, Lane, Fox, Johnston, and McSweeney (1997) found the peptide [fl02-199] from bovine α_{s1}-casein in the electrophoregrams of cheeses curdled with chymosin, as a band that increased in intensity throughout ripening.

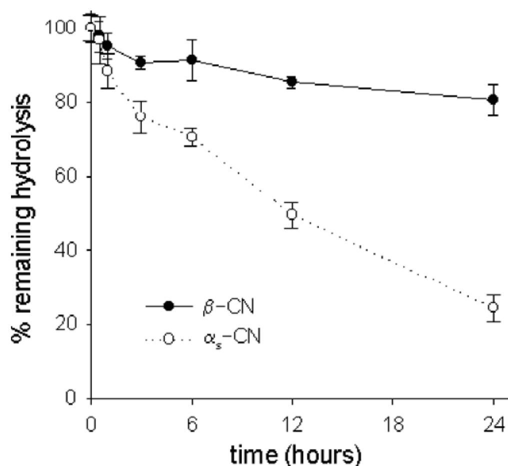

Fig. 6. Kinetics of hydrolysis of ovine α_s- and β-casein.

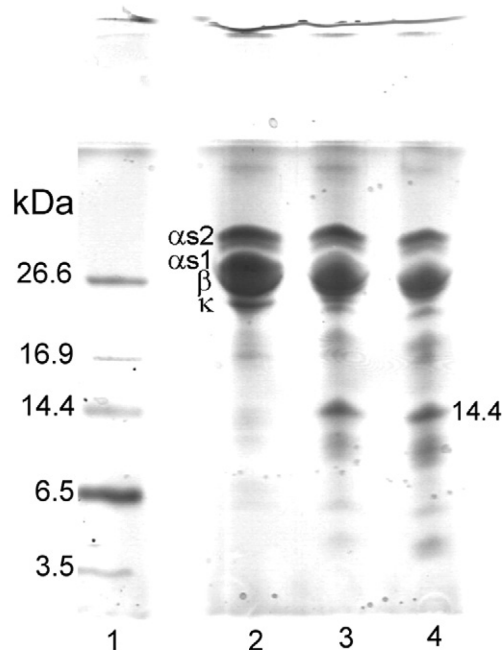

Fig. 7. Tricine SDS-PAGE electrophoretogram (resolution gel of $T = 14.5\%$, $C = 3\%$, overlaid by separating gel of $T = 10\%$, $C = 3\%$, and then by stacking gel of $T = 4\%$, $C = 3\%$). Degradation patterns of ovine caseins; lane 1: polypeptide molecular weight markers (Bio-Rad): bacitracin (1.4 kDa), insulin b chain, oxidized (3.5 kDa), aprotinin (6.5 kDa), α-lactalbumin (14.4 kDa), myoglobin (17.0 kDa) and triose-phosphate isomerase (26.6 kDa); lane 2: intact casein; lanes 3–5: whole casein after 6 h, 12 h and 24 h of digestion, respectively. Proteins were stained with Coomasie Blue G.

4. Conclusions

Distinct peptide profile were observed in goat's and ewe's caseinates throughout hydrolysis brought about by extracts of *S. marianum*; however, lot caseinates underwent less extensive degradation than bovine caseins, for the same enzyme extract. On the other hand, caprine and ovine Na-caseinates were hydrolyzed more extensively with the plant extract than the same caseinates by calf commercial rennet, under the same conditions.

Caprine α_{s1}- and β-caseins were degraded up to 68%, and 40%, respectively during 24 h of incubation. Only one important and well-defined band – characterized by a molecular weight of 14.4 kDa, was observed by 12 h of hydrolysis, which is likely a fragment of goat's β-casein. By 24 h of incubation, ovine α_s- and β-caseins were degraded up to 76% and 19%, respectively.

In terms of specificity, the major cleavage site of ovine caseinate was Leu99-Arg100 of α_{s1}-casein.

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