Degradation of Caseins from Milk of Different Species by Extracts of *Centaurea calcitrapa*

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Two purified aqueous extracts of *Centaurea calcitrapa*, a plant from the Compositae family, were shown to degrade bovine, ovine, and caprine milk caseinates. The corresponding hydrolysis patterns were monitored by urea−polyacrylamide gel electrophoresis and compared with those of a commercial mixture of chymosin and pepsin. The plant proteases degraded both α_s- and β-caseins more extensively than the commercial rennet, thus yielding proteolytic patterns where different specificities toward such substrates are apparent. The animal rennet was found to display the greatest clotting power per milligram of protein but the least specificity toward ovine and caprine caseins. It is, therefore, suggested that the said plant extracts be used as an alternative to commercial animal rennets especially in the manufacture of caprine and ovine milk cheeses.

Keywords: *Centaurea calcitrapa; plant rennet; proteolysis*

INTRODUCTION

The high price of traditional rennets and ethical considerations associated with their use have prompted research toward alternative milk coagulants produced either from plants or from native or genetically modified microorganisms. In the Iberian Peninsula, aqueous crude extracts from the flowers of the thistle (*Cynara cardunculus*, *Cynara scolymus*, or *Cynara humilis*) have been traditionally used for the manufacture of raw ovine and/or caprine milk cheeses (Sá and Barbosa, 1972). Alternative rennets of plant origin that have been the focus of previous studies include extracts of the following: (1) crude papaya (Cabezas *et al.*, 1981); (2) pineapple (Cattaneo *et al.*, 1994); (3) *Dieffenbachia maculata* (Padmanabhan *et al.*, 1993); (4) sodom apple (*Calotropis procera*) (Ibiama *et al.*, 1987); and (5) ash gourd (*Benincasa cerifera*) (Gupta and Eskin, 1977). However, unlike proteases from *Cynara* spp., none of the aforementioned plant proteases has been successfully used in cheesemaking due either to an excessively low ratio of caseinolytic to proteolytic activity, resulting in bitter peptides in ripened cheese when starter bacteria are absent, or to an excessively low clotting power, which gives rise to low cheese yields. The aqueous extracts of *Cynara* spp. were further shown to possess three active proteinases which have been isolated, purified, and partially characterized in terms of activity (Heimgartner *et al.*, 1990; Faro, 1991; Cordeiro *et al.*, 1992) and specificity toward pure bovine caseins (Macedo, 1993). More recently, two additional aspartic proteinases were isolated from fresh stigmas of *C. cardunculus* and were named cardosin A and cardosin B (Faro *et al.*, 1995; Veríssimo *et al.*, 1996); it was shown that one of them is similar to chymosin whereas the other resembles pepsin in terms of catalytic specificity (Veríssimo *et al.*, 1996; Ramalho-Santos *et al.*, 1996).

In principle, a rennet suitable for cheese manufacture is characterized by a highly specific caseinolytic activity (i.e., the enzyme should promptly break the Phe105−106 bond in κ-casein) and a small generalized proteolytic activity (i.e., it should have a relatively low affinity toward other bonds in κ-, α_s-, and β-caseins) (Pires *et al.*, 1994). High rates of nonspecific proteolysis, associated with rennets of bacterial and plant origin, give rise to potentially bitter peptides but favor soft-bodied cheeses and development of unique flavors in some of the more appreciated farm cheeses manufactured in the Mediterranean basin.

When a potential rennet substitute is studied, it is of chief importance to adequately evaluate the degradation patterns of the caseins because of their effects on yield, consistency, and flavor of the final cheese (Fox, 1989). One of the most accurate and expeditious methods to monitor proteolytic processes is urea−polyacrylamide gel electrophoresis (urea−PAGE) (Creamer, 1991), which is thus suitable to characterize the performance of rennets. The purpose of this work was to preliminarily assess the hydrolysis of bovine, ovine, and caprine milk caseinates by crude extracts of *Centaurea calcitrapa* (a member of the Compositae family as is *Cynara* spp.) and to qualitatively and quantitatively compare degradation patterns obtained with the extracts thereof at various incubation times with those of a commercial rennet consisting of chymosin and pepsin.

MATERIALS AND METHODS

Preparation of Rennets. Two crude enzyme fractions, hereafter denoted as *C. calcitrapa* extracts A and B, were obtained from fresh flowers of *C. calcitrapa* and further purified by chromatographic methods as described by Domingos *et al.* (1996); such fractions corresponded to two perfectly resolved peaks in gel permeation chromatography. A com-

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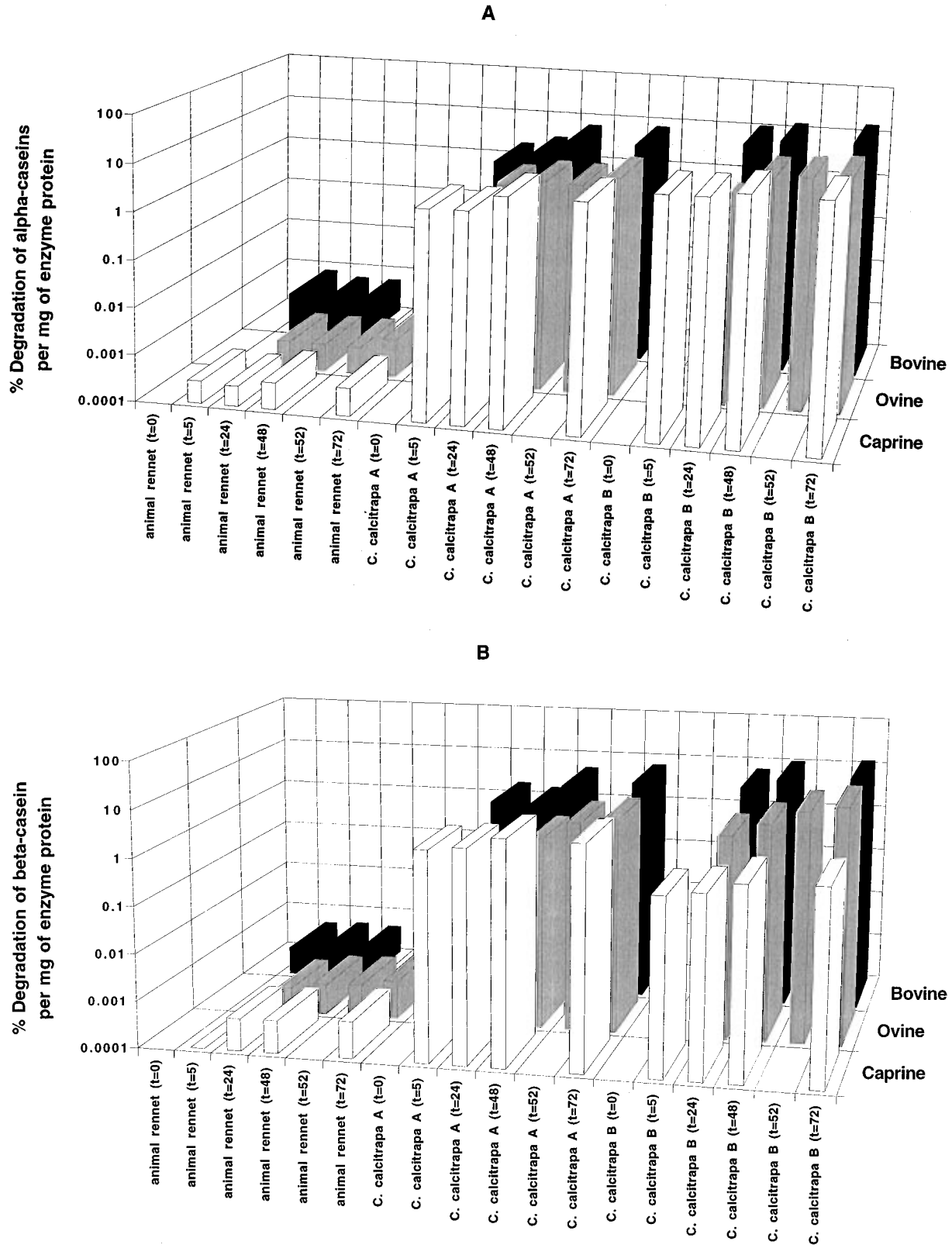

Figure 1. Percentage of hydrolysis of α_s-casein (A) and β-casein (B) in sodium caseinates from bovine, ovine, and caprine origin by commercial animal rennet, *C. calcitrapa* extract A, and *C. calcitrapa* extract B. Values are averages obtained from densitograms of three independent runs of urea−PAGE; the standard error of the mean was 3.56 for α_s-casein and 2.65 for β-casein.

mercial animal rennet (Stabo 230, Chris. Hansen's, Denmark), made up of 25% chymosin and 75% bovine pepsin, was used as reference.

Clotting Activity. Low-heat skim milk powder, Nilac (Nizo, Ede, The Netherlands), was reconstituted by dissolving 12 g in 100 mL (final volume) of 10 mM $CaCl_2$ (pH 6.5) at 30 °C. Enzyme extracts were added at a rate of 100 mL/2 mL of reconstituted skim milk and their specific activity expressed as clotting unit (CU) per milliliter of extract preparation; one CU was defined as the amount of enzyme needed to coagulate 10 mL of reconstituted skim milk in 100 s at 30 °C.

Preparation of Caseinates. Whole bovine, ovine, and caprine caseins were obtained as sodium caseinates by isoelectric precipitation from the corresponding milks following acidification at pH 4.2 and 37 °C. After precipitation, the samples were centrifuged at 6000*g* for 10 min and the

Table 1. Kinetic Parameters Describing the Degradation of α_s- and β-Casein in Bovine, Ovine, and Caprine Sodium Caseinates

	bovine		ovine		caprine	
	α_s-CN	β-CN	α_s-CN	β-CN	α_s-CN	β-CN
			C. calcitrapa Extract A			
$v_{max,s}$ (g L^{-1} h^{-1})	7.44×10^{-4}	2.90×10^{-4}	0.47×10	3.27×10^{2}	5.98×10^{7}	1.42×10^{5}
$K_{m,s}$ (g/L)	2.24×10^{-1}	1.00×10^{-4}	6.93×10^{3}	6.81×10^{5}	5.92×10^{10}	1.62×10^{8}
$v_{max,s}/K_{m,s}$ (h^{-1})	3.32×10^{-3}	0.29×10	6.73×10^{-4}	4.80×10^{-4}	1.01×10^{-3}	8.75×10^{-4}
			C. calcitrapa Extract B			
$v_{max,s}$ (g L^{-1} h^{-1})	8.24×10^{4}	7.72×10^{-3}	2.21×10^{2}	6.41×10^{-4}	5.58×10^{6}	6.58×10^{4}
$K_{m,s}$ (g/L)	3.16×10^{7}	1.49×10^{-2}	1.45×10^{5}	1.00×10^{-5}	2.12×10^{9}	3.34×10^{8}
$v_{max,s}/K_{m,s}$ (h^{-1})	2.61×10^{-3}	5.17×10^{-1}	1.52×10^{-3}	6.41×10	2.64×10^{-3}	1.97×10^{-4}
			Commercial Rennet			
$v_{max,s}$ (g L^{-1} h^{-1})	3.32×10^{-4}	1.00×10^{-7}	1.57×10^{-3}	2.00×10	5.30×10^{-5}	2.00×10^{-7}
$K_{m,s}$ (g/L)	3.00×10^{3}	1.80×10^{7}	1.00×10^{-7}	1.84×10^{8}	8.00×10^{2}	1.28×10^{9}
$v_{max,s}/K_{m,s}$ (h^{-1})	1.10×10^{-7}	5.55×10^{-15}	1.57×10^{4}	1.09×10^{-7}	6.63×10^{-8}	1.56×10^{-16}

precipitate was recovered by filtration and washed several times in distilled water. The caseinates were then resuspended to a common volume in distilled water, and the pH was adjusted to 7.0 with 10 M NaOH. The solutions were held overnight at 4 °C and then lyophilized and stored at −30 °C until use.

Proteolytic Activity. Ten grams of each type of sodium caseinate was dissolved in 1000 mL (final volume) of 100 mM phosphate buffer (pH 6.5) and allowed to dissolve completely at 30 °C. Sodium azide (0.1%) was added to inhibit microbial growth. Hydrolysis of caseins was initiated by addition of 525 mL of each enzyme extract (which corresponded to 80.8 CU of the commercial animal rennet, 8.0×10^{-6} CU of extract A, and 3.2×10^{-5} CU of extract B) to 5 mL of the caseinate solution. Samples of the hydrolysates were taken at 0, 5, 24, 48, 52, and 72 h and the hydrolysis reaction therein was quenched in double concentrated sample buffer 50% (v/v) (McSweeney *et al.*, 1993) prior to electrophoretic analysis. Controls containing sodium caseinate and sodium azide at the same concentrations but without enzyme added were also sampled at several times and analyzed to double check the absence of microbial proteolytic action.

Electrophoresis. Urea−PAGE (12.5% T, 4% C) was performed in triplicate using a Protean II XI vertical slab-gel unit (Bio-Rad Laboratories, Watford, U.K.) according to the method of Andrews (1983) with the modifications of Shalabi and Fox (1987). The gels were stained with Coomassie Blue G-250 (Bio-Rad, Richmond, CA) using the method of Blakesley and Boezi (1977). Quantification of stained bands accounted for by intact portions of α_s- and β-caseins was done by densitometry using a Model CD60 densitometer (Desaga, Heidelberg, Germany); the density of the corresponding band at 0 h of hydrolysis was employed as reference.

Intrinsic Kinetic Constants. Modeling of the experimental data pertaining to proteolytic breakdown of the caseins was based on the assumption of Michaelis−Menten kinetics for the enzyme-mediated degradation of substrates in a batch, well-stirred system, viz.

$$-\frac{dC_s}{dt} = \frac{v_{max,s}C_s}{K_{m,s} + C_s} \quad (1)$$

where C_s denotes concentration (g/L) of substrate s (where subscript s denotes α_s-CN or β-CN), t denotes hydrolysis time (h), $v_{max,s}$ denotes the reaction rate (g/(L·h)) under saturation of enzyme by substrate s, and $K_{m,s}$ denotes the corresponding Michaelis−Menten constant (g/L). Nonlinear fit of the integrated form of the model conveyed by eq 1 to the experimental data allowed estimation of kinetic parameters; $v_{max,s}$ was also normalized by the clotting activity of the enzyme in question (which is virtually equivalent to $v_{max,\kappa-CN}$, i.e., v_{max} for the clotting activity upon κ-casein).

RESULTS AND DISCUSSION

The specific clotting activity of each rennet, which is a measure of its strength as coagulant, was 0.63 CU/mg of protein for the commercial rennet, 0.00077 CU/mg of protein for *C. calcitrapa* extract A, and 0.0050 CU/mg of protein for *C. calcitrapa* extract B.

Data on the extents of degradation of α_s-CN and β-CN from bovine, ovine, and caprine origin by the three types of rennets tested are depicted in Figure 1. A split ANOVA revealed that the commercial rennet and *C. calcitrapa* extract A did not show any significant differences between each other when acting upon the substrates (in terms of type and source); however, *C. calcitrapa* extract B acted in a significantly different way ($p < 0.05$) upon caprine and ovine β-caseins, on the one hand, and upon caprine and bovine β-caseins, on the other. From the full ANOVA, it was concluded that both the source of proteolytic enzymes and the nature of the substrate yielded significant effects in the percent degradation of all β-caseins but only the enzyme used had a significant effect upon the percent degradation of α_s-caseins.

The estimates of kinetic parameters are tabulated in Table 1. As can be seen, extract B contained the enzyme with the greatest K_m toward bovine and ovine α_s-caseins. Since lower K_m values reflect stronger binding within the ES complex, the higher K_m of extract B enzymes for some caseins implies weaker binding within the ES complex. In constrast, enzymes in *C. calcitrapa* extract B had a lower K_m (stronger affinity) for ovine β-caseins. The commercial rennet binds tightly to both ovine and caprine α_s-caseins, as indicated by the relatively low K_m values, but shows rather weak affinity toward all three β-caseins. When comparing the *C. calcitrapa* extracts with one another, it is noticeable that extract A had a lower K_m toward bovine β- and α_s-caseins than did extract B, thus reflecting a stronger affinity toward these caseins. Comparing all three enzyme extracts when the enzyme is well apart from saturation with substrate, it was found that the commercial animal rennet exhibited the highest proteolytic activity toward ovine α_s-casein (as reflected by the higher coefficient v_{max}/K_m in Table 1) and the lowest proteolytic activity toward all other types and sources of caseins.

With respect to *C. calcitrapa,* extract A degraded all substrates slower and less extensively than extract B, except for caprine β-casein, which could be partially due to the strong binding affinity of extract B toward this substrate (see Table 1). In addition, α_s-caseins were degraded to a higher extent than β-caseins, an observation that is in agreement with the literature because β-casein has been claimed to be more resistant to proteolysis than α_s-caseins in cheese manufactured with raw ovine milk and extracts from flowers of *C. cardun-*

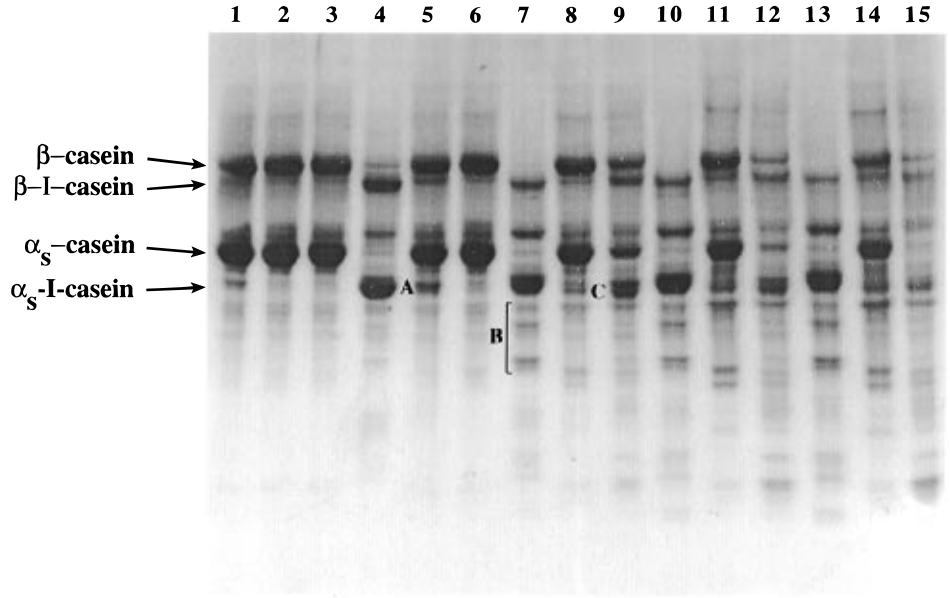

Figure 2. Electrophoretogram of bovine sodium caseinate hydrolyzed by commercial rennet (lanes 1, 4, 7, 10, and 13), *C. calcitrapa* extract A (lanes 2, 5, 8, 11, and 14), and *C. calcitrapa* extract B (lanes 3, 6, 9, 12, and 15) for 0, 5, 24, 48, and 72 h, respectively.

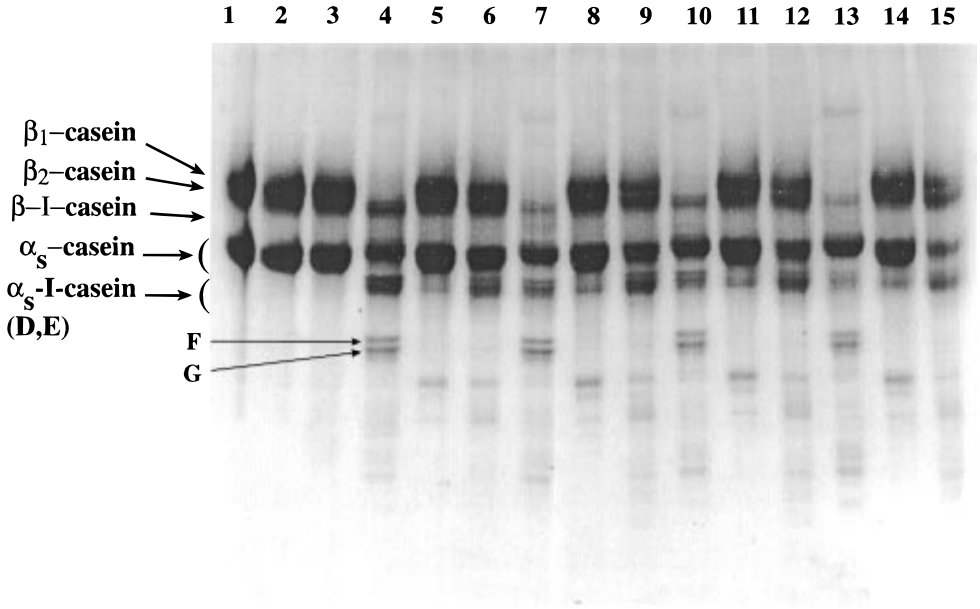

Figure 3. Electrophoretogram of ovine sodium caseinate hydrolyzed by commercial rennet (lanes 1, 4, 7, 10, and 13), *C. calcitrapa* extract A (lanes 2, 5, 8, 11, and 14), and *C. calcitrapa* extract B (lanes 3, 6, 9, 12, and 15) for 0, 24, 48, 52, and 72 h, respectively.

culus (Mora and Marcos, 1981; Sousa and Malcata, 1997) and in cheese manufactured with caprine milk and calf rennet (Carretero *et al.*, 1994). Furthermore, although major differences do not exist among the three types of milk with respect to the amino acid sequence of α_s-casein, and between bovine and ovine milks with respect to β-casein (Trujillo *et al.*, 1995), a significant difference in caprine β-casein when compared to its bovine and ovine counterparts, owing to deletion of the dipeptide Pro179−Tyr180, has been reported by Trujillo *et al.* (1995); such difference may account for the specificity and concomitant binding affinity of extract B toward this casein. After 24 h of hydrolysis, *C. calcitrapa* extract A degraded bovine and ovine β-caseins to half the extent of α_s-caseins, but the opposite was observed for caprine caseinate (Figure 1); for the same hydrolysis time, extract B degraded bovine and caprine α_s-caseins more than β-caseins, but the opposite was observed for ovine caseinate. By 72 h of hydrolysis, *C. calcitrapa* extract A degraded α_s- and β- bovine caseins to almost the same extent, whereas α_s-casein was degraded to a higher extent in the other two caseinates. For the same hydrolysis time, extract B degraded bovine and caprine α_s-caseins further than β-caseins, but the opposite was observed with ovine caseinate. Structural differences between micelles in the various milk types can explain the aforementioned results; in fact, ovine and caprine milks have higher amounts of fat and form smaller micelles than bovine milk, thus causing β-casein, the most hydrophobic fraction, to be more superficially located, presumably around the fat globules (Morgado, 1990). In addition, studies by Richardson and Creamer (1974), latter complemented with comparative studies on micelle structure and characterization of major ovine caseins (Richardson and Creamer, 1976), have shown that caprine and bovine caseins have a single major α_s-casein whereas ovine casein has two and, sometimes, three major α_s-caseins with a composition more similar to that of bovine α_{s1}-casein than to caprine's.

Typical electrophoretograms of bovine, ovine, and

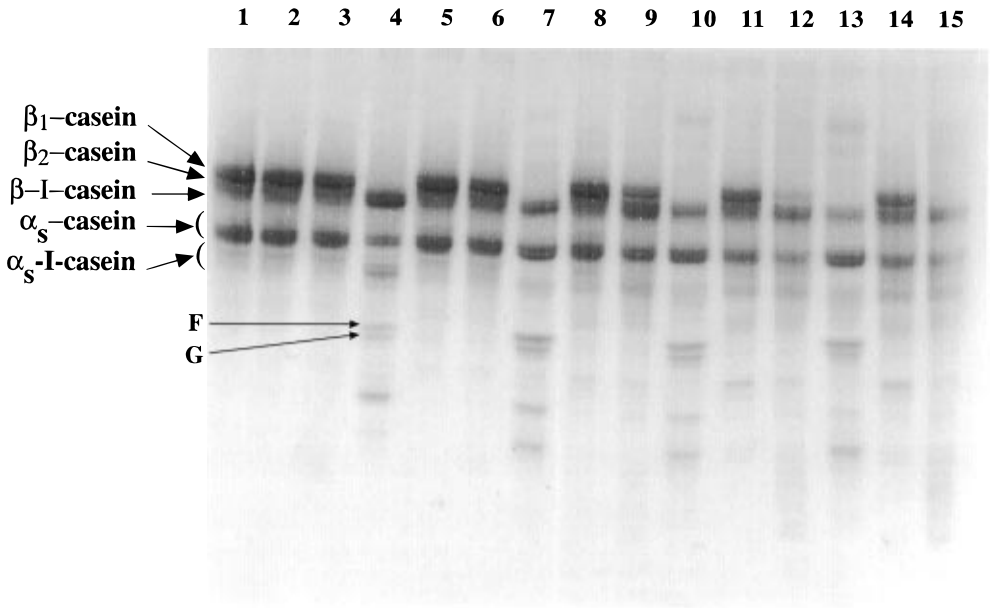

Figure 4. Electrophoretogram of caprine milk caseinate hydrolyzed by commercial rennet (lanes 1, 4, 7, 10, and 13), *C. calcitrapa* extract A (lanes 2, 5, 8, 11, and 14), and *C. calcitrapa* extract B (lanes 3, 6, 9, 12, and 15) for 0, 5, 24, 48, and 72 h, respectively.

caprine caseinates after hydrolysis for various times using the three types of enzymes are displayed as Figures 2−4. From observation of these figures, it can be concluded that β-casein was almost completely degraded by 24 h by the commercial rennet irrespective of the milk source, giving rise to a major peptide, identified as β-I-casein (Gripon *et al.*, 1975; McSweeney *et al.*, 1994), via fragmentation of the Leu192−Tyr193 and Ala189-Phe190 bonds (Sousa, 1993; Macedo, 1993). Bovine β-casein experienced limited degradation by *C. calcitrapa* extract A after 24 h of hydrolysis, but a greater degree of degradation was exhibited when extract B was used. α_{s1}-Casein was rapidly and extensively degraded by the commercial rennet in bovine milk by 5 h of hydrolysis, with concomitant appearance and accumulation of α_{s1}-I-casein, i.e., the f24−199 of α_{s1}-casein (Visser, 1981; McSweeney *et al.*, 1994). *C. calcitrapa* extract A degraded bovine α_{s1}-casein to a very limited extent with subsequent appearance of a band of greater mobility but low intensity (A in Figure 2), which appeared at a later time in the case of *C. calcitrapa* extract B. Four low-intensity bands (B in Figure 2) with greater mobility than α_{s1}-I-casein were produced by action of the animal rennet. Of these bands, the one with mobility greater than that of α_{s1}-I also appeared in the electrophoretograms of *C. calcitrapa* extracts A and B, but the other three bands could not be visualized (Figures 3 and 4). By 72 h, the patterns of degradation of β-casein brought about by all three enzyme preparations were similar; however, α_{s1}-casein was more extensively degraded by *C. calcitrapa* extract B, thus producing more intense bands (C in Figure 2) with relative mobilities similar to those of α_{s1}-I-casein. In ovine caseinate, the α_s-caseins showed limited degradation by either the commercial rennet or the *C. calcitrapa* extracts, with appearance of two bands of low intensity with mobilities similar to those of α_{s1}-I-casein (D and E in Figure 3). Ovine α_s-casein, subject to hydrolysis by chymosin at pH 6.5, was degraded to a set of bands of higher electrophoretic mobility, which may correspond to bovine α_{s1}-I-casein (Whyte, 1995). The bond most susceptible to chymosin attack in the ovine α_s-casein region is Phe23−Val24, and therefore, the electrophoretic band designated as α_{s1}-I-casein is probably the peptide Val24−Trp199 (Sousa and Malcata, 1997). The same two bands with greater electrophoretic mobility than α_{s1}-I-casein (F and G, in Figure 3) were produced by the commercial rennet but not by either of the *C. calcitrapa* extracts. However, extra bands with higher mobilities were produced by the two *Centaurea* extracts, which tended to prevail up to 72 h. In caprine caseinate, the α_s-caseins were degraded only by the commercial rennet with concomitant appearance of bands F and G (Figure 4) and two other bands with higher mobility.

CONCLUSIONS

C. calcitrapa extract B has the highest generalized proteolytic activity per milligram of protein when compared with extract A and the animal rennet. It binds strongly to ovine β-casein although it preferentially degrades α_s-casein. On the other hand, *C. calcitrapa* extract A mimicks the commercial rennet in terms of greater affinity toward bovine caseins, displaying similar binding and catalytic properties but lower clotting power. Although *C. calcitrapa* extracts A and B show higher proteolytic activity (as reflected by the percent degradation) than the commercial rennet, they exhibit higher specificity toward ovine and caprine caseinates and so it is suggested that they be used as alternative rennet to produce high-quality cheeses from caprine and/or ovine milk where a relatively high proteolysis rate is required in the initial stages of the ripening period.

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