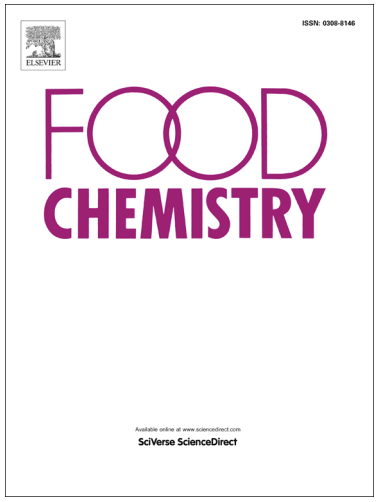

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Miniature cheeses made with blends of chymosin and a vegetable rennet from flowers of *Silybum marianum*: enzymatic characterization of the flower-coagulant peptidase

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ABSTRACT

Binary blends of *S. marianum*–flower extract and chymosin, as coagulant preparations, enabled the manufacture of miniature cheeses with distinctive characteristics compared to those of chymosin-renneted cheeses. The physicochemical parameters, sensory attributes of the cheeses, and *in-vitro* water-soluble antioxidant activity were analyzed and compared to those properties obtained from control chymosin-renneted cheeses. The preponderant proteolytic constituent in the flower extract was isolated in a two-step-purification protocol. The thus purified aspartic peptidase was maximally active at acidic pHs and exhibited a preference for peptide bonds between hydrophobic residues. Enzymologic characterization revealed differences in the kinetic parameters and specificity compared to other enzymes employed, such as rennet. *S. marianum*–flower extract, as a source of peptidase with distinctive characteristics, is a suitable substitute for chymosin in miniature-cheese production. The addition of vegetable rennet contributed to the development of an intense aroma and conferred antioxidant activity to the cheeses and wheys.

Keywords: Plant rennet, Miniature cheese, Antioxidant activity, Aspartic peptidase

1. INTRODUCTION

The relative abundance, attractive biochemical features, and biotechnologic potential of plant peptidases have prompted a screening of those enzymes in different wild plant species (Moreno-Hernández et al., 2016). The application of plant peptidases to different industrial processes had been previously successful. Peptidases from *Carica papaya* (papain) and *Ananas comosus* (bromelain) have high commercial significance: the uses of these enzymes have included meat tenderizing, beer chill-proofing, laundry- and dishwashing-detergent formulation, among others (Malek et al., 2016; Moreno-Hernández et al., 2016).

The flowers of cardoon (*Cynara cardunculus*) are used in the Mediterranean countries for traditional artisanal-cheese production because of the aspartic peptidases (APs) in those organs with the ability to coagulate milk. A *C. cardunculus* coagulant is used as rennet in the elaboration of high-quality ovine and caprine cheeses, whereas the manufacture of bovine cheeses in this manner is accompanied by an excessively bitter character (Almeida Malaquias et al., 2017; Rincón et al., 2017). Cardoon is a thistle from the genus *Cynara*, tribe Cardueae, family Asteraceae. The distinctive properties of the APs from thistle flowers have made them useful in a wide variety of industrial processes. Apart from cheese-making, other promising applications such as the production of bioactive peptides with antimicrobial and cytotoxic activity, the remodelling of the bone extracellular matrix, immobilization in chitosan sponges for drug delivery, and catalysis in organic media have been explored (Vairo Cavalli, Lufrano, Colombo, & Priolo, 2013).

Along with pepsin-like peptidases from various sources, typical APs from thistle belong to the A1A (pepsin) subfamily. All the peptidases in the pepsin subfamily are endopeptidases, mainly active at acidic pH, and with two aspartic residues responsible for the catalytic activity (Rawlings et al., 2014). Thistle APs cleave the Phe105-Met106 peptide bond in bovine κ-casein to coagulate milk enzymatically (Llorente, Obregón, Avilés, Caffini, & Vairo-Cavalli, 2014). With a stronger proteolytic action than chymosin, these enzymes eventually lead to an extensive breakdown of the caseins; thus producing cheeses with a soft buttery texture, a typical aroma, and slightly piquant flavor (Galán, Prados, Pino, Tejada, & Fernández-Salguero, 2008). O'Mahony et al. (2003) investigated the use of binary coagulant blends of *C. cardunculus* peptidases and chymosin for the manufacture of miniature Cheddar-type cheeses and reported that the incorporation of small amounts of *C. cardunculus* peptidases substantially altered the patterns of proteolysis in those cheeses.

The antioxidant activity of foods is significant for two reasons: protection from oxidative damage for consumers and food shelf life. Consumer responsiveness to the health implications

regarding food consumption is growing significantly, especially owing to the increasing literacy of consumers and their concerns towards healthier life-styles. Antioxidant activity has been considered a desirable nutritional property because an increased oxidative stress is involved in the onset of most age- and/or diet-related chronic diseases (Carocho et al., 2016; Fardet & Rock, 2017).

Even when the use of other vegetable rennets extracted by aqueous maceration has been described, only a few of those preparations have been confirmed as suitable for making commercial cheese (Rincón et al., 2017). Milk-clotting activity was previously reported for an aqueous extract (at pH 3.0) of *Silybum marianum* flowers, Cardueae tribe; and these flowers were proposed as a source of vegetable rennet potentially useful in the dairy industry (Vairo-Cavalli, Claver, Priolo, & Natalucci, 2005). In the present work, we hypothesized that blends of *S. marianum*–flower extract and chymosin, as coagulant preparations, might enable the manufacture of miniature cheeses with distinctive characteristics relative to those of chymosin-renneted cheeses. We also proposed that the major proteolytic enzyme present in those flower extracts had specific enzymatic features. In view of these hypotheses, the objectives of the work reported here were to utilize *S. marianum*–flower extract and chymosin blends as coagulant preparations to obtain miniature cheeses and to characterize those cheeses according to their physicochemical parameters, sensory attributes, and *in-vitro* antioxidant activity. In addition, we also aimed at purifying and characterizing the preponderant proteolytic constituent in the aqueous extract of *S. marianum*. To the best of our knowledge, this work constitutes the first example of this enzyme extract being added as an alternative to rennet in the manufacture of cheeses.

2. MATERIALS AND METHODS

2.1. Flower-enzyme extract

Flowers of *S. marianum* (L.) Gaertn. were collected from plants grown in La Plata, Buenos Aires, Argentina (voucher specimen LPE 1162, Facultad de Ciencias Exactas, UNLP). The enzyme extract (EE) was obtained according to Vairo Cavalli et al. (2005) with slight modifications. The flowers were ground in a mortar under liquid nitrogen, homogenized in 25 mM citric acid, 25 mM sodium citrate buffer (pH 3.0) at 1 g per 3 ml, stirred for 30 min. and centrifuged at 5,000 × *g* for 20 min at 4 °C.

2.2. **Formulation of miniature Cheddar-type cheese from bovine milk**

First, the milk-clotting activity (MCA) of 100-µL of EE or of dilutions of a commercial protease (chymosin: Chy-Max® Extra rennet powder, 2,080 international milk-clotting units (IMCUs) per g (Christian. Hansen, Inc.; Hørsholm, Denmark) was measured in 1 mL of 12.5 % (v/v) skim milk (Ilolay, Argentina) in 30 mM $CaCl_2$ at 37 °C, following the procedure described by the International Dairy Federation (1992).

Miniature (20 g) Cheddar-type cheeses were manufactured from ultrapasteurized whole milk (Armonía, Mastellone Hnos SA, Argentina) after the procedure of Shakeel-Ur-Rehman et al. (1998) with modifications. A direct vat-set culture, Mesophilic Chr-Hansen R-703™ (Christian Hansen, Inc.), was used as the starter culture at a concentration of 0.013 g/L, with $CaCl_2$ being added to the milk at final concentration of 300 mM; next, the milk was placed in a water bath at 37 °C until a pH of 5.4 was reached. Then one of three coagulants was used: 100% (w/v) chymosin (1/3000 dilution from a stock solution with 50 IMCUs/mL Chy-Max® Extra rennet powder), 90% chymosin/10% EE, and 75% chymosin/25% EE; hereafter abbreviated Q100:S0, Q90:S10, Q75:S25, respectively. With each preparation having an equal total MCA, four miniature cheeses were manufactured for each one. Those cheeses were produced in wide-mouth plastic centrifuge bottles (100 mL) filled with 100 mL of milk and inoculated with the renneting preparation; the bottles were covered immediately and mixed by inversion three times and then kept until a firm coagulum was formed (50 min). The coagulum in each bottle

was next cut manually with a plastic tool at room temperature and left for 5 min; finally, the preparation was stirred slowly for 5 min with a glass rod. The temperature was increased gradually from 33 to 42 °C for 35 min, and the bottles were then centrifuged at room temperature for 30 min at 1,700 × *g*. The whey was separated from each tube and the curds in the centrifuge bottles placed in a water bath at 36 °C; after 15 min, the cheeses thus produced were inverted in the bottles and centrifuged again at 1,700 *g* for 20 min to obtain a regular smooth surface on both sides. In this manner, the resulting miniature cheeses, of spherical shape (3.9 cm), were obtained. The cheeses were brine-salted (20% NaCl, 0.05% $CaCl_2.2H_2O$ [w/v]) for 40 min at room temperature, wiped with tissue paper, and ripened at 4 °C and 60% relative humidity for 1 month. During the first week, the cheeses were inverted every day and afterwards every two days.

2.3. **Analysis of cheeses**

At the end of the ripening period, the cheeses were analyzed for total protein content by the micro-Kjeldahl method, moisture by oven drying, water activity by dew-point hygrometry (Aqualab series 3, Decagon Devices Inc., USA), and internal pH by direct contact of the cheese with a lance-tipped pH electrode (Hanna Instruments, Bs As, Argentina). The surface color L*, a*, and b* parameters (Hunter parameters) were measured with a hand-held chromometer CR-400™ (Konica Minolta, Japan), where L* indicates lightness and a* and b* the respective red-green and yellow-blue coordinates. To determine the total color difference (ΔE*) between Q100:S0 and Q90:S10 or Q75:S25, the following formula was used:

$$\Delta E^* = [\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}]^{1/2}$$

Each analysis was performed in duplicate, except for the pH and the color—those being evaluated in triplicate and quintuplicate, respectively.

The protein profile was assayed in the cheeses by tricine sodium-dodecyl-sulfate–polyacrylamide-gel electrophoresis (SDS-PAGE) according to the method of Schägger & von Jagow (1987). For visualization of the bands, the gels were stained with Coomassie blue G-250.

A hedonic test of the cheeses was performed by a panel of 18 judges (all inexpert individuals with no formal cheese training), the participants being students and employees of the University of La Plata who reported liking cheese and consuming that commodity frequently. None of the participants had previous or present taste or smell disorders. Informed consent was obtained from all those subjects. In order to detect acceptability for a given attribute, a 5-point scale was used—with 1 being the worst and 5 the best quality—to yield data on the magnitude of liking or disliking the resulting sensations. Three samples, one of each product, were presented to each panellist. The appearance, texture, aroma, and flavor of the cheeses were evaluated at the end of the ripening period. Precautions were taken to randomize the samples, mask the identity, and minimize the effects of contrast and adaptation.

2.4. **Antioxidant activity**

The ABTS radical cation [2,20-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] was generated according to Luo et al. (2013) by mixing a 7.0 mM ABTS stock solution with 2.45 mM potassium persulphate in the dark at room temperature for 16 h. The assay was made in 96-well microplates. The samples were the Q100:S0, Q90:S10, Q75:S25 cheeses and the corresponding wheys. The whey samples were prepared by separation in centrifugal filter tubes (Amicon® Ultra Centrifugal Filters with Ultracel-3–kDa membrane, Millipore, USA) to obtain fractions with molecular masses above and below 3 kDa). The cheese samples were obtained by disaggregation of 0.5 g in 5 mL of water, centrifugation at 4,000 × *g* for 20 min, and collection of the supernatants. Different blanks were prepared by mixing the enzyme blends with water in place of milk in the same ratio as employed in the manufacture of the cheeses. Aliquots of 2 µL of the reactions were mixed with 200 µL of sample. After 10 min, the absorbance was measured at 734

nm in an Infinite Pro M200-TECAN™ plate reader (Tecan Trading AG, Switzerland). The results were expressed as trolox equivalents through the use of a calibration curve involving 0.05–2.50 mg/mL trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic-acid). In addition, the ABTS percent scavenging activity (ABTS-SA%) was calculated by the following equation:

$$\text{ABTS-SA\%} = [(AB_{0\,min} - AS_{10\,min}) - (AB_{0\,min} - AB_{10\,min})\,] \times 100/AB_{0\,min}$$

where $AB_{0\,min}$ and $AB_{10\,min}$ are the absorbances without sample at 0 and 10 min, respectively, and $AS_{10\,min}$ is the absorbance with sample at 10 min.

2.5. **Enzyme purification**

EE was analyzed by zymogram after isoelectric focussing. For both assays the methods described by Brutti et al. (2012) were followed. An EE (4 mL) was applied to a HiLoad Superdex 200™ column equilibrated with 25 mM Tris-HCl, pH 7.6 (Buffer A) and eluted at 1 mL/min. Each peak of absorbance at 280 nm was collected and assayed for enzymatic activity. The active fraction was applied to a Mono Q HR 5/5™ column preequilibrated with Buffer A. The proteins were eluted with a linear NaCl gradient (0–0.4 M) in Buffer A (flow rate of 0.75 mL/min) and the protein peaks collected and assayed for activity. The protein concentration was determined in triplicate by bicinchoninic-acid method (Smith et al., 1985) with bovine-serum albumin as standard. SDS-PAGE (14%) was performed according to Laemmli (1970).

2.6. **Enzyme characterization**

2.6.1. Enzyme assay and kinetics

The synthetic peptide 7-methoxycoumarin-4-acetyl- (MCA-)KPAEFFAL-DNP (-dinitrophenyl) was used as the substrate for determining endopeptidase activity (Vairo Cavalli et al., 2008). The rate of hydrolysis of the peptide bond Phe-Phe was monitored by excitation of the MCA fluorophore at 328 nm. Light emission was detected at 393 nm in a Perkin Elmer Luminescence Spectrometer LS 50 B™. The effect of pH on enzyme activity was measured with

the synthetic peptide as substrate at different pH values [50 mM acetic acid, 50 mM sodium acetate plus 140 mM NaCl (pH 3.5 – 5.6); 50 mM MES-NaOH plus 140 mM NaCl (pH 6.0 – 6.6); and 25 mM Tris-HCl plus 140 mM NaCl (pH 7.6)]. The experimental conditions for the kinetic studies were: 37 °C and pH 5.6, with substrate concentrations ranging from 0.27 to 3.24 µg/mL. The kinetic parameters were calculated through the use of the Sigma Plot Enzyme Kinetic Module™ (Systat Software Inc., Chicago, IL, USA).

All the experiments were performed in triplicate, and the values were treated as separate data points.

2.6.2. Digestion of the oxidized insulin β chain

The insulin β chain (1 mg/mL) was digested at 37 °C with the purified EE enzyme (enzyme:substrate ratio, 1:50) in 0.1 M formic acid, pH 3.1, or in 0.1 M citric – 0.1 M phosphate buffer, pH 6.0. Aliquots of 100 µl were removed at appropriate time intervals (0, 1, 3, 6, 12, and 18 h) and subjected to high-performance liquid chromatography in a Merck-Hitachi system with a L-6200A pump, an L-4000 ultraviolet detector, and a D-2500 integrator on a BioRad BioSil 318-10™ C-18 column (250 x 4.6 mm). The peptides were eluted at 1 mL/min with a linear gradient of acetonitrile (0 to 80%) in 0.1% (v/v) aqueous trifluoroacetic acid. The effluent was monitored at 215 nm. Aliquots of the resulting peptide fractions after hydrolysis at pH 3.1 were dissolved in water and subjected to an Edman degradation of the N-terminal amino-acid sequences, in an Applied Biosystem 473-A™ protein sequencer.

2.6.3. Determination of the catalytic mechanism

The mechanistic class was determined through the use of a set of specific protease inhibitors on the digestion of the insulin β chain. The enzyme isolated was preincubated (15 min) at room temperature with different specific peptidase inhibitors: 90 µM pepstatin A, 24 µM E-64 (a cysteine-protease inhibitor), 2.5 mM PMSF (phenylmethylsulfonyl fluoride, a serine-protease

inhibitor), or 12 mM ethylenediaminetetraacetic acid (EDTA, a metallopeptidase inhibitor). The detection of remnant proteolytic activity was assessed by insulin β-chain degradation at pH 3.1 under the experimental conditions described in Section 2.6.2 above.

2.7. **Statistical analysis**

All the data were reported as the means ± the standard deviations of three or five technical replicates according to the method used. The results of the physicochemical parameters, antioxidant activity, and sensory analyses were evaluated by the one-way analysis of variance (ANOVA; GraphPad Prism 6, GraphPad Software Inc., La Jolla, CA 92037 USA). Significant differences between the means of each parameter were determined by Dunnett's or Tukey's test (p <0.05), depending on whether the data comparisons were made to one particular group (reaction blank) or to a set of groups of data against each other, respectively.

3. RESULTS AND DISCUSSION

3.1. ***Cheese properties and sensory analysis***

The presence of APs in aqueous extracts of *S. marianum* flowers with milk-clotting activity has been previously demonstrated (Vairo Cavalli, Claver, Priolo, & Natalucci, 2005). In the present work, we used binary coagulating blends of aqueous extracts of this flower species with chymosin for the manufacture of miniature bovine-milk cheeses. Both the plant extract and the selected dilution of a stock commercial rennet coagulated milk in about 35 min at 37 °C. Regardless of the rennet used (*i. e.*, Q100:S0, Q90:S10, Q75:S25), the curd was firm and elastic and the whey produced clear and slightly yellow. As a by-product, a small amount of ricotta was obtained when the process included the EE because of the acid pH of that EE. At the end of the ripening time, the cheeses tended to be crumbly. Table 1 compares the yield and main physicochemical properties of those cheeses. The greater was the proportion of EE used in the coagulant blends, the lower was the yield of cheese obtained. The Q100:S0 cheeses contained

the lowest percentage of dry matter and the highest water activity; with both parameters being significantly different (p <0.05) from the cheeses manufactured with the blends containing EE. On the contrary, variance analysis and a comparison of the means by Tukey's test revealed no significant pH differences between the different treatments (p >0.05), with those values being consistent with the data reported by Shakeel-Ur-Rehman *et al*. (1998) for miniature Cheddar-type cheeses.

In terms of the color parameters, a* was clearly less negative at an increased ratio of EE in the coagulants (with +a* being the red direction and –a* the green), while b* showed no significant differences (p >0.05). Thus, the incorporation of EE in coagulant blends made the cheeses more red and less green than with pure chymosin as the renning agent, with that difference possibly being attributable to the fuchsia color of the EE. A significant decrease in the parameter L* occurred in the cheeses renneted with Q75:S25 relative to the values for the Q100:S0 or Q90:S10 cheeses (Table 1); moreover, to the naked eye a uniform lightness was not perceived for the Q75:S25 cheeses. This difference would also be expected from the standard-deviation value. The total color difference (□E) of 11.0 revealed the greatest discrepancy between the Q100:S0 and the Q75:S25 cheeses, whereas the difference between the Q100:S0 and Q90:S10 cheeses was considerably lower at a value of 1.70 (Table 1).

Even though the same amount of calcium had been added in the preparation of all the cheeses, a lower final calcium content was obtained at the highest proportion of EE used in the coagulant blends (Table 1). Since the residual rennet activity may be higher in cheeses with a low calcium content, calcium can impact on cheese ripening by affecting the rate of proteolysis and also the cheese's final textural properties (Soodam, Ong, Powell, Kentish, & Gras, 2015; Upreti & Metzger, 2006).

Fig. 1 depicts the cheese-protein profiles at the end of ripening time upon resolution by tricine SDS-PAGE; the α_{s1}-, α_{s2}-, and β-caseins were identified, as well as whey proteins entrapped within the cheese matrices (*i. e.*, lactoferrin, seroalbumin, α-lactoalbumin, and β-

lactoglobulin). The same electrophoretic profile was obtained regardless of the coagulant blend used. This result is consistent with the protein content of the cheeses, which level was to some extent lower for only the Q75:S25 cheeses. The lower yield, as well as the lower protein content in the Q75:S25 cheeses, can be attributed to the proteolytic activity of the APs within the EE.

Table 1 summarizes the *in-vitro* antioxidant activity of the exuded wheys and cheeses. The whey from Q100:S0 cheeses did not contain a significant difference in antioxidant activity from that of the reaction blank (Dunnet's test; p >0.05). With the exuded-whey >3-kDa fraction, significant differences in antioxidant activity (Tukey's test; p <0.05) were observed between those obtained with the Q90:S10 or Q75:S25 coagulants, with the level of the latter sample being 12.3% higher than that of the the former. The percentage of antioxidant activity corresponding to the whey >3-kDa fraction from the Q75:S25 cheeses was equivalent to 0.87 ± 0.08 mg/mL of trolox and was 17.3% higher than that of the enzyme blank. Moreover, this whey fraction exhibited 18.3% more antioxidant activity than the whey <3-kDa fraction. For this reason, a part of total antioxidant activity could be attributed to peptides released by proteolysis, especially those with masses greater than 3 kDa. Nevertheless, we need to mention here that the enzyme blank (of the Q75:S25 blend) manifested residual antioxidant activity probably caused by the presence of phenolic pigments (of low molecular weight). Because of the large volumes produced and the nutritional composition, the design of new processes that take advantage of the potential value of whey, a principal by-product of cheese manufacturing, represents a major challenge to the dairy industry (Parra Huertas, 2009); especially since this commodity, in addition, has health-benefitting properties, such as the antioxidant capability. Finally, an analysis of the soluble antioxidant activity of the cheeses indicated significant differences between the Q75:S25 and the Q100:S0 coagulant combinations (Tukey's test; p <0.05). The value of the antioxidant activity of the Q75:S25 cheeses was 7.1 ± 0.9%. From this value and upon consideration of the dilution employed in sample preparation, we estimated the specific antioxidant activity to be equivalent to about 0.64 mg of trolox per g of cheese. In

cheeses, different compounds can contribute to the antioxidant activity in the manufactured product. In addition, the plant-enzyme preparations employed to clot milk can possess pigments as well as other antioxidant species. The review published by Brewer (2011) describes the presence of antioxidant compounds in different plants (*e. g.*, phenolic acids, phenolic diterpenes, flavonoids, volatile oils, anthocyanins, and anthocyanidins), as well as their mechanisms of action and their potential application to foods. Moreover, clotting enzymes can contribute a residual proteolytic activity during the manufacturing, ripening, and storage of cheeses. Thus, antioxidant peptides can be released from milk proteins and can remain in the cheese or in the sweet whey. According to the review of Fardet and Rock (2017), cheeses present the highest antioxidant potential among all the dairy products, probably because of their higher protein content along with the nature of the fermentation process. In fact, Silva et al. (2006) found peptides with antioxidant activity in ovine and caprine cheese-like systems, manufactured with proteases from *C. cardunculus*–flower APs.

In the present work, a sensory panel evaluated the Q100:S0, Q90:S10, and Q75:S25 cheeses in terms of the following attributes: appearance, texture, aroma, and flavor, as well as with respect to the cheeses' overall acceptability. The evaluators mostly considered that the appearance, texture, and general acceptability attributes of the three types of cheeses pleased them or did so very much (Fig. 2). As regards the aroma (Fig. 2, Panel C), most of the evaluators were indifferent to that of the cheeses produced with chymosin alone, whereas the aromas of the Q90:S10 and Q75:S25 cheeses were very pleasing to them. This result is similar to that reported by Rincón et al. (2017), who found a higher odor intensity for *C. cardunculus*–renneted cheeses from goat milk than for those renneted with chymosin. That evaluation is also in agreement with the broader literature on thistle-flower cheeses. The greatest dispersion of appreciations for the three types of cheeses was observed regarding taste (Fig. 2, Panel D), where a net pattern of pleasure or dislike could not be established. In addition to those hedonic scales, the evaluators expressed their specific perceptions on the particular attribute of taste,

where approximately one-third of the panelists found a slight bitter savor in the cheeses, regardless of the rennet used in the manufacture. Aroma is one of the first stimuli to be perceived before consumption and is a principal component of the sensory properties of cheeses for consumer acceptance and preference (Niimi et al., 2015). Cheeses manufactured with vegetable rennet exhibited a special and intense aroma according to the evaluators' perception, and thus the coagulant blends with EE contributed to the development of such a favorable character of aroma.

3.2. **Enzyme purification (Fig. 3)**

Isoelectric focussing followed by zymogram development enabled an estimation of the isoelectric point of the APs present in EE. Most of the proteins in the crude extract had isoelectric points considerably lower than 7.0 (Fig. 3, Panel A, Lane 2), and zymogram development revealed a single active band corresponding to a pI of 4.8 (Fig. 3, Panel A, Lane 3). This result is in agreement with those obtained for other Asteraceae APs. Similar observations had been reported for flower-enzyme preparations of *Arctium minus, Cynara scolymus*, and *Onopordum acanthium* (Brutti et al., 2012; Cimino, Liggieri, Priolo, Bruno, & Vairo Cavalli, 2010; Llorente, Brutti, & Caffini, 2004; Tamer, 1993).

The purification strategy included a step of size-exclusion chromatography to eliminate the substantial amount of phenolic compounds and anthocyanin pigments present in the EE of *S. marianum* flowers. This filtration through a molecular sieve enabled the separation of an endopeptidase-active fraction (Fraction a, Fig. 3, Panel B). This fraction hydrolyzed insulin at pH 3.1 and the synthetic peptide substrate MCA-KPAEFFAL-DNP at pH 4.7. Based on the pI value of the proteolytic fraction, as estimated by the zymogram development after isoelectric focussing depicted in Panel A, anion-exchange chromatography was selected for further purification. Three protein fractions were eluted by application of a linear NaCl gradient (Fig. 3, Panel C). The main protein fraction (Peak I), eluted at 0.21 M NaCl, exhibited proteolytic activity on both insulin and

the fluorogenic peptide as the substrates. Table 2 summarizes the scheme used for the purification of the enzymatic activity of this fraction. Veríssimo et al. (1995), studying flowers of *C. cardunculus*, found that APs constituted 60% of the total protein of mature stigmas, which figure could explain the poor fold purification obtained in the present work. Fig. 3, Panel D illustrates the SDS-PAGE of the purified enzyme under reducing and nonreducing conditions. While under nonreducing conditions a single band of 44 kDa was obtained, reduction with 2-mercaptoethanol produced two bands of 13 and 29 kDa (Lane 1 of the panel). This result is consistent with the molecular weights of other aspartic peptidases from Asteraceae flowers. The cyprosins and cardosins from *C. cardunculus* comprise two subunits, one small (13–16 kDa) and one large (31–35 kDa), as a result forming a dimeric enzyme of ~45 kDa (Heimgartner et al., 1990; Veríssimo et al., 1996).

3.3. **Proteolytic specificity, catalytic mechanism, and kinetic parameters**

The Supplementary Material (sections 1 and 2) summarizes the cleavage patterns of insulin hydrolyzed with the purified AP under different conditions. The proposed catalytic mechanism for the purified *S. marianum* peptidase was confirmed by preincubation with group-specific inhibitors. E-64, EDTA, and PMSF produced no enzyme inhibition (Supplementary Material Section 2). That EDTA, in fact, activated the enzyme by about 3-fold was indeed notable; and that phenomenon needs to be further investigated, though such an activation might be readily explained by a chelation of some ion that otherwise suppresses enzyme activity. In contrast, pepstatin A produced a 75% inhibition of the purified AP under those same assay conditions. This last result is consistent with the well documented characteristic inhibition of phytepsins by pepstatin A (Kervinen & Wlodawer, 2013).

The same insulin-cleavage pattern was obtained after 1, 3, or 6 h of incubation with the purified AP at pH 3.0. The concentration of two of the released peptides increased over time, whereas new products of hydrolysis were detectable only after 12 h of reaction (Supplementary

Material Section 1). The most susceptible cleavage site after prolonged incubation times with insulin as the substrate was at Tyr16-Leu17, followed by Thr27-Pro28 and Phe25-Tyr26. According to these results the specificity of this AP is different from that of other related Asteraceae APs employed as vegetable rennets (Supplementary Material Section 3). The cleavage at position Phe25-Tyr26 had been reported previously for cardosins (Sarmento et al., 2009; Veríssimo et al., 1995). Cardosins A and B are also capable of splitting insulin at the bonds Leu15-Tyr16 and Leu17-Val18; while cardosin B, though less specifically, also hydrolyzes other peptide bonds (Veríssimo et al., 1995). Although all milk-clotting APs share a preference for peptide bonds with hydrophobic residues, *S. marianum* AP has a more restricted specificity than calf chymosin—the reference milk coagulant—and human pepsin—it a model AP (Athauda & Takahashi, 2002; Nedjar, Humbert, le Deaut, & Linden, 1991).

The insulin-cleavage profiles were found to be significantly different at the various pHs assayed, with the hydrolysis at pH 3.1 being more specific and efficient than at pH 6.0 (Supplementary Material Section 1, panels B and F, respectively). This difference is to be expected since alterations in specificity at different pH values depend on the degree of protonation of the amino-acid side chains at the catalytic subsites in the peptidase (Dunn et al., 1986). The cleavage specificity of several pepsin-like APs has also evidenced significantly altered specificities between different pH values (Athauda & Takahashi, 2002). The pH dependence of the purified AP activity in the present work was studied following the hydrolysis of the model substrate MCA-KPAEFFAL-DNP with a single scissile bond (Phe-Phe [*i. e.*, FF]). An optimum pH of 5.6 with this substrate was detected, but no activity was recorded at pHs below 4.0. A maximum activity at pH 4.5–5.0 had been reported for cardosin A with the same substrate (Castanheira et al., 2005), whereas barley phytepsin manifested maximum activity with the substrate PTEFRL-Nph (where Nph = p-nitrophenylalanyl) between pH 3.5 and 4.1 (Kervinen, Sarkkinen, Kalkkinen, Mikola, & Saarma, 1993). Contrary to expectations, at pH 7.6 the purified AP from *S. marianum* still retained about 50% of the maximal activity.

The kinetic data were well described by the classical Michaelis-Menten model (21 degrees of freedom and a correlation coefficient squared of 0.96). For substrate cleavage (pH 5.6), the Km and Vmax were 25.4 μM and 0.604 $\mu M.min^{-1}.mg^{-1}$ of enzyme, respectively. For a chromophoric derivative of the same peptide, the Km values for cardosins A and B (Veríssimo et al., 1996) were about 4 times higher than that obtained for the purified AP from *S. marianum*. The use of a set of four chromophoric peptides for the study of cyprosin 3 had previously indicated Km values between 15 and 280 mM (Cordeiro et al., 1998).

4. CONCLUSIONS

The use of binary coagulant blends of *S. marianum* aqueous extract and chymosin were suitable for the manufacture of miniature cow cheeses. Lower cheese yields and calcium contents were obtained with greater proportions of EE used in the coagulant blends. The Q100:S0 cheeses exhibited the highest aqueous activity and the lowest percentage of dry matter, but no significant pH differences were found among the different treatments. The incorporation of EE into coagulant blends made the cheeses redder and less green than the color of the Q100:S0 preparations. The same protein electrophoretic profile of the insoluble fractions of the cheeses was obtained independently of the coagulant blend used. Antioxidant activity was detected in both the Q75:S25 cheeses and in their exuded wheys. Cheeses manufactured with vegetable rennet exhibited an intense characteristic aroma according to the evaluators' perception.

A heterodimeric peptidase was isolated from the aqueous extract used as the rennet substitute by size-exclusion and anion-exchange chromatography. Only pepstatin A produced a significant inhibition of the peptidase isolate. Kinetic and specificity studies revealed distinctive characteristics for the purified peptidase.

The hypotheses proposed in this work were confirmed. *S. marianum*–flower extract, as a source of an AP with distinctive characteristics, was suitable for replacing part of the animal

rennet normally used in the production of miniature cheeses and in so doing resulted in the development of an intense aroma in the cheese and at the same time conferred an *in-vitro* antioxidant activity to both the cheese and the whey.

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CONFLICT OF INTEREST STATEMENT

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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LEGENDS TO THE FIGURES

Fig. 1. Tricine SDS-polyacrylamide-gel electrophoresis of the insoluble fractions of miniature Cheddar-type cheeses manufactured through the use of 100% chymosin, 90% chymosin/10% *S. marianum*–enzyme extract, or 75% chymosin/25% *S. marianum*–enzyme extract as coagulant (lanes 1, 2, and 3, respectively) after 1 month of ripening. Lane 4: molecular weight markers low-range–protein-ladder standards (3.4–100 kDa, Thermo).

Fig. 2. Histogram of the subjective-reaction categories with respect to the experience of the cheeses (expressed as) I to V (I, I dislike it a lot; II, I dislike it; III, I am indifferent to it; IV, I like it; and V, I like it a lot) in relation to the attributes of appearance (Panel A), texture (Panel B), aroma (Panel C), taste (Panel D), and general acceptability (Panel E). In the panels, the percent of the evaluators is plotted on the *ordinate* for each of the five categories of evaluation on the *abscissa*. Bar shades denoting the cheeses prepared by the indicated renneting mixtures: Q100:S0, black bars; Q90:S10, light-gray bars; and Q75:S25, dark-gray bars

Fig. 3. Purification and characterization of the main aspartic-protease fraction from *S. marianum*–flower extract. The peptidases present were purified by size-exclusion

chromatography followed by ion-exchange chromatography; and the enzymatic activity was assayed by the milk-clotting ability and the hydrolysis of both the substrates insulin and the typical AP fluorogenic synthetic peptide MCA-KPAEFFAL-DNP.

Panel A: Isoelectric focussing (lanes 1 and 2) and zymogram (lane 3) of *S. marianum*–flower extracts. Lane 1: pI markers (3.6–9.3): *Aspergillus niger* amyloglucosidase (pI 3.6), soybean trypsin inhibitor (pI 4.6), bovine-milk ß-lactoglobulin A (pI 5.1), bovine-erythrocyte carbonic anhydrase II (pI 5.9), human-erythrocyte carbonic anhydrase I (pI 6.6), species-heart myoglobin (pIs 6.8 and 7.2), *Lens culinaris*–lentil lecitin (pIs 8.2, 8.6, and 8.8), bovine-pancreas trypsinogen (pI 9.3); lanes 2 and 3: crude enzyme extract (EE). The zone of proteolytic activity appeared as a clear area when the hemoglobin-agarose film was stained with Coomassie brilliant blue (indicated with an arrow).

Panel B: Size-exclusion chromatography. A sample (4 ml) from an acidic extract was applied to a HiLoad Superdex 200™ column equilibrated with 25 mM Tris-HCl, pH 7.6. In the figure, the absorbance at 280 nm is plotted on the *ordinate* as a function of the elution volume on the *abscissa*. The *a* indicates the endopeptidase-active peak that was further characterized.

Panel C: The active fractions eluated from Superdex 200 (Peak a in Panel B) were pooled and further purified by ion-exchange chromatography in an HR 5/5 Mono Q™ column equilibrated with 25 mM Tris-Cl, pH 7.6. The elution was performed with a linear gradient of 0.5 M NaCl in the equilibration buffer at a flow rate of 0.75 ml/min. In the figure, the absorbance at 280 nm (blue solid curve), to the left of the *ordinate*, and the molarity of NaCl in the elution gradient (pink broken curve), to the right, are plotted as a function of the elution volume on the *abscissa*. The assay for endospeptidase activity on MCA-KPAEFFAL-DNP determined at pH 4.7, insulin hydrolysis at pH 3.1, and milk-clotting activity of peaks I–V eluted from the column revealed only one active fraction (indicated between dotted vertical lines).

Panel D: The purified peptidase was analyzed by SDS-PAGE under reducing (R, lanes 1 and 2) and nonreducing (NR, lanes 3 and 4) conditions. The Mono-Q™-ion-exchange column eluate

(Peak I from Panel C) was run in lanes 1 and 3 and the molecular-weight markers Precision Plus Protein Standards™ (10–250 kDa, BioRad) in lanes 2 and 4.

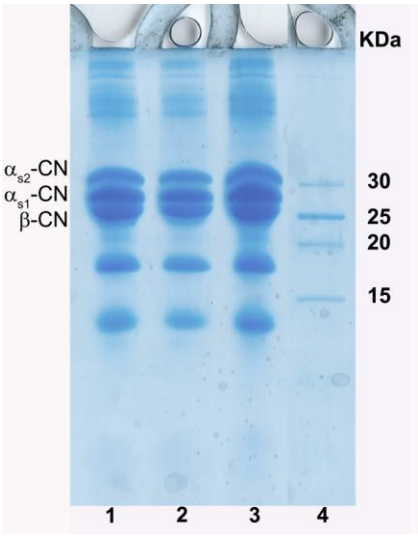
Tables

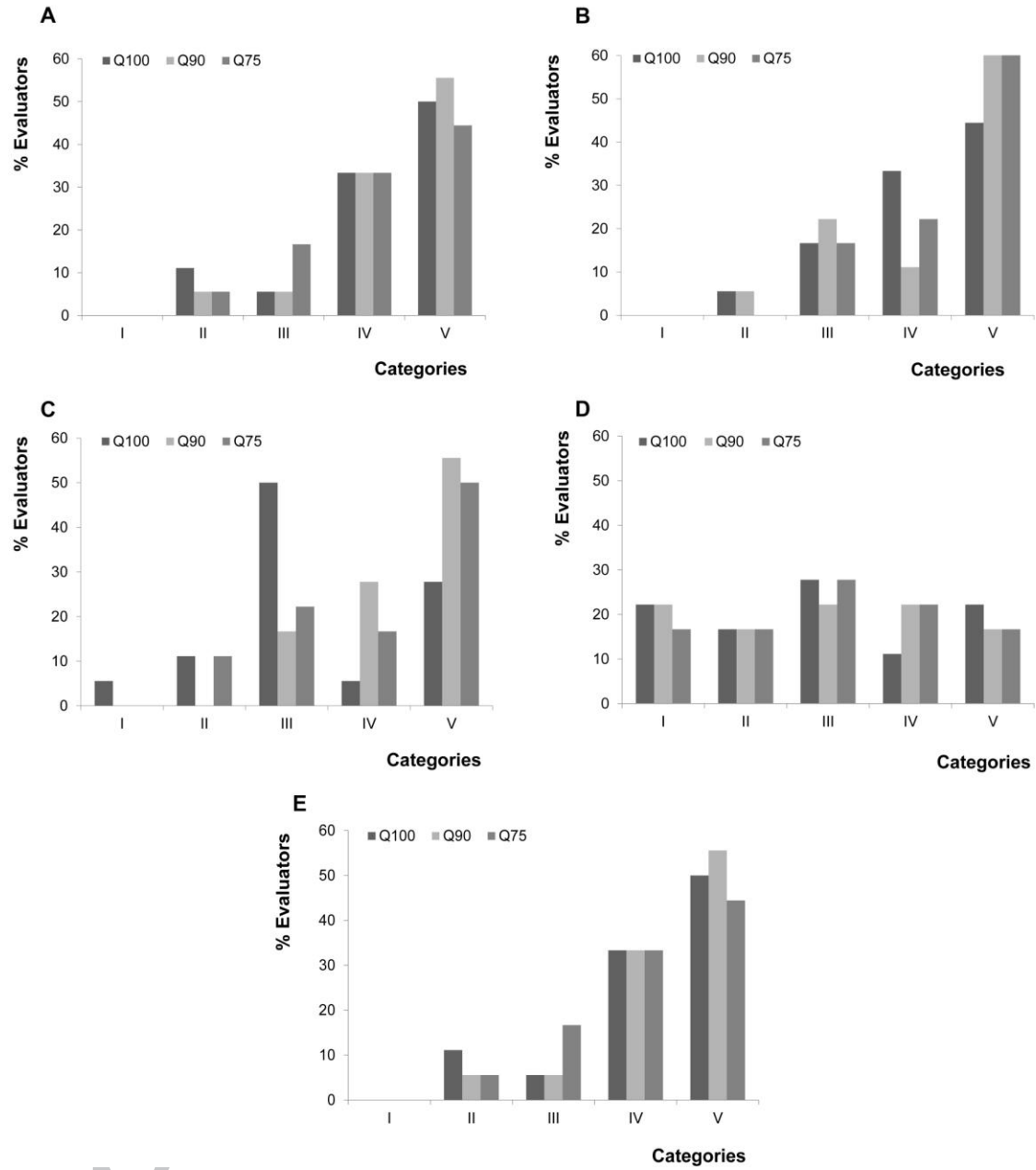
Table 1. Comparison of yield, percent dry weight, water activity, internal pH, surface color, and total protein and calcium content of miniature cheeses**

Table 2. Protease activity during the purification steps of the enzyme from flowers of *S. marianum*

Highlights

- Blends of *S. marianum* extract and chymosin were used for manufacture of cheeses
- Cheeses manufactured with vegetable rennet exhibited a special and intense aroma
- *In-vitro* antioxidant activity was detected in cheeses and exuded whey
- Distinctive kinetic and specificity was detected for *S. marianum* flower peptidase

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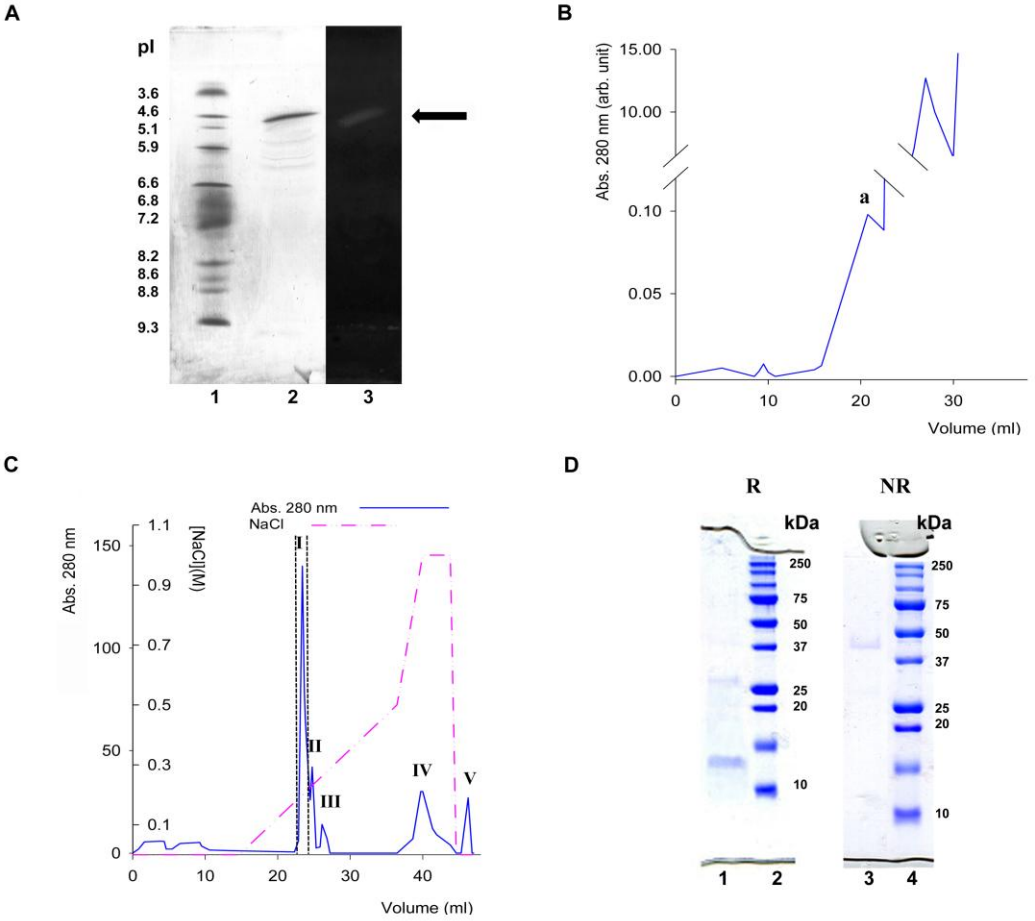

Parameter	Q100:S0[1]	Q90:S10[2]	Q75:S25[3]
Yield (kg/L)[4]	0.212 ± 0.006[a]	0.188 ±0.008[b]	0.162 ± 0.003[c]
Dry weight (%)	36.9 ± 0.3[a]	40.82 ± 0.03[b]	42 ± 1[b]
Water activity	0.967 ± 0.001[a]	0.948 ± 0.004[b]	0.953 ± 0.004[b]
pH	5.30 ± 0.01[a]	5.22 ± 0.03[a]	5.34 ± 0.08[a]
Color[†] L*	95.6 ± 0.6[a]	94.0 ± 0.9[a]	84 ± 10[b]
a*	–2.07 ± 0.07[a]	–1.77 ± 0.06[b]	–1.05 ± 0.19[c]
b*	13.6 ± 0.2[a]	13.9 ± 0.7[a]	12.5 ± 1.7[a]
Protein (%)[5]	15.0 ± 0.9	15.0 ± 0.9	14.4 ± 0.3
Calcium (%)[6]	2.53	2.34	1.98
AA EW >3k-Da fraction (%)[7]	n.d.[¶]	14.7 ± 3.2	27.0 ± 1.9
AA EW <3k-Da fraction (%)[8]	n.d.	n.d.	8.7 ± 1.6
AA cheeses (mg TE/mg)[9]	n.d.	n.d.	0.64

**All the cheeses were made on the same day from the same milk and the analysis performed after 1 month of ripening. The results represent the mean ± the standard deviation. Treatments followed by the same letter do not differ significantly (p >0.05).

[†]Hunter parameters: L* indicates lightness and a* and b* the respective red-green and yellow-blue coordinates.

[¶]Not detectable

[1]Cheese renneted with 100% chymosin

[2]Cheese renneted with 90% chymosin/10% *S. marianum* enzyme extract

[3]Cheese renneted with 75% chymosin/25% *S. marianum* enzyme extract

[4]Yield expressed as kg of cheese per L of milk

[5]Protein content expressed as g of protein per 100 g of sample

[6]Calcium content expressed as g of calcium per 100 g of sample.

[7]Antioxidant activity (AA) of exuded whey (EW) >3k-Da fraction, expressed as percentage

[8]Antioxidant activity of exuded whey, <3k-Da fraction, expressed as percentage [9]Antioxidant activity of cheeses expressed as mg trolox equivalent (TE) per mg cheese

Purification step	Total protein (mg)	Endopeptidase activity (mU)[a]	Specific activity (mU/mg)	Fold purification	Yield (%)
Superdex 200 chromatography	1.32 ± 0.07	6.5	4.9	1	100
MonoQ-Sepharose chromatography	0.38 ± 0.01	4.5	0.12	2.45	70

[a]Endopeptidase activity determined with the substrate MCA-KPAEFFAL-DNP