

Bioactive Peptides in Ovine and Caprine Cheeselike Systems Prepared with Proteases from *Cynara cardunculus*

S. V. Silva,* A. Pihlanto,† and F. X. Malcata*¹

*Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, P-4200-072 Porto, Portugal

†MTT Agrifood Research Finland, Food Research, FIN-31600 Jokioinen, Finland

ABSTRACT

The potential angiotensin-converting enzyme (ACE)–inhibitory and antioxidant activities of peptides in water-soluble extracts, obtained from raw and sterilized ovine and caprine cheeselike systems coagulated with enzymes from the plant *Cynara cardunculus*, were assessed. Prior to the assay, the 3,000-Da permeate from 45-d-old cheeselike systems was fractionated by tandem chromatographic techniques. Several peaks were obtained in each chromatogram, but only some were associated with ACE-inhibitory or antioxidant activity or both. Peptides Tyr-Gln-Glu-Pro, Val-Pro-Lys-Val-Lys, and Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-* from β -casein, as well as Arg-Pro-Lys and Arg-Pro-Lys-His-Pro-Ile-Lys-His-* from α_{s1} -casein exhibited ACE-inhibitory activity. Peptides released upon cleavage of the peptide bond Leu190-Tyr191 (either in ovine or caprine β -casein), and corresponding to the β -casein sequence Tyr-Gln-Glu-Pro-*, possessed antioxidant activity.

Key words: plant protease, ovine cheese, caprine cheese, angiotensin-converting enzyme inhibition

INTRODUCTION

Biologically active peptides are of particular interest in food science and nutrition, because they can play several physiological roles. Hidden (or at least inactive) within the AA sequence of dairy proteins, a class of such peptides can be released (or activated) in vivo during gastrointestinal digestion, or upstream during food processing via specific, enzyme-mediated proteolysis (Silva and Malcata, 2005a), and eventually be absorbed into the bloodstream.

Ripened cheese is one of the most important dairy products worldwide. It typically contains numerous peptides that originate mainly from CN breakdown during ripening. Such peptides contribute to the flavor, taste, and texture of the final product. Among the bioac-

tivities attributed to those peptides are angiotensin-converting enzyme (ACE)–inhibitory activity, opioid activity, and the ability to sequester calcium and other minerals; thus acting as biocarriers (Addeo et al., 1992; Meisel et al., 1997; Smacchi and Gobetti, 1998; Saito et al., 2000; Gómez-Ruiz et al., 2002; Sforza et al., 2003).

In the Iberian Peninsula, several raw ovine and caprine milk cheeses, which are highly appreciated for their unique organoleptic characteristics, have been manufactured on the farm level since ancient times, with extracts of the flowers of *Cynara cardunculus* as the coagulant. After flowers are collected from mature plants, they are dried in the open air in the shade before use, stored in a dry place, and sold at local markets. Their milk-clotting activity is caused by 2 aspartic proteases, cardosins A and B, which resemble chymosin and pepsin, respectively, in activity and specificity (Verissimo et al., 1995). Cardosin A is a proteolytic enzyme that is specific toward the Phe105-Met106 bond of κ -CN, whereas cardosin B is a nonspecific, highly proteolytic enzyme. No toxicity to humans has ever been found for either of those enzymes. To our knowledge, no data are currently available concerning bioactive peptides from ovine or caprine milk cheeses manufactured with plant enzymes; only the research efforts encompassing the total antioxidant content of breast milk (Vander-Jagt et al., 2001), the antioxidant activity of bovine milk following 2 different analytical methods (Chen et al., 2003), and evaluations of the antioxidant activity of CN and CN-derived peptides (Suetsuna et al., 2000; Rival et al., 2001).

In particular, ACE (EC 3.4.15.1) is important in blood pressure regulation, so its inhibition may actually prevent hypertension (Meisel, 1998). This dipeptidyl carboxypeptidase is a glycoprotein peptidyl dipeptide hydrolase, which is able to release histidyl-leucine from angiotensin I (a relatively inactive decapeptide) into angiotensin II, thus increasing the blood pressure and the aldosterone content, and inactivating the depressor action of bradykinin. Peptides bearing ACE-inhibitory activity have been found in several kinds of cheeses (Addeo et al., 1992; Meisel et al., 1997; Smacchi and Gobetti, 1998; Saito et al., 2000; Gómez-Ruiz et al., 2002, 2004; Sforza et al., 2003). Their C-terminal

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¹Corresponding author: fxmalcata@esb.ucp.pt

tripeptide sequence is the structural feature governing such inhibitory response (Maruyama et al., 1987). In addition, Meisel (1993) indicated that the ACE-binding pocket exhibits a preference for hydrophobic AA. To measure ACE activity and inhibition thereof in vitro, the method of Cushman and Cheung (1971) is often used. This particularly sensitive spectrophotometric assay determines the amount of hippuric acid formed from hippuryl-L-histidyl-L-leucine via the action of ACE. Recently, a (spectrophotometric) diagnostic assay designed to measure ACE activity was transformed into an enzyme inhibition assay and further optimized, which led to a more sensitive yet less expensive assay; it takes advantage of furanacryloyl-Phe-Gly-Gly as the substrate, and uses rabbit lung acetone extract as standard of activity (Vermeirssen et al., 2002).

On the other hand, antioxidant activity is accounted for by compounds able to protect biological systems against the potential harmful effects of processes or reactions that cause excessive degrees of oxidation, and that may thus be responsible for cellular damage. Several chemical and physicochemical methodologies have been proposed and used to monitor oxidation processes; some allow direct quantification of free radical production and its inhibition by antioxidants, whereas others indirectly assess the effectiveness of antioxidants in preventing oxidative damage (Antolovich et al., 2002). Accelerated stability tests, measurement of the ability to bring about diene conjugation, the thiobarbituric acid-reactive substance assay, measurement of hexanal and related end products, and quantification of free radicals are but a few examples. Among those of the latest type, the 2,2'-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid (**ABTS**) assay is one of the most effective, and hence is commonly used.

The aim of this work was thus to find and characterize peptides with potential ACE-inhibitory and antioxidant activities in raw and sterilized ovine and caprine cheeselike systems, manufactured with enzymes from *C. cardunculus* as the clotting agent.

MATERIALS AND METHODS

Enzyme Source

The plant proteases were obtained via maceration of 0.6 g of dry flowers of *C. cardunculus* in 10 mL of water. The solution was then kept refrigerated until use (no filtration was needed, as it remained limpid).

Cardosins in the aforementioned crude extract of *C. cardunculus* were isolated and purified, following the procedure described by Silva et al. (2003). The lyophilized enzymes were reconstituted in water, just prior to use, up to 0.0035 kg/L.

Cheese Making and Sampling

Ovine (or caprine) milk was collected from the sheep (or goat) flocks on the morning of cheese making. Each batch of milk was then divided in 2 equal portions: one portion was heated at 110°C for 10 min, whereas the other received no thermal treatment. Milk sterility (for the first batch) was checked as the absence of microorganisms on plate count agar incubated at 30°C for 48 h (Tavaria and Malcata, 1998). Cheeses were manufactured on the same day from raw ovine (or caprine) milk, and from sterilized ovine (or caprine) milk. Portions of 50 mL of each kind of milk (ovine or caprine) were placed in 100-mL sterilized flasks; then 0.200 mL of test enzyme solution (crude aqueous extract, or purified cardosin A or B) was added to the milk under sterile conditions and incubated at 28°C until coagulation occurred (approximately 45 min). The curd was then cut, stirred, and allowed to set to permit draining, again under sterile conditions. The whey produced was removed aseptically by opening the flasks every 15 min throughout a period of 1.5 h. The flasks were placed in a chamber maintained at 10°C. For further analyses, 45-d-old cheeses were selected at random.

Preparation of Water-Soluble Extracts

Water-soluble extracts (**WSE**) and water-insoluble extracts of cheeselike systems were prepared following the procedure of Kuchroo and Fox (1982). The cheese-like system was homogenized in a stomacher (Seward, London, UK) at 20°C for 10 min with twice its weight of water. The slurry was held at 40°C for 1 h, and then centrifuged at $3,000 \times g$, at 4°C for 30 min. The resulting supernatants were filtered through glass wool, freeze-dried, and kept at -20°C until use.

A 20-mg quantity of WSE was reconstituted in 600 μ L of water, and 500 μ L of this solution was then filtered through a Centriprep hydrophilic 3,000-Da cutoff membrane (Amicon, Beverly, MA) prior to analysis.

Purification of Peptides

The freeze-dried WSE samples (10 mg) were dissolved in 1 mL of 50% (vol/vol) trifluoroacetic acid (**TFA**) in water and filtered through a 0.45- μ m cellulose acetate filter. This solution was used to further fractionate the peptides present in the WSE. Aliquots (100 μ L) were applied to a reversed-phase column (SuperPak Pep-S, 4.0×250 mm, 5 μ m, after a Pep-S precolumn, 4.0×10 mm, 5 μ m, both from Pharmacia, Uppsala, Sweden). Elution was at 40°C via a mobile phase of 2 solvents—solvent A: 0.05% (vol/vol) TFA in water; and solvent B: 0.05% (vol/vol) TFA in acetonitrile:water (9:1)—at a flow rate of 1.0 mL/min, starting with 98%

Table 1. Average angiotensin-converting enzyme-inhibitory activity (%) of water-soluble extracts of raw and sterilized ovine and caprine cheesecake systems, manufactured separately with cardosin A, cardosin B, or a crude aqueous extract of *Cynara cardunculus*

Coagulant	Ovine milk				Caprine milk			
	Raw		Sterilized		Raw		Sterilized	
	Protein, mg/mL	Inhibition, %	Protein, mg/mL	Inhibition, %	Protein, mg/mL	Inhibition, %	Protein, mg/mL	Inhibition
Cardosin A	0.67	6.21 ± 0.07	0.99	6.21 ± 0.09	2.32	44.95 ± 0.20	1.33	16.57 ± 0.04
Cardosin B	1.34	50.86 ± 0.15	0.80	12.57 ± 0.11	2.38	79.43 ± 0.08	1.43	33.71 ± 0.11
Crude aqueous extract of <i>C. cardunculus</i>	1.84	62.29 ± 0.06	1.34	40.11 ± 0.08	2.30	63.43 ± 0.04	0.34	48.00 ± 0.13

(vol/vol) solvent A, passing through a linear gradient from 2 to 60% (vol/vol) solvent B over 45 min, another linear gradient from 60 to 100% (vol/vol) solvent B for 2 min, 100% (vol/vol) solvent B for 5 min, and a linear gradient from 98 to 2% (vol/vol) solvent B for 2 min, and ending with 2% (vol/vol) solvent B for 2 min. Detection was by spectrophotometry at 214 nm. The fractions were collected based on their peak assignments. This step was repeated 5 times, and the fractions from the various chromatographic runs were pooled and dried in a vacuum.

Estimation of ACE-Inhibitory Activity

Inhibition of ACE was assayed by the method of Vermeirssen et al. (2002). The ACE reagent (containing 0.5 mmol/L of *N*-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycylglycine, with stabilizer and buffer at pH 8.2; Sigma, St. Louis, MO) was reconstituted in 5 mL of water. The ACE from rabbit lung (Sigma) was reconstituted with demineralized water (0.2 U/mL) and diluted to 1:3 (vol/vol) before use.

Each aliquot (125 µL) of sample was mixed and preincubated, at 37°C for 2 min, with 125 µL of ACE reagent. After adding 25 µL of ACE reagent to this suspension, the reaction mixture was further incubated at 37°C for 5 min. Subsequently, absorbance at 340 nm was measured against demineralized water (control) in the heated cuvette holder (37°C) of a UV-visible spectrophotometer, over a time interval of 10 min.

The inhibitory activity was expressed as the percentage of ACE inhibition per milligram of protein, except for WSE, in which the activity was calculated as the amount of water-soluble nitrogen needed to inhibit 50% of ACE activity (IC₅₀). The activity of each sample was tested in triplicate, and their average was used as a datum point. A positive control (1 mM captopril; Sigma), which is known to inhibit ACE, was also included in the assay program.

Estimation of Antioxidant Activity

The antioxidant activity of either WSE or the isolated peptides thereof was assayed according to the method

described by Re et al. (1999). 2,2'-Azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid was dissolved in water to a 7 mM concentration. To prepare the ABTS^{•+} radical specifically needed for this test, the ABTS aqueous solution was oxidized via treatment with 2.45 mM potassium persulfate (1:0.5) for 12 to 16 h in the dark, and then diluted in 0.1 mol/L of phosphate buffer (pH 7.4) prior to assaying, which yielded a typical absorbance of 0.70 ± 0.02 at 734 nm after equilibration at 30°C. A 10-µL quantity of sample (or Trolox, as positive control) was added to 1 mL of diluted ABTS^{•+} solution and incubated at 30°C for 10 min. Scavenging of the ABTS^{•+} radical was followed by monitoring the decrease in absorbance at 734 nm. A reading was taken 1 min after initial mixing and then periodically up to 6 min. A solvent blank was run in each assay (negative control). All determinations were carried out in triplicate, and their average was used as a datum point. The percentage inhibition of absorbance at 734 nm was calculated and plotted as a function of the concentration of the reference antioxidant (Trolox).

Sequencing of Peptides

The peptides that exhibited ACE-inhibitory activity were sequenced by Edman degradation using a model 491 automated, pulsed liquid-phase protein-peptide sequencer (Applied Biosystems, Foster City, CA). The AA released were detected as their phenylthiohydantoin derivatives. The partial sequence thus obtained was checked against the (known) sequence of CN in reference databases (BLAST at the National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/BLAST>), in attempts to identify the specific peptide bonds cleaved by the enzymes under consideration.

Determination of Protein Content

The protein (peptide) concentrations were determined by the phenol reagent method for biological fluids using the Micro Protein Determination kit (Sigma).

Table 2. Molecular characteristics of peptides identified in the permeate of water-soluble extracts of raw and sterilized ovine cheeselike systems, manufactured separately with cardosin A, cardosin B, or a crude aqueous extract of *Cynara cardunculus*, and corresponding angiotensin-converting enzyme (ACE)-inhibitory and antioxidant activities¹

Peak	Peptide bond cleaved	Sequence	Protein concentration, mg/mL	ACE-inhibitory activity, IC ₅₀ (μg/mL)	Antioxidant activity, TEAC (mM)
Raw system					
A	ND	ND	0.180	112.76 ± 0.15	ND
B	ND	ND	0.660	661.04 ± 0.07	0.095 ± 0.06
C	β-CN [Gly94-Val95]	Val-Pro-Lys-Val-Lys	0.090	93.75 ± 0.06	0.079 ± 0.15
D	α _{s1} -CN	Arg-Pro-Lys	0.170	169.86 ± 0.10	0.102 ± 0.10
	β-CN [Leu190-Tyr191]	Tyr-Gln-Glu-Pro			
	β-CN [Met102-Val103]	Val-Pro-Lys-His			
	β-CN [Asn73-Ile74]	Ile-Leu-Pro			
Sterilized system					
E	ND	ND	0.070	65.46 ± 0.05	ND
F	β-CN [Leu190-Tyr191]	Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-*	0.500	499.99 ± 0.04	0.124 ± 0.11
Captopril	—	—	—	0.00 ± 0.12	—

¹Peaks shown in Figure 1. IC₅₀ = amount of water-soluble nitrogen needed to inhibit 50% of ACE activity; TEAC = Trolox-equivalent antioxidant capacity; ND = not determined.

RESULTS AND DISCUSSION

Data pertaining to the ACE-inhibitory activity of the WSE from ovine and caprine milk cheeselike systems manufactured with the various coagulants, namely cardosin A, cardosin B, and crude aqueous extracts of *C. cardunculus*, are presented in Table 1. Typical elution profiles of the 3,000-Da permeate from raw and sterilized ovine and caprine milk cheeselike systems, manufactured with the aforementioned coagulants, are shown in Figures 1 and 2. The results obtained via sequencing, as well as those referring to protein content, ACE-inhibitory activity, and antioxidant activity

of the peptides isolated by HPLC, are depicted in Tables 2 and 3.

The most obvious differences between the peptide profiles in Figures 1 and 2 can be ascribed to the use of raw vs. sterilized milk. Heat treatment of cheese-making milk is intended to ensure the safety of the feedstock and the final product via elimination of adventitious, pathogenic microflora (Hermier and Cerf, 2000). Furthermore, cheese makers sometimes heat milk prior to cheese manufacture to increase cheese yield (Calvo et al., 1992). However, interactions of whey proteins with CN micelles brought about by heating milk do

Table 3. Molecular characteristics of peptides identified in the permeate of water-soluble extracts of raw and sterilized caprine cheeselike systems, manufactured separately with cardosin A, cardosin B, or a crude aqueous extract of *Cynara cardunculus*, and corresponding angiotensin-converting enzyme (ACE)-inhibitory and antioxidant activities¹

Peak	Peptide bond cleaved	Sequence	Protein concentration, mg/mL	ACE-inhibitory activity, IC ₅₀ (μg/mL)	Antioxidant activity, TEAC (mM)
Raw system					
G	ND	ND	0.710	705.70 ± 0.11	0.064 ± 0.05
H	ND	ND	0.140	143.59 ± 0.13	0.069 ± 0.03
I	ND	ND	0.220	216.63 ± 0.15	0.073 ± 0.02
J	β-CN [Leu190-Tyr191]	Tyr-Gln-Glu-Pro	0.690	689.40 ± 0.18	0.072 ± 0.06
K	α _{s1} -CN	Arg-Pro-Lys-His-Pro-Ile-Lys-His-*	0.503	892.83 ± 0.11	ND
Sterilized system					
L	ND	ND	0.001	0.580 ± 0.04	ND
M	β-CN [Gly94-Val95]	Val-Pro-Lys-Val-Lys	0.004	2.45 ± 0.13	ND
	α _{s1} -CN	Arg-Pro-Lys	0.062	36.68 ± 0.13	ND
	ND	ND			
N	ND	ND			
O	α _{s2} -CN [Phe89-Tyr90]	Tyr-Gln-Lys-Phe-Pro-Gln-Tyr	0.100	95.46 ± 0.18	0.068 ± 0.02
P	ND	ND	0.291	161.41 ± 0.07	ND
Q	ND	ND	0.202	116.92 ± 0.24	ND
R	β-CN [Leu190-Tyr191]	Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-*	0.650	653.98 ± 0.22	0.191 ± 0.05
Captopril	—	—	—	0.00 ± 0.12	—

¹Peaks shown in Figure 2. IC₅₀ = amount of water-soluble nitrogen needed to inhibit 50% of ACE activity; TEAC = Trolox-equivalent antioxidant capacity; ND = not determined.

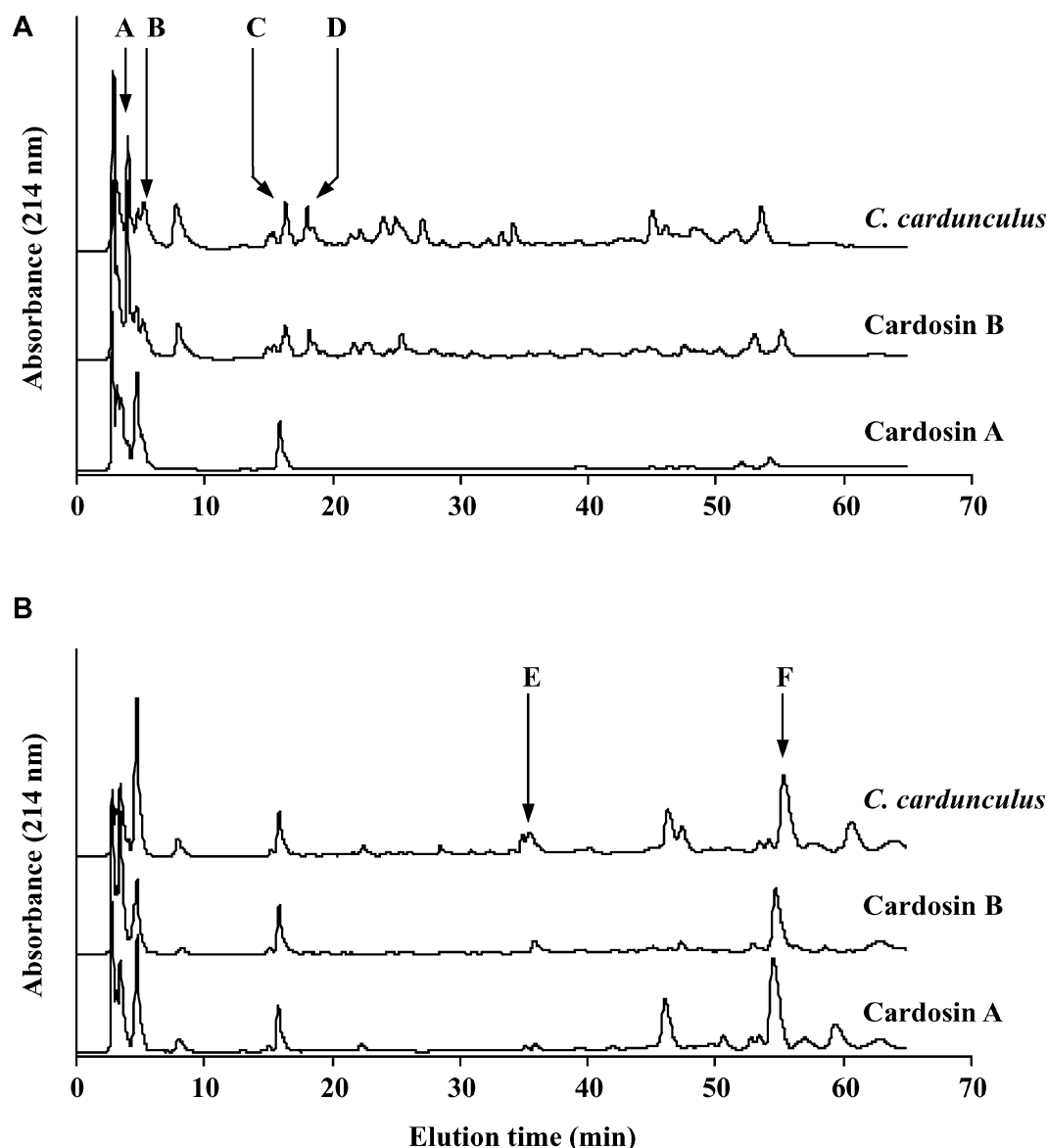


Figure 1. Reversed-phase HPLC chromatograms of the 3,000-Da permeate of water-soluble extracts of (A) raw and (B) sterilized ovine cheeselike systems, manufactured separately with cardosin A, cardosin B, or a crude aqueous extract of *Cynara cardunculus*. Peaks labeled A to F correspond to peptides identified in Table 2.

interfere with the rennet coagulation process, hence leading to longer coagulation times as well as weaker curd structures (Dagleish, 1987; Banks, 1990; Singh and Waungana, 2001). The resulting cheese is known to tend to develop an atypical flavor and texture afterward during ripening, which in turn depends quantitatively mainly on the extent of proteolysis and qualitatively on the profile of proteolysis. In such a case, the coagulant retained in the curd is mainly responsible for CN breakdown, which may account for the results obtained as compared with those of raw milk, in which the mi-

croflora also play an important role (especially in terms of secondary proteolysis).

Higher ACE-inhibitory activity was found in the WSE of cheeselike systems obtained from raw rather than sterilized milk, and a higher IC_{50} against ACE was detected for caprine rather than ovine cheeselike systems (in which the extent of enzyme-mediated proteolysis was also higher). Similar results were found by Silva and Malcata (2005b) concerning proteolysis of ovine vs. caprine milk CN via enzymes contributed by *C. cardunculus*.

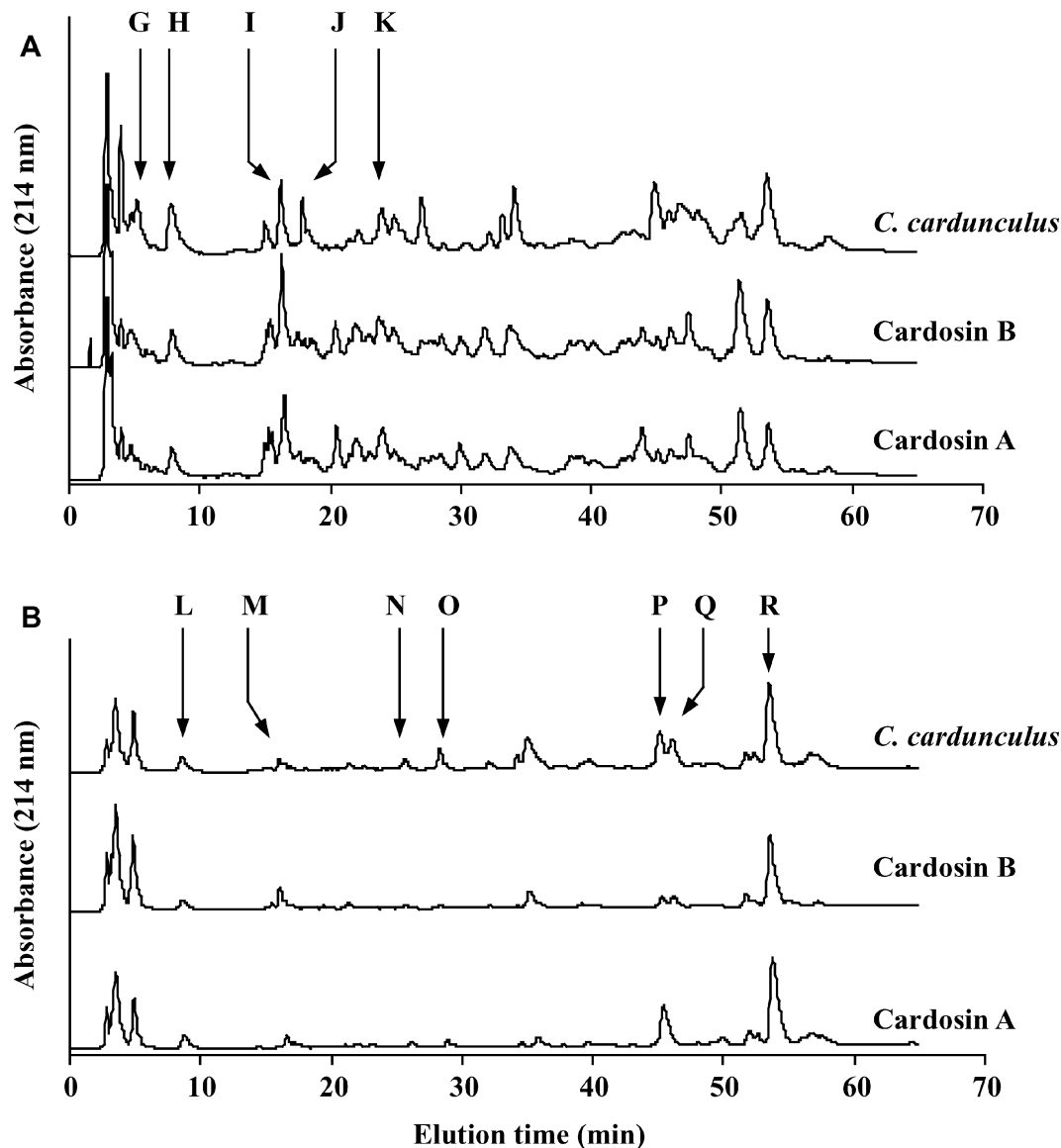


Figure 2. Reversed-phase HPLC chromatograms of the 3,000-Da permeate of the water-soluble extracts of (A) raw and (B) sterilized caprine cheeselike systems, manufactured separately with cardosin A, cardosin B, or a crude aqueous extract of *Cynara cardunculus*. Peaks labeled G to R correspond to peptides identified in Table 3.

Among the 3 types of coagulants tested, the lowest effect was noticed in cheeselike systems produced with cardosin A, and the highest with the crude aqueous extract. Cardosin A is the most abundant cardosin in crude aqueous extracts of *C. cardunculus* (Frazão et al., 1999); its specificity is believed to be similar to that of chymosin. Cardosin B resembles pepsin in terms of kinetic behavior (Veríssimo et al., 1995) and is also known to possess nonspecific proteolytic activity. In the crude aqueous extract, a synergistic action apparently takes place between the 2 cardosins, which could ex-

plain the actual results obtained. Concerning the peptide profile of the corresponding WSE (Figures 1 and 2), several peaks appear in each chromatogram, but only some of them show ACE-inhibitory or antioxidant activities or both (Tables 2 and 3).

In raw ovine cheeselike systems, only 4 peptides were found to have ACE-inhibitory activity. All of them were produced via the action of cardosin B, either as such or as a crude mixture in the aqueous extract of *C. cardunculus*, whereas only one was formed in cheeselike systems manufactured with cardosin A. The peptide de-

noted as C had the highest activity ($IC_{50} = 93.75 \mu\text{g/mL}$), whereas the peptide denoted as B possessed the lowest ($IC_{50} = 661.04 \mu\text{g/mL}$).

Very few peptides were present in the 3,000-Da permeate of sterilized ovine cheeselike systems, and only 2 peptides were shown to possess ACE-inhibitory activity ($IC_{50} = 65.46$ for the peptide denoted as E, and $IC_{50} = 499.99 \mu\text{g/mL}$ for the peptide denoted as F). Several peptides were found in the corresponding permeate of raw caprine cheeselike systems, but only 5 inhibited ACE activity. The peptide denoted as K possessed the lowest activity ($IC_{50} = 892 \mu\text{g/mL}$), whereas the peptide denoted as H exhibited the highest activity ($IC_{50} = 143.70 \mu\text{g/mL}$).

Seven peptides present in the WSE of sterilized caprine cheeselike systems manufactured via crude aqueous extracts of *C. cardunculus* (denoted as L through R) were shown to have ACE-inhibitory activity. The highest activities were accounted for by peptides L ($IC_{50} = 0.58 \mu\text{g/mL}$) and M ($IC_{50} = 2.45 \mu\text{g/mL}$), whereas peptide R exhibited the lowest activity ($IC_{50} = 653.98 \mu\text{g/mL}$). Peptide Q ($IC_{50} = 116.92 \mu\text{g/mL}$) was not found in cheeselike systems made with cardosin A.

Chemical sequencing permitted us to determine the peptide bonds cleaved by the coagulating enzymes within CN, from (putative) identification of the peptides (or at least by part of their sequence) released. However, some chromatographic peaks were accounted for by a major peptide accompanied by other minor components (i.e., a mixture of peptides), which made the analysis increase considerably in complexity.

Peptide bonds in β -CN cleaved by enzymes from *C. cardunculus* included Leu190-Tyr191, Gly94-Val95, Met102-Val103, and Asn73-Ile74, which gave rise to the peptides Tyr-Gln-Glu-Pro, Ile-Leu-Pro, Val-Pro-Lys-Val-Lys, and Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro* (see Tables 2, 3). Peptide Tyr-Gln-Glu-Pro-Val-Leu, also found in hydrolyzed bovine milk, was demonstrated to have ACE-inhibitory activity, characterized by an IC_{50} of $280 \mu\text{M}$ (Pihlanto-Leppälä et al., 1998).

In the case of α_{s1} -CN, N-terminal peptides were identified that exhibited ACE-inhibitory activity, namely, Arg-Pro-Lys [α_{s1} -CN (1-3)] and Arg-Pro-Lys-His-Pro-Ile-Lys-His* [α_{s1} -CN (1-*). When studying a new type of ripened, low-fat cheese, Ryhänen et al. (2001) also reported that similar peptides from α_{s1} -CN [i.e., α_{s1} -CN (1-9), α_{s1} -CN (1-7), and α_{s1} -CN (1-6)] possessed ACE-inhibitory activity. Moreover, Saito et al. (2000) found α_{s1} -CN (1-9) from Gouda cheese among the peptides bearing ACE-inhibitory activity ($IC_{50} = 13.4 \mu\text{M}$), which also showed a weak antihypertensive activity in spontaneously hypertensive rats.

Structure-activity studies involving various peptides that can inhibit ACE indicate that their binding thereto

is strongly influenced by the hydrophobicity and steric size of the AA in the C-terminal position of the peptide (Gobetti et al., 2004; Pripp et al., 2004, 2006). In addition, Cheung et al. (1980) have claimed that ACE prefers substrates or competitive inhibitors that contain such hydrophobic AA residues as Pro, Phe, and Tyr in the C-terminus. Among the peptides experimentally proven to possess ACE-inhibitory activity, only the sequence Tyr-Gln-Glu-Pro has those characteristics; however, Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro* may be seen as a precursor of the previous shorter sequence.

Finally, one should emphasize that bioactivity per unit weight of cheese might be more relevant for consumers than the inhibitory strength of peptide fractions isolated from cheese, which will make a difference concerning any health claims of ACE inhibition (Pripp et al., 2006).

The Trolox-equivalent antioxidant capacity (TEAC) assay is based on scavenging of ABTS^{•+}, with concomitant conversion thereof to a colorless product. The degree of decolorization induced by a compound relates to that induced by Trolox, thus yielding a TEAC value, which is often used as a useful tool to detect (unknown) antioxidants (Arts et al., 2004). The TEAC values found for peptides isolated from WSE of raw and sterilized ovine and caprine cheeselike systems are shown in Tables 1 and 2. Although most peptides exhibit low TEAC values compared with those of α -tocopherol and vitamin C (0.97 and 0.99 mM, respectively, by 6 min; Miller et al., 1993), higher TEAC values were generally found for peptides in WSE from ovine cheeselike systems.

Peptides D, F, and R, from WSE of raw ovine, sterilized ovine, and sterilized caprine cheeselike systems, respectively, presented the highest TEAC values (0.102, 0.124, and 0.191 mM, respectively). A comparison of the TEAC values of such peptides with the TEAC values of others found in the literature (using the ABTS^{•+} method) indicates that their antioxidant capacity is comparable to that of some sherry wines (Villano et al., 2004). The aforementioned peptides arise from cleavage of the peptide bond Leu190-Tyr191, either in ovine or caprine β -CN, and correspond to the sequence Tyr-Gln-Glu-Pro*. Such a realization is of great importance, because there are no reports to date concerning peptides from cheeses bearing this kind of bioactivity. This provides useful information toward the development of functional food products in the near future, and of additives with proven antioxidant properties.

Difficulties in peptide identification constrain the knowledge encompassing bioactive peptide formation and their release from the precursor proteins. In fact, milk protein hydrolysates are highly complex mixtures that may contain up to hundreds of different peptides. Therefore, identification of such peptides in fermented

dairy products or in milk protein hydrolysates generated by the action of nonspecific enzymes is a labor-intensive and difficult task. This task comprises several purification steps, normally a combination of distinct (and complementary) chromatographic techniques. Each separation step in turn requires solvent removal and assessment of biological activity. In most cases, the final fractions still contain multiple components, which can cause a discrepancy between the activity exhibited by the purified fractions and that by their original counterparts (Gómez-Ruiz et al., 2002; Gobetti et al., 2004).

CONCLUSIONS

Raw and sterilized ovine and caprine cheeselike systems, manufactured with proteases from *C. cardunculus*, are a source of peptides with ACE-inhibitory and antioxidant activities. The peptides Tyr-Gln-Glu-Pro, Val-Pro-Lys-Val-Lys, and Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-* from β -CN, as well as Arg-Pro-Lys and Arg-Pro-Lys-His-Pro-Ile-Lys-His-* from α_{s1} -CN exhibited ACE-inhibitory activity. Peptides released upon cleavage of the peptide bond Leu190-Tyr191 (either in ovine or caprine β -CN), and corresponding to the β -CN sequence Tyr-Gln-Glu-Pro-*, possessed antioxidant activity. Despite their moderate bioactivity, they intrinsically behave as functional foods, so they may eventually be recommended for inclusion in one's daily diet as a more appealing, health-promoting vector than the direct use of more potent drugs. Moreover, possibilities exist for designing new dietary products as well as for exploiting novel natural drugs, because peptide sequences not previously described in the literature possess such desired bioactivities.

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