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Use of artichoke (*Cynara scolymus*) flower extract as a substitute for bovine rennet in the manufacture of Gouda-type cheese: Characterization of aspartic proteases

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ABSTRACT

Artichoke (*Cynara scolymus* L.) flower extract was assayed with the aim of replacing animal rennet in the manufacture of Gouda-type cheeses from bovine milk. Floral extract coagulated milk within a suitable time for use on an industrial scale, while the yield of cheese obtained was equal to that achieved with bovine abomasum. Five proteolytic fractions with milk-clotting activity were isolated in a two-step purification protocol, three belonging to the cardosin group. Cheeses made with *C. scolymus* proteases must be brined for a longer period (40 h) to prevent overproteolysis and avoid the development of a background flavor. The type of coagulant (bovine or vegetable) had no significant effect on the cheeses' chemical parameters analyzed throughout ripening, and no significant organoleptic differences were detected between those manufactured with *C. scolymus* or animal rennet. The results indicate that *C. scolymus* flower extract is suitable for replacing animal rennet in the production of Gouda-type cheeses.

1. Introduction

Cheese is traditionally produced through the use of commercial calf rennet or rennet substitutes as enzymes of microbial origin, recombinant proteases metabolized by genetically modified microorganisms, and plant proteases (Jacob, Jaros, & Rohm, 2011). Milk has a natural pH of 6.5, while the pH optimum for the hydrolysis of κ-casein in the primary phase of rennet coagulation is 5.1–5.3 (Shalabi and Fox, 1982). The primary function of the enzymes in rennets is to destabilize the casein micelle and thus cause the gelation or coagulation of milk, but those hydrolases also play a major role in proteolysis during maturation of cheeses (Lane, Fox, Johnston, & McSweeney, 1997). Substitutes with cheese-making potential should mimic calf rennet specific properties: a high ratio of clotting activity to proteolytic activity at pH and temperature of cheese-making as well as a sufficient thermolability to ensure whey products without remnants of active coagulant (Jacob et al., 2011). Many proteases that coagulate milk do not fulfil these requirements and are therefore unsuitable for cheese making. The generally used milk-clotting enzymes belong to the class of aspartic peptidases (APs), which are successful in bringing about the specific cleavage of the bovine–κ-casein Phe105–Met106 bond. The great majority of APs are optimally active at acidic pH. During the processes of cheese manufacture and ripening, the limited action of the APs within the pH range of 4.5–6.5 is of particular interest. The primary function of the milk-clotting enzyme is to initiate the coagulation of the milk to which it has been added. This includes the rapid and highly specific cleavage of the hydrophilic caseinomacropeptide part from κ-casein molecules located at the periphery of the casein micelles (Dalgleish, 1987; Visser, 1993).

Vegetable enzymes from higher plant organs have been extensively investigated as potential coagulants in cheese making; nevertheless, some of them have been found to be unsuitable for cheese production owing to a characteristic excessive proteolytic activity that lowers cheese yield and produces bitter flavors in the final product. Among the vegetable enzymes that have been reported to produce satisfactory final products, cardosins and cyprosins (the proteases present in flowers of *Cynara cardunculus* L.) deserve special mention. Aqueous extracts of this thistle flower have been used for centuries in traditional artisanal farm production of ewe- and/or goat-milk cheeses such as Serra da Estrela,

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Manchego, La Serena, or Serpa in Portugal and Spain, some of them having Protected-Designation-of-Origin status (Jacob et al., 2011; Reis & Malcata, 2011; Silva & Malcata, 2005). Although artisan-cheese makers are small-scale producers, their production makes a substantial socioeconomical contribution to the dairy sector at regional areas of each country and thus makes a significant contribution to the local agricultural economy (Roseiro, Barbosa, Ames, & Wilbey, 2003).

Cardosins A and B—the two APs mainly responsible for the clotting activity of such extracts—have been shown to act on κ-casein in a similar way to chymosin, cleaving the Phe105–Met106 peptide bond on bovine and ovine κ-casein, whereas caprine κ-casein is preferentially cleaved at Lys116–Thr117 (Silva & Malcata, 2005). The two enzymes can also hydrolyze both α- and β-casein to produce cheeses characterized by a soft buttery texture, a typical aroma, and a slightly piquant and creamy flavor (Galán, Prados, Pino, Tejada, & Fernández-Salguero, 2008). APs from *C. cardunculus* thistle are encoded by a multigene family (Pimentel, Van Der Straeten, Pires, Faro, & Rodrigues-Pousada, 2007) and to date nine APs—the cyprosins A, B, and C and the cardosins A, B, E, F, G, and H—have been isolated, purified, and biochemically characterized from this plant (Sarmento et al., 2009). Cardosins and cyprosins—synthesized as single-chain zymogens with the subsequent conversion into the two-chain mature enzymes being a crucial step in the regulation of their activity—are dimeric, glycosylated, pepstatin-sensitive APs, active at acidic pHs (optimum at pH 4.5), and with preferential specificity for peptide bonds between hydrophobic amino-acid residues (Sampaio, Fortes, Cabral, Pais, & Fonseca, 2008; Sarmento et al., 2009). The cardosin C and D genes have also been described, although the predicted proteins have not yet been identified (Pimentel et al., 2007).

It is found in the literature that aqueous extracts from dried flowers of *C. cardunculus*—and such related species as *C. scolymus* and *C. humilis*, have been used for ages in the Iberian Peninsula, apparently ever since the Roman occupation in premedieval times (Lourenço, de Castro, Martins, Clemente, & Domingos, 2002; Silva & Malcata, 2000; Tejada, Vioque, Gómez, & Fernández-Salguero, 2008). In addition to the species of the genus *Cynara*, *Arctium minus*, *Centaurea calcitrapa*, *Cirsium vulgare*, *Onopordum acanthium, Onopordum turcicum*, and *Silybum marianum*—all species included within the tribe: Cardueae Cass. = Cynareae Less, Asteraceae family—contain APs in their flowers with milk-clotting activity but without excessive proteolytic action (Brutti, Pardo, Caffini, & Natalucci, 2012; Cimino, Liggieri, Priolo, Bruno, & Vairo-Cavalli, 2010; Domingos et al., 2000; Lufrano et al., 2012; Silva & Malcata, 2000; Tamer, 1993; Vairo Cavalli et al., 2008; Veríssimo, Esteves, Faro, & Pires, 1995). In the case of *C. scolymus*, maximum proteolytic activity at pH 5.0 as well as easy enzyme inactivation by moderate heating, make this crude protease extract potentially useful for cheese production (Chazarra, Sidrach, López-Molina, & Ródriguez-López, 2007; Llorente, Brutti, & Caffini, 2004; Sidrach, García-Cánovas, Tudela, & Rodríguez-López, 2005).

According to the United Nations Food and Agricultural Organization (FAO), in 2009 Argentina was the 9th cheese-producing country in the world. This industrial sector is by far the principal dairy product, accounting for approximately 45% of national milk production. The country has approximately 1000 cheese-manufacturing plants, and many plants of the leading companies along with several medium-sized enterprises operate according to the standards set by the International Organization for Standardization and observe the criteria for hazard analysis and critical control points (Cappellini, 2011). In 2010, Argentina's cheese exports amounted to about 52,000 metric tons, and in the first semester of 2013 its cheese exports had reached 24,189 metric tons, an output representing *ca.* 120 million dollars (free on board), according to the Undersecretary of Dairy Products (Under Secretary of Dairy, Ministry of Agriculture, Livestock, Fisheries -SAGPAyA, 2010). Semi-hard cheeses (Gouda and Edam) represented 48% of the total volume, followed by soft (mainly Mozzarella) and hard (Goya and Reggianito) cheeses, with 33% and 18%, respectively (Cappellini, 2011).

In this research we thus attempted to utilize aqueous extracts of the *C. scolymus* flower as a rennet substitute in the manufacture of Gouda-type cheese on a pilot scale and to characterize the APs present in those extracts. Even when the extracts of flowers of *C. scolymus* have been studied biochemically and its milk-clotting and proteolytic activities have been reported, to our knowledge, this is the first time that cheeses manufactured with this vegetable rennet are studied and characterized.

2. Materials and methods

2.1. Plant material

Mature flower heads of *C. scolymus* L. were obtained from farm plants grown in Nogoyá (Entre Ríos, Argentina) and La Plata (Buenos Aires, Argentina) collected in December and identified by Dr. Nancy Apóstolo, from the Plant Anatomy Laboratory, National University of Luján, Argentina.

2.2. Reagents

Bovine-serum albumin, cysteine, ethylenediaminetetraacetic acid (EDTA), glycine, pepstatin, and tris(hydroxymethyl)aminomethane were purchased from Sigma Chemical Co. (St. Louis, MO, USA), bicine and molecular-weight markers from GE Healthcare Biosciences (Uppsala, Sweden), casein (Hammarsten type) from Research Organics Inc. (Cleveland, OH, USA), bromophenol-blue dye marker from Mallinckrodt Chemical Works (Paris, KY, USA), acrylamide, bisacrylamide, Coomassie brilliant blue G-250, and polyvinylidene-fluoride membrane from Bio-Rad (Hercules, CA, USA), Trypsin Gold from Promega Corporation (Madison, WI, USA), peptide calibrants and α-cyano-4-hydroxycinnamic acid from Bruker Daltonics (Bremen, Germany), and San Regime™ powdered skim milk from SanCor (Sunchales, Argentina). All other chemicals were obtained from commercial sources and were of the highest purity available.

2.3. Vegetable rennet

Enzyme extracts were prepared following the method of Llorente et al. (2004). Artichoke flowers were ground under liquid nitrogen with a mortar and pestle and homogenized at a ratio of 1 g per 3 ml of 0.1 M potassium phosphate buffer (pH 6.0) containing 1.0 mM EDTA. The homogenized extracts were then filtered through two layers of gauze and finally centrifuged at 16,000×*g* for 20 min at 4 °C.

2.4. Milk-clotting activity

Milk-clotting activity was measured following the procedure described by the International Dairy Federation *Standard no. 157 (1992)*. A 100 μl-aliquot of sample was added to 1 ml of skim milk reconstituted at 12% (w/v) with 10 mM $CaCl_2$ containing 0.02% (w/v) sodium azide as a bactericide. The time elapsing at 37 °C between the mixing of the reagents and the initial appearance of solid material was recorded.

2.5. Enzyme isolation

Enzyme purification was carried out according to a modification of the procedure of Llorente et al. (2004) involving a two-step chromatography in an Äkta Purifier system (GE Healthcare Life Sciences Biosciences, Uppsala, Sweden). The extract was loaded onto a HiTrap Desalting column (Sephadex G25 Superfine, GE Healthcare Life Sciences) preequilibrated with 25 mM Tris–Bicine buffer (pH 6.0). The resulting depigmented-protein extract was recovered in the void volume and then loaded onto a HiTrap Q column (GE Healthcare Life Sciences), previously equilibrated with 25 mM Tris-Bicine buffer (pH 6.0). The bound material was eluted with two consecutive linear NaCl gradients (0.10–0.35 M, 0.35–0.50 M) followed by two steps of 0.5 and 1 M NaCl in the same buffer and the fractions containing milk-clotting activity pooled. Fractions from anion exchange chromatography were analyzed by SDS–PAGE according to the method described by Laemmli (1970); samples were separated on a 12.5% (w/v) gel using a Miniprotean III cell system (Bio-Rad). Gels were run at 60 mA for 45 min and then stained with Coomassie Blue G-250.

2.6. Protein quantification

The protein concentration was determined by the method of Bradford (1976) with bovine-serum albumin as reference standard for the calibration curve.

2.7. Peptide-Mass Fingerprints (PMFs)

Bands corresponding to the purified peptidases were cut out from the gel, washed first with Milli Q™ water and then with acetonitrile several times to remove the dye before drying under vacuum. For Cys-sulfhydryl alkylation, the gel fragments were treated with 0.1 M NH_4HCO_3 containing 10 mM dithiothreitol for 30 min at 37 °C, centrifuged, washed with acetonitrile for 5 min, and finally incubated in the dark in 0.1 M NH_4HCO_3 with 50 mM iodoacetamide for 20 min at 25 °C. Digestions were carried out with 4 ng/ml trypsin for 12 h at 37 °C. The peptides obtained were recovered by extraction with a half volume of acetonitrile, dried in a SpeedVac vacuum centrifuge, and redissolved in 10 μl of 1 ml/l trifluoroacetic acid. Each sample was spotted on a polished-steel plate and mixed with freshly prepared matrix solution (10 mg/ml of α-cyano-4-hydroxycinnamic acid in aqueous solution containing 30% acetonitrile and 0.1% trifluoroacetic acid by volume). PMFs were obtained by matrix-assisted-laser-desorption-and-ionization–time-of-flight mass spectrometry (MALDI-TOF) in an UltraFlex MALDI-TOF mass spectrometer (BrukerDaltonics, Bremen, Germany). Spectra were obtained by means of Flex Control Software within a range of *ca.* 1000–3500 *m*/*z*. The external calibration was performed with peptide calibration standards.

MASCOT search tool (URL http://www.matrixscience.com) was used for identification of the related tryptic maps. The probability-based mowse (protein) score was calculated as $10 * \text{Log}(P)$, where P is the probability that the observed match is a random event. Protein scores greater than 56 were considered significant ($p < 0.05$).

2.8. Digestion of α-, ß-, and κ-casein by artichoke-flower APs

The alpha, beta, and kappa caseins purified by the method of Rusconi, Priolo, López, and Vairo Cavalli (2011) were kindly provided by Elina Rusconi, MSc (LIProVe, Faculty of Exact Sciences, University of La Plata). Reactions were started by the addition of 300 μl of 0.80 mg/ml α- or β-casein or 0.65 mg/ml of κ-casein to 30 μl of each purified enzyme (which volume corresponded to 60 ± 4 μg of peptidase/ml). At 30 and 60 min the reactions were quenched by the addition of 2x sample buffer for sodiumdodecylsulfide-polyacrylamide–gel electrophoresis (SDS–PAGE); then samples were heated at 100 °C for 5 min. Controls containing each casein, at the same concentration but without addition of enzyme, were also included.

Hydrolysis was analyzed by SDS–PAGE according to Laemmli (1970); samples were separated on 12.5% acrylamide gels using a Mini Protean III cell system (Bio-Rad). Gels were run at 60 mA for 45 min and then stained with Coomassie Blue G-250. After separation, the bands were blotted onto a polyvinylidene-fluoruide membrane through the use of the Mini Trans-Blot system (Bio-Rad) in 10 mM CAPS buffer (N-cyclohexyl-3-aminopropanesulfonic acid) plus 10% (v/v) methanol, pH 11.0. N-terminal sequencing was performed by the Sequencing Services of LANAIS-PRO (CONICET–UBA) via Edman degradation.

2.9. Formulation of Gouda-type cheese from bovine milk

The extracts obtained in Section 2.3 were used as plant coagulants to produce semihard cheeses. Standard manufacturing conditions for bovine-rennet–curdled cheeses—according to the FAO of the World Health Organization (1986)—were followed through the entire cheese-making process by a protocol optimized at the Pilot Plant of Research and Development Center of National University of Luján (CIDETA-UNLu). Milk produced from Holando Argentino dairy cows was obtained from National University of Luján dairy (Dairy Production Unit; Teaching, Research and Outreach Center for Farming Production) and pasteurized at 72 °C for 15 s before use. Two hundred l of pasteurized milk (fat and protein content 34.0 and 31.4 g per l, respectively) were transferred to a 500-l cheese vat (Bauducco, El Trébol, Santa Fe, Argentina) and the acidity adjusted to 16° Dornic with food-grade acetic acid. The milk was then inoculated with a mesophilic starter culture CHN11 (CHR Hansen, Quilmes, Argentina) and incubated to 32 °C for 1 h before rennet addition. Bovine rennet (0.6 ml per 10 l of milk) (Cortafor-T, Tuteur SACIFIA, Buenos Aires, Argentina) or artichoke-flower extract (30 ml [54 mg of protein] per 10 l of milk) was added to curdle the milk at 35 °C. The milk was kept undisturbed until coagulation was complete. The curd was cut with blades left to heal for 5 min, and then gently stirred mechanically for 5 min. The temperature was raised gradually to 40 °C over a period of 15 min, and the curd was then washed with chlorine-free water before a second step of cutting and stirring was performed under the same conditions.

The curd was packed into cylindrical molds and pressed (2.0 kg/cm^2) at 20 °C for 1 h. Five cheeses were produced with animal rennet (AR) and 10 with vegetal rennet (VR). The curds were salted by brining in NaCl solution (22 °B). Vegetable-curd cheeses were separated into two groups; the first group was brined for 30 h (VR-30) and the second group for 40 h (VR-40). AR cheeses were salted for 30 h. Finally cheeses were transferred to a ripening chamber for a 44-day storage at 14 °C.

After this treatment the cheeses were analyzed for total and water-soluble nitrogen content (TN and WSN, respectively) by the semimicro-Kjeldahl method (AOAC, 1984), moisture by oven drying (AOAC, 1984), and internal and external pH by direct contact of the cheese with an electrode having a protective plastic cover (Hanna Instruments, Buenos Aires, Argentina). A determination of the change in those parameters over time was performed on three samples chosen at random for each treatment, whereas the pH and humidity were measured at the end of ripening on two samples of every cheese. The casein-hydrolysis profile was analyzed by urea-PAGE according to the method of Shalabi and Fox (1987). The TN and soluble-casein content were determined in duplicate with three cheeses from each treatment. A quantification of the intact caseins was done by gel scanning followed by

densitometry through the use of the software Scion Image Beta v. 4.02 for Windows (Scion Corporation, Frederick, MA, USA).

Triangular discriminative tests (Lawless & Heymann, 1999) were performed in order to detect differences between the bovine-rennet cheeses and those made with artichoke-flower extract salted for either 30 or 40 h. A sensory analysis was performed by a panel of 12 trained judges. Three samples of each product were presented to each panelist, who tested two equal samples and a different one. Both the external and the internal appearance, as well as the texture and flavor of the cheese at the end of the ripening process, were evaluated. Precautions were taken to randomize the samples, to mask the identity, and to minimize the effects of contrast and adaptation.

2.10. Statistical analysis

Statistical evaluation by the one-way analysis of variance (ANOVA) was performed by means of statistical software (IBM-SPSS v19). Tukey's comparison was used to determine the significance between groups. A p-value $\leqslant 0.01$ was considered significant. The results are reported as the mean ± standard error of the mean, with the latter being used as an indication of the uncertainty in each measurement.

3. Results and discussion

3.1. Purification

Crude extract from fresh flowers of *C. scolymus* L. (artichoke), exhibited milk-clotting activity (time < 2 min). Llorente, Brutti, Natalucci, and Caffini (1997) originally provided evidence that the curdling activity of extract from the inflorescence of *C. scolymus* at various stages of development is caused by APs. In subsequent studies, Llorente et al. (2004), using fresh or frozen artichoke flowers, obtained peptidase homogenates at pH 6.0 with maximal proteolytic activity at pH 5.0 and a pI of around 4.0. Employing adsorption with activated charcoal followed by anion-exchange and affinity chromatography, those authors isolated two main protein heterodimeric fractions consisting of a 30- plus a 15-kDa subunit, both with acid-proteolytic and milk-clotting activities. In contrast, three proteinases with milk-clotting activity were isolated from the stigmas of artichoke flowers by Sidrach et al. (2005); these proteases were later partially characterized by Chazarra et al. (2007), who named them cynarases A, B, and C. The two-step purification procedure used in the present work, based on that chromatographic procedure of Llorente et al. (2004), resulted in 5 fractions (*a*, *b*, *c*, *d*, and e) with milk clotting activity (Fig. 1A). All the enzymes were heterodimeric proteins with two subunits of apparent molecular masses of 30 and 14.4 kDa (Fig. 1B) as estimated by SDS–PAGE. The five isolated enzyme fractions, at concentrations of 50 μg/μl, exhibited milk-clotting activity: fractions *b*, *c*, *d*, and *e* did so at times of less than 30 min, while Fraction *a* coagulated milk only after 20 h. We cannot conclude that the enzymes purified in the present work correspond to the cynarases cited in the literature, as even when the authors characterized those enzyme fractions, no DNA-sequential nor the N termini analyses were made. The increase in the number of purified fractions in this work is not unexpected, since, as demonstrated for cardoon APs, these enzymes apparently belong to a multigene family (Pimentel et al., 2007).

3.2. Enzyme characterization

3.2.1. PMFs

The Coomassie-blue-stained bands of the 30-kDa subunits from each fraction were excised from the polyacrylamide gels and digested with trypsin. The resulting peptides were then analyzed by mass spectrometry (Fig. 1 in Supplementary Data). The PMFs were compared to protein sequences present in the nonredundant database as summarized in Table 1. Several peaks were found to be repeated in the different spectra analyzed. It is quite common to see several proteins getting the same high score. Even if the protein sequences in the database are nonidentical since the same group of matched-mass values may nevertheless occur in multiple proteins (http://www.matrixscience.com/help/pmf_summaries_help.html). Six experimental tryptic fragments of Fraction *a* matched peptides of cardosin H (score, 90) with a sequence coverage of 57% and a satisfactory peptide distribution. A good correlation was also found between the number of matching peptides, the score and the percentage of coverage with cardosin F (score, 69; major subunit sequence coverage, 41%), and procardosin A (score, 57; major subunit sequence coverage, 36%). Eight digested peptides of Fraction *b* matched cardosin H (score, 98.90; sequence coverage, 45%) and procardosin A (score, 78%; sequence coverage, 23%). The Fraction-*c* PMFs were well correlated with cardosin H (score, 97; sequence coverage, 45.3%), procardosin A (score, 87; sequence coverage, 23.8%), and cardosin E (score, 58; sequence coverage, 39.7%). The peptides with m/z 1303.55; 1708.71, and 2,994.35 from the tryptic digest of Fraction *b* and the peptides with the same m/z of 1303.59, 1708.76, and 2994.39 from Fraction *c* comprised the N-terminal (residues 1–29) of mature cardosin A and cardosin H, consistent with the first 20 residues (*i. e.*, the N-terminal sequence) obtained by Llorente et al. (2004) through Edman sequencing of the species in their peak IV. The sequence identity between the major subunits of procardosin A and cardosin F (*C. cardunculus*) is 86% (with 88% positives), while the correspondence between the heavy subunits of procardosin A and cardosin H is 82% (with 82% positives) and between the former and cardosin E 60% (with 61% positives). It is also noteworthy that the available sequences of cardosin H, F, and E are not complete, but rather contain several gaps. Mature flowers of *C. cardunculus* (cardoon) produce two identified groups of typical plant APs—cardosins and cyprosins (Sandra Vairo Cavalli, Lufrano, Colombo, & Priolo, 2013). In view of the foregoing data, *C. scolymus* fractions *a, b,* and *c* clearly belong to the cardosin group. Further investigation, however, is needed in order to elucidate the complete sequences of these enzymes.

The PMFs of fractions *d* and *e* were identical to each other but even with a high peptide-mass tolerance employed (1000 ppm), no matches were found, indicating the presence of significant differences in the primary sequences from those available in the database. Nevertheless, some of the spectral peaks of fractions *d* and *e* are coincident with the PMF peaks of the other fractions or even with the m/z values corresponding to a theoretical digest of the cardosins available in the data base (*e.g.*, peak m/z 969.4 is present in fractions *b, c, d,* and *e*; procardosin A; and cardosins H and E).

3.2.2. Casein-hydrolytic profile

The degradation pattern of α_s-, β-, and κ-caseins hydrolyzed by fractions *b*, *c*, and *e* was visualized by SDS–PAGE (Fig. 2). A breakdown of either β- or κ-casein was observed after 30 min with each enzymatic fraction; but the hydrolytic profiles showed a specific activity for all the fractions without excessive proteolysis, and in all instances a principal degradation fragment was present. Conversely, the α-caseins were less susceptible to proteolysis by the isolated enzyme fractions, and after 60 min the protein degradation was only partial under the assay conditions used.

Peptides produced by κ-casein digestion were analyzed by Edman sequencing. The isolated APs fractions from *C. scolymus* extract possessed a specificity such that, in addition to hydrolyzing the κ-casein Phe105-Met106 bond, fractions *b*, *c*, and *e* cleaved the Tyr42-Tyr43 bond and Fraction *e* also split the Leu50-Ile51 bond, both of the hydrophobic N-terminal peptide, para-κ-casein; as

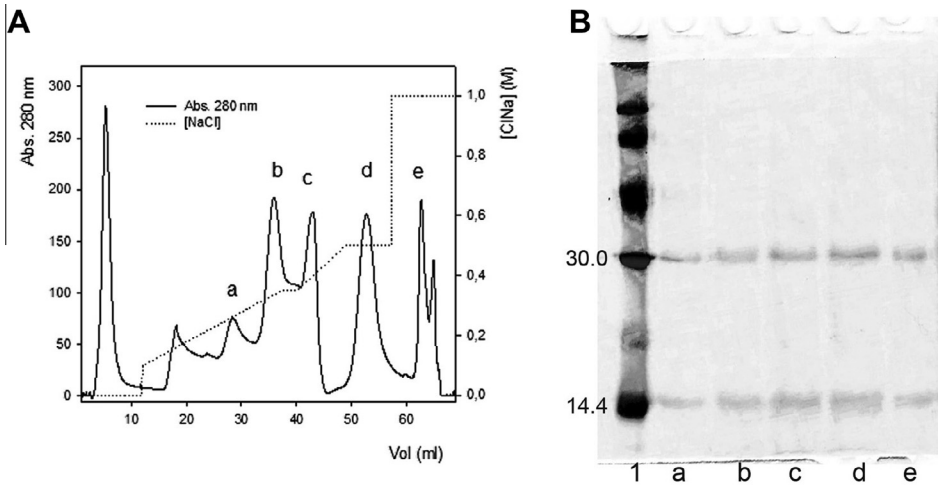

Fig. 1. (A) Anion-exchange chromatography on a HiTrap Q column. The bound material was eluted with two consecutive linear NaCl gradients (0.10–0.35 M, 0.35–0.50 M) followed by first 0.5 and then 1 M NaCl. Fractions a, b, c, d, and *e* contained milk-clotting activity. (B) SDS–PAGE. Lane 1, molecular-weight standards (phosphorylase b, 97.0 kDa; albumin, 66.0 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 30.0 kDa; trypsin inhibitor, 20.1 kDa; α-Lactalbumin, 14,4 kDa). Lanes a–e, fractions a–e, respectively.

Table 1
Identification of trysin-digested peptides from *fractions a–c.*

Exp. PMF*	Theoretical PMF						Peptide fragment sequence
Fraction a							
	Cardosin H		Cardosin F		Procardosin A		
m/z	*m/z*	F.P.	*m/z*	F.P.	*m/z*	F.P.	
1125.55	1125.59	108–115	1125.59	122–129	1125.59	218–225	RFSFWLNR
1303.62	1303.64	1–13			1303.64	69–81	DSGSAVVALTNDR
1724.82			1724.83	15–30			DTSYYGEIGIGTPPQK
1969.02	1969.02	30–47	1969.02	31–48	1969.02	98–115	FTVIFDTGSSVLWVPSSK
2178.99	2178.99	62–79					EQDFIEATDETDNVFLHR
2329.06	2329.06	116–136	2329.06	130–150	2329.06	226–246	NVDEEEGGELVFGGLDPNHFR
3033.48	3033.45	137–162	3033.45	151–176	3033.45	247–272	GDHTYVPVTYQYYWQFGIGDVLIGDK
Fraction b							
	Cardosin H		Procardosin A				
m/z	*m/z*	F.P.	*m/z*	F.P.			
969.42	969.49	109–115	969.49	219–215			FSFWLNR
1125.52	1125.59	108–115	1125.59	218–225			RFSFWLNR
1303.55	1303.64	1–13	1303.64	69–81			DSGSAVVALTNDR
1708.71	1708.83	14–29	1708.83	82–97			DTSYFGEIGIGTPPQK
1968.88	1969.02	30–47	1969.02	98–115			FTVIFDTGSSVLWVPSSK
2147.83			2147.98	170–187			EQDFIEATDEADNVFLHR
2328.91	2329.06	116–136	2329.06	226–246			NVDEEEGGELVFGGLDPNHFR
2994.35	2994.46	1–29	2994.46	69–97			DSGSAVVALTNDRDTSYFGEIGIGTPPQK
3033.35	3033.45	137–162	3033.45	247–272			GDHTYVPVTYQYYWQFGIGDVLIGDK
Fraction c							
	Cardosin H		Procardosin A		Cardosin E		
m/z	*m/z*	F.P.	*m/z*	F.P.	*m/z*	F.P.	
969.45	969.49	109–115	969.49	219–225	969.49	67–73	FSFWLNR
1125.54	1125.59	108–115	1125.59	218–225	1125.61	66–73	RFSFWLNR
1303.59	1303.63	1–13	1303.63	69–81			DSGSAVVALTNDR
1708.76	1708.83	14–29	1708.83	82–97	1709.76	14–29	DTSYFGEIGIGTPPQK
1968.93	1969.02	30–47	1969.02	98–115			FTVIFDTGSSVLWVPSSK
2178.89	2178.99	62–79			2178.99	48–65	EQDFIEATDETDNVFLHR
2329.95	2330.07	116–136			2330.07	74–94	NVDEEEGGELVFGGLDPNHFR
2994.39	2994.46	1–29	2994.46	69–97			DSGSAVVALTNDRDTSYFGEIGIGTPPQK
3033.38	3033.45	137–162	3033.45	247–272	3034.45	95–120	GDHTYVPVTYQYYWQFGIGDVLIGDK

Cysteines have been treated with iodoacetamide to form carbamidomethyl-cysteine (Cys-CAM). For the theoretical PMF Cys-CAM, the oxidized Cys and monoisotopic masses of the occurring amino acid residues obtained were considered. The experimental and theoretical peptide matches were selected with a mass tolerance of 50 ppm.
* PMF, peptide-mass fingerprints.

determined from an N-terminal sequencing of the 13.0- and 12.5-kDa bands. The APs generally display specificity for amino-acid residues on either side of the scissile bond containing large hydrophobic side chains such as those involved in the aforementioned cleavages. For example, when the cleavage specificity of the cardosin A from *C. cardunculus* and *C. humilis* was evaluated with bovine α_{s1}-casein and caprine α_{s2}-casein, the (Tyr165-Tyr166) and (Tyr186-Tyr187) bonds, respectively, were found to be hydrolyzed (Ramalho-Santos, Veríssimo, Faro, & Pires, 1996; Silva & Malcata, 2000). Although these APs can cleave those bonds in a simplified

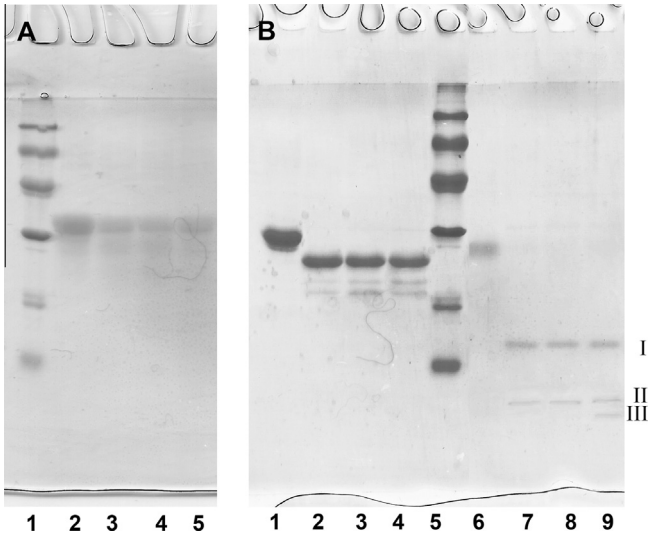

Fig. 2. Hydrolysis of caseins by fractions from *C. scolymus* flower extract. SDS–PAGE electrophoretogram (12.5% [w/v], preceded by a stacking gel of 4% [w/v], polyacrylamide). (A) Degradation patterns of α-casein by fractions *b–d*. Lane 1, molecular-weight standards (phosphorylase b, 97.0 kDa; albumin, 66.0 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 30.0 kDa; trypsin inhibitor, 20.1 kDa; α-Lactalbumin, 14.4 kDa); Lane 2, α-casein after incubation for 0 min; lanes 3–5, α-casein after 30 min of hydrolysis by fractions *b–d*. (B) Degradation patterns of κ- and β-bovine caseins by fractions *b–d*. Lanes 1 and 6, β- and κ-caseins, respectively, after incubation for 0 min; lanes 2–4, β-casein after 30 min of hydrolysis by fractions *b–d*, respectively; Lane 5, molecular-weight standards (same as Panel A, Lane 1); lanes 7–9, κ-casein after 30 min of hydrolysis by fractions *b–d*, respectively. The κ-casein major digestion product is indicated by I, (15 kDa); II and III indicate the 13.0- and 12.5-kDa products resulting from κ-casein cleavage at the Tyr42-Tyr43 and Leu50-Ile51 bonds, respectively.

experimental system where the κ-casein is isolated, the same peptide bonds will not necessarily be split during coagulation since casein micelles are a much more complex system.

3.3. Cheese production and evaluation

Since in this study cheese making with vegetable (VR) and animal (AR) was performed simultaneously with the same equipment and reagents, the observed differences were mainly attributable to the action of the coagulant used. Both the floral extract and the commercial rennet coagulated milk in about 30 min at 35 °C, a time suitable for use on an industrial scale. The curd was firm and elastic in all instances and the whey produced was clear, indicating a high efficiency of the enzymes in coagulating the caseins. The change in pH of the mass obtained was slight, thus permitting a correct pressing. Cheese yields were equal—9% at the end of ripening—regardless of the type of coagulant used. At the beginning of ripening the VR cheeses showed no differences from the AR cheeses in either texture or color.

For all treatments, the acidity of the whey was 11 °D when the curd was cut; while the pH of the mass was 6.37 and 6.49 with VR or with AR, respectively. After cutting, stirring, and washing, the acidity was 9° D (pH 6.44) and 8.5 °D (pH 6.58) when using VR and AR, respectively.

In Fig. 3, panels A and B show the respective changes in pH at the surface and at the center of the cheeses over time during the ripening period, while Panel C presents the moisture profile during that ripening. Additional analyses of repeated measurements over time revealed significant differences (Tukey's test, $p < 0.01$) between the three treatments with respect to both the surface and the center pHs indicating a higher value with the use of the AR than with either of the VR preparations, with the VR-30 continually producing lower pH values than those of VR-40. At the end of ripening the pH at the surface of the cheeses was 5.31 ± 0.03 for the AR cheeses, and 5.10 ± 0.05 and 5.00 ± 0.06 for the VR cheeses brined for 30 (VR-30) and 40 h (VR-40), respectively; whereas the three respective pHs in the center of those cheeses was 5.30 ± 0.04, 5.10 ± 0.04, and 5.00 ± 0.04 (Fig. 3, Panels A,B). Similar values of pH were reported by Fernández-Salguero and Sanjuán (1999) and Sousa and Malcata (1997a) in an investigation of the biochemical evolution of cheeses made with extract of cardoon flowers.

Variance analysis and a means comparison (Tukey's test) showed no significant moisture differences ($p < 0.01$) at the end of ripening between the VR-40 cheeses and the AR cheeses (Fig. 3, Panel C). An increase in the brining time of the VR cheeses resulted in a reduction in moisture content, thus approximating the percentages of the AR control and at the same time being within the average moisture content of Gouda-type cheeses specified in the FAO Cheesemaking Manual (FAO, 1986).

During the maturation period, the degradation of the caseins was higher in the VR cheeses than in the AR cheeses. This difference meant that the WSN content (as a percentage of the TN, which is a measure of proteolytic activity) of the VR cheeses was approximately twice that of the AR cheeses (Fig. 4). Thus, the average WSN of the last three samples taken (at 30, 35, and 44 days) of the VR cheeses (at 21.8% of the TN) was 108% higher than the value for the AR cheeses (at 10.5% of the TN). Vioque et al. (2000) had observed differences in the WSN contents of Los-Pedroches cheeses after renneting by different plant coagulants of the Cardue tribe: for example, the WSN levels of cheeses renneted with extract of *C. cardunculus* were higher than the values for those treated with *C. humilis* preparations, but the WSN contents of the latter cheeses was, in turn, much higher than the values obtained for cheeses coagulated through the use of chymosin (Fernández-Salguero & Sanjuán, 1999). Equivalent results were reported by Sousa and Malcata (1997b) after comparing the production of ovine cheeses renneted with either flower extracts of *C. cardunculus* or AR prepared on a pilot scale. The proteolysis of the present VR cheeses was also influenced by the duration of exposure to salt (*cf.* Fig. 4): after the sixth day of maturation cheeses brined for the longer period of 40 h exhibited lower levels of the WSN/TN ratio than those brined for 30 h.

Electrophoretic analyses of the water-insoluble fractions from cheeses manufactured with either type of rennet were performed in order to assess which casein was the more susceptible to proteolysis in these systems. Before curdling, the intact caseins were quantified by gel scanning and densitometry analysis and the amounts of α- and β-caseins expressed as a percentage of the total insoluble-protein content during cheese ripening (Fig. 5). The degradation rate of both caseins in the AR and VR-40 cheeses was pronounced up to between 10 and 15 days of ripening, respectively, thereafter remaining almost constant until the end of the ripening. In contrast, the rate of casein hydrolysis of the VR-30 cheeses was almost linear throughout the entire 45 days. At the end of ripening, variance analysis and the comparison of means (Tukey's test) indicated no significant differences ($p < 0.01$) between the percentages of α-casein degradation after each treatment. With respect to the hydrolysis of β-casein, no differences were likewise observed between AR and VR-40 cheeses, but both were significantly different from the VR-30 cheeses (Table 1 in Supplementary Data). These results also confirm that proteolysis is influenced by the conditions of brine treatment.

Although slight differences were noted in pH values and chemical parameters studied for AR and VR cheeses, most data were comparable throughout the ripening process, especially with respect to those salted for 40 h. These results are in agreement with those reported by Galán et al. (2008), who compared the influence of different amounts of vegetable coagulant from cardoon and calf rennet on the production of ovine cheeses. These researchers

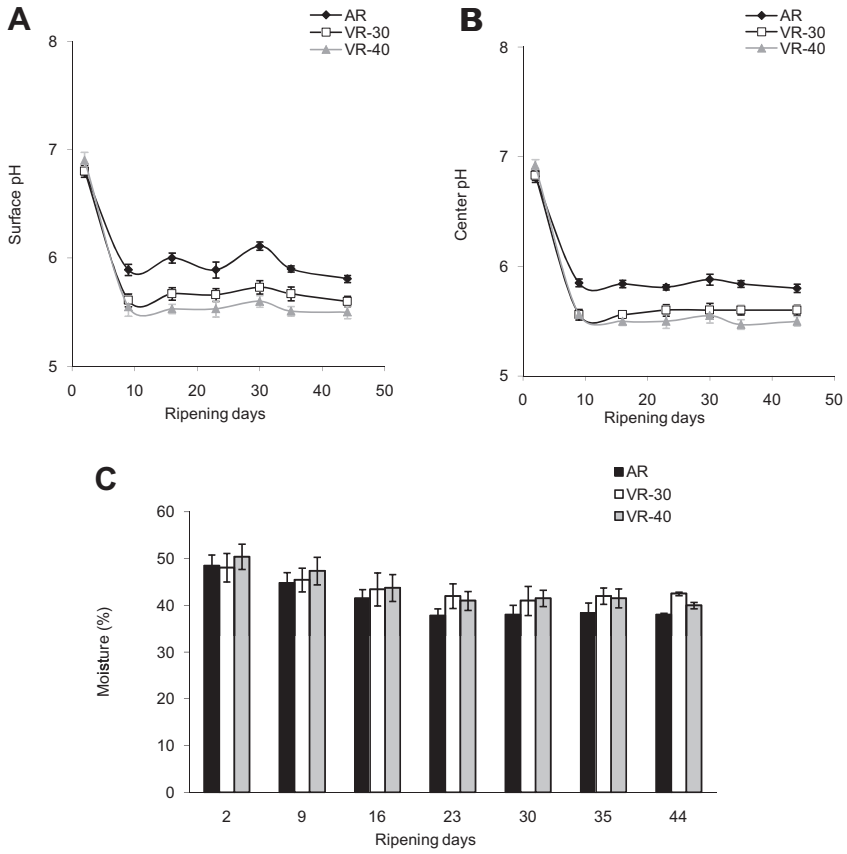

Fig. 3. Acidity (pH) and moisture of the different cheeses during ripening. (A) pH values at the surface of the cheeses, (B) pH values at the center of the cheeses, (C) cheese moisture content. Means ± standard error.

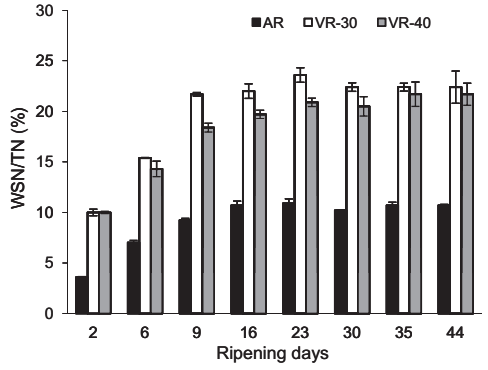

Fig. 4. Water-soluble nitrogen as a percent of the total nitrogen (WSN/TN × 100) during the ripening of AR and VR cheeses salted for 30 or 40 h. Means ± standard error.

observed no differences between the coagulants assayed for most of the chemical parameters they studied. However, significantly higher levels of casein hydrolysis were observed by them in cheeses produced with the double amount of vegetable rennet compared with those made with normal amount of the same rennet.

A trained sensory panel detected no significant differences between the three manufacturing conditions in the overall external and internal characteristics (in terms of appearance; uniformity; color; and the quantity, the size, and the distribution of the eyes present) and the texture (*i.e.*, elasticity, hardness, shear strength, and cohesiveness in the mouth). The sensory panel detected a bitter flavor in cheeses elaborated with the VR that was perceived noticeably in the VR-30 cheeses but only slightly in the cheeses kept in brine for 40 h. Similar results had been found by Brutti et al. (2012) when a trained sensory panel compared two commercial semihard cheeses with a cheese manufactured by treatment with an extract of *O. acanthium*. Cheese flavor is the end product of complex biochemical reactions, where in fact the proteolysis of cheeses during ripening plays a key role in the development of textural changes within the cheese matrix—*i.e.* agreeable flavor *versus* an off-flavor (Sousa, Ardö, & Mcsweeney, 2001). The concerted action of a protease carried over from milk clotting, the indigenous milk proteases, and the starter proteases provides suitable substrates for the starter peptidases, whose hydrolysis ultimately generates small peptides and free amino acids (Visser, 1993). The quantity of coagulant that is retained in cheese curd varies with the variety of cheese. With the Gouda type, the residual rennet activity could be about 15% of the original amount added, but also depends on the class and ratio of enzymes in the particular rennet used, those enzymes' stabilities to pH and temperature during the cheese making, and the influence of pH on the ability of the enzymes to bind to the caseins (Bansal, Fox, & Mcsweeney, 2009). Immersing the VR-curdled cheeses in brine for the longer period (*i.e.*, 40 h) prevented overproteolysis in these cheeses, thus avoiding the development of a background flavor. A balanced breakdown of the curd proteins into small peptides and free amino acids is necessary for the development of acceptable flavors and is determined by the choice of ripening conditions—*i. e.*, the storage temperature, relative humidity in the ripening room, and the duration of the ripening period (Forde & Fitzgerald, 2000). The presence of sodium chloride influences the flavor of cheeses not only because of its salty taste but also indirectly by affecting the intrinsic catalytic activity of cell-free enzymes (Macedo & Malcata, 1997),

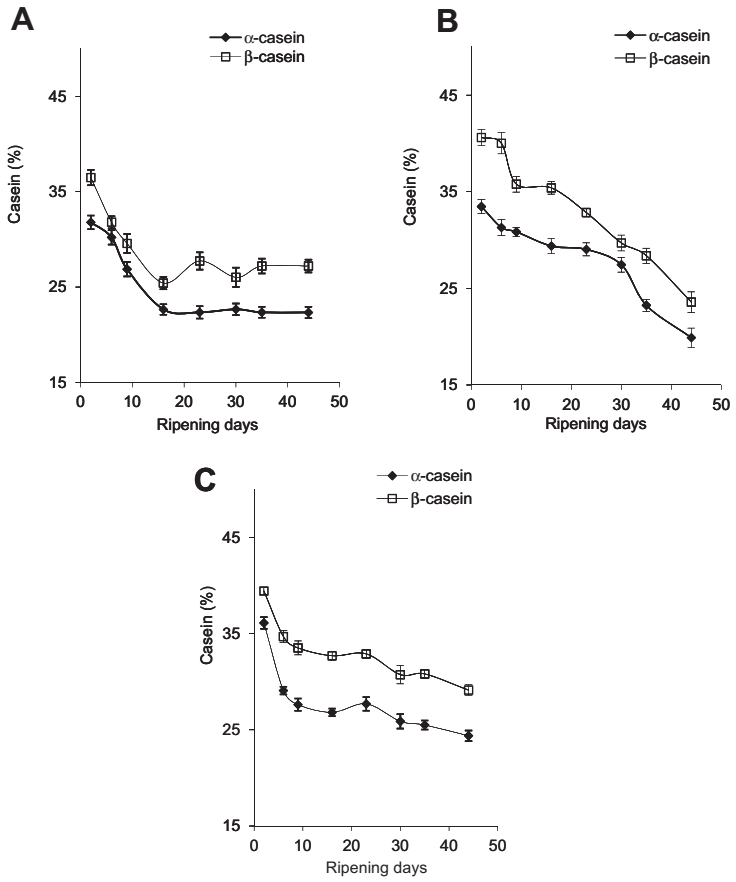

Fig. 5. Evolution of α- and β-casein during cheese ripening in: (A) AR cheeses, (B) VR-30 cheeses, and (C) VR-40 cheeses. In the figure the percent casein content is plotted as a function of the ripening time in days. Means ± standard error.

whose differing degrees of hydrolysis could explain the individuality of each VR cheese.

4. Conclusions

The results obtained in this study indicate that *C. scolymus*–flower extract is suitable as substitute for AR in Gouda-type cheese manufacturing, whith capability to be utilized by our domestic dairy industry since those floral extracts coagulated milk within a time appropriate for use on an industrial scale. Five proteolytic fractions (*a, b, c, d,* and *e*) with milk-clotting activity were isolated. Fractions *a, b,* and *c* belonged to the cardosin group as determined by peptide-mass fingerprinting.

We observed a higher level of casein-degradation products in cheeses made with *C. scolymus* proteases and brined for 30 h along with an accompanying bitter taste. Immersing the VR cheeses in brine for a longer period (40 h), however, prevented overproteolysis in these cheeses, thus almost entirely avoiding the development of that background bitterness. The type of coagulant had no significant effect upon the chemical parameters analyzed and pH values of the cheeses throughout ripening, and no significant differences were detected in the organoleptic properties between cheeses manufactured with *C. scolymus* brining for 40 h or animal rennet.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.03.007.

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