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Insights into milk-clotting activity of latex peptidases from *Calotropis procera* and *Cryptostegia grandiflora*

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ABSTRACT

Latex fractions from *Calotropis procera, Cryptostegia grandiflora, Plumeria rubra,* and *Himatanthus drasticus* were assayed in order to prospect for new plant peptidases with milk-clotting activities, for use as rennet alternatives. Only *C. procera* and *C. grandiflora* latex fractions exhibited proteolytic and milk-clotting activities, which were not affected by high concentrations of NaCl and $CaCl_2$. However, pre-incubation of both samples at 75 °C for 10 min eliminated completely their activities. Both proteolytic fractions were able to hydrolyze k-casein and to produce peptides of 16 kDa, a similar SDS-PAGE profile to commercial chymosin. RP-HPLC and mass spectrometry analyses of the k-casein peptides showed that the peptidases from *C. procera* or *C. grandiflora* hydrolyzed k-casein similar to commercial chymosin. The cheeses made with both latex peptidases exhibited yields, dry masses, and soluble proteins similar to cheeses prepared with commercial chymosin. In conclusion, *C. procera* and *C. grandiflora* latex peptidases with the ability to coagulate milk can be used as alternatives to commercial animal chymosin in the cheese manufacturing process.

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1. Introduction

Enzymes are among the most studied molecules in industry, because they have several biotechnological and biomedical applications (González-Rábade, Badillo-Corona, Aranda-Barradas, & Oliver-Salvador, 2011). In 2009, the estimated value of the world market for these proteins was $220 billion (Bhunia, Basak, & Dey, 2012). Among all enzymes, peptidases represent around 60% of all of this market (González-Rábade et al., 2011; Ningthoujam, Kshetri, Sanasam, & Nimaichand, 2009). In the food industry, the use of peptidases can confer new chemical, physical, and biological properties to protein-hydrolyzed, which can improve or exhibit new nutritional characteristics, delay deterioration, decrease allergenicity, change solubility, texture, taste, and smell, and also destroy toxic or inhibitory proteins (Tavano, 2013).

Animal peptidases with milk-clotting activities are the major enzymes used in cheesemaking. The most extensively used peptidase is chymosin (EC 3.4.23.4), which is responsible for specific cleavage between bovine k-casein Phe_{105}–Met_{106} bond, resulting in the disruption of casein micelles and milk coagulation (Egito et al., 2007). Besides peptidase, another important parameter for the gelation of milk is the temperature. In general, coagulants can hydrolyse the casein at different temperatures; however, the milk does not clot at temperatures below 18 °C (Esteves, Lucey, Hyslop, & Pires, 2003).

Since the global production volumes of cheese are increasing, peptidases with milk-clotting activity have also been obtained from other animals besides fungi, bacteria and plants. Furthermore, some plant peptidases with milk-clotting activity have also been proposed as rennet substitutes because the use of animal coagulants can be limited for religious (Judaism and Islam) and/or dietary (vegetarianism) reasons (Jacob, Jaros, & Rohm, 2011; Shah, Mir, & Paray, 2014).

Latex has been described as a fluid of milky aspect found in >20,000 plant species (Lewinsohn, 1991). Certainly, among all latex proteins, the peptidases are the most abundant and studied. Papain, isolated from *Carica papaya* latex, is the best known (Konno, 2011). Biotechnological applications of latex peptidases are numerous, such as in medicine: plasma-clotting (Viana et al., 2013), anti-cancer, antihelminthic, and anti-inflammatory activities (Salas, Gomes, Hernandez, & Lopes,

2008); in agriculture: defense against insects and fungi (Konno et al., 2004; Ramos et al., 2014); in the food industry: tenderization of meat (Rawdkuen, Jaimakreu, & Benjakul, 2013) and milk-clotting activities (Badgujar & Mahajan, 2014).

Here, the milk-clotting activity of latex cysteine peptidases from different species is reported about. The effects of peptidase concentration, NaCl, CaCl₂, and temperature on the milk-clotting activity are described, as well as the specificity on the hydrolysis of α, β and k-caseins. Two new cheeses were formulated, manufactured and partially characterized. The results presented here show the possible application of latex cysteine peptidases as alternatives to chymosin for the production of new cheeses with new flavors, and textures.

2. Material and methods

2.1. Chemicals and reagents

Azocasein, k-casein, papain, L-cysteine, Freund's complete and incomplete adjuvant, goat anti-rabbit IgG conjugated with alkaline phosphatase, and p-nitrophenyl phosphate disodium were purchased from Sigma-Aldrich (São Paulo, SP, Brazil). Dithiothreitol (DTT) and molecular mass markers were from GE HealthCare (São Paulo, SP, Brazil). Commercial chymosins (Coalhopar® and Halamix®) were obtained at local markets (Fortaleza, CE, Brazil). All other chemicals were obtained from commercial sources and were of analytical grade.

2.2. Plant material

Peptidases from the latex fluids of *Calotropis procera, Cryptostegia grandiflora, Plumeria rubra* and *Himatanthus drasticus* were obtained as described by Freitas et al. (2007, 2010). The latex fluids were collected from non-cultivated healthy plants growing around Fortaleza-CE, Brazil. The peptidases from the latex of *C. papaya* green fruits was obtained as described by Teixeira, Ribeiro, Gomes, Lopes, and Salas (2008). The soluble proteins were quantified as described by Bradford (1976).

2.3. Protein profile and proteolytic activity

The protein profiles of all studied latex fractions were performed by SDS-PAGE as described by Freitas et al. (2007, 2010). The total proteolytic activities were determined using azocasein as a non-specific substrate and by zymogram containing 0.1% gelatin (Freitas et al., 2007, 2010). In the colorimetric assays, the reaction mixtures were constituted of different doses (20, 30, 40, 50, 60, 70, 80, 90, and 100 μg) of latex proteins dissolved in 300 μL of 50 mM Tris-HCl buffer (pH 6.5) containing 1 mM DTT and 200 μL of 1% azocasein. One unit of specific proteolytic activity (SPA) was defined as the amount of enzyme (mg) required to produce an absorbance change of one unit per minute under the assay conditions. The effect of different reducing agents on proteolytic activities test was performed by pre-incubating each proteolytic fraction (10 min at 25 °C) with different concentrations of the reducing agents DTT, L-cysteine, and ascorbic acid (0–3 mM), as described above.

2.4. Autolysis assays

The proteolytic fractions of *C. procera* and *C. grandiflora* (2 mg/mL in 50 mM Tris-HCl pH 6.5, containing 1 mM L-cysteine) were incubated at 37 °C for 30 min, 1, 4, 8, 24, and 48 h. Aliquots (10 μL) of each time were used for determination of residual proteolytic activities (as described in Section 2.3) and the protein autolysis was achieved by SDS-PAGE, as described by Laemmli (1970).

2.5. Milk-clotting activity

The milk-clotting activity of the latex peptidases was assayed as described by Arima, Yu, and Iwasaki (1970). Different doses (20, 30, 40, 50, 60, 70, 80, 90, and 100 μg) of the proteolytic fractions were dissolved in 200 μL of 50 mM Tris-HCl buffer (pH 6.5) containing 1 mM L-cysteine and mixed with 2 mL of 10% skimmed milk in 10 mM CaCl₂ at pH 6.5. Brazilian commercial chymosins (Coalhopar® and Halamix®) were used as positive controls, as described by suppliers.

Clotting time was recorded when discrete particles were visible in the milk. One specific milk-clotting activity (SMCA) was defined as the amount of enzyme (mg) that clotted 10 mL of milk within 40 min (Ahmed, Babiker, & Mori, 2010), using the formula:

$$SMCA\,(MCA/mg) = [(2400/clotting\ time(s)) \times dilution\ factor]/mg\,(enzyme)$$

The effect of different temperatures (37, 45, 55, 65, and 75 °C for 10 min), concentrations of NaCl, and CaCl₂ (10, 50, 100, 200, 500, and 1000 mM) on the milk-clotting activity of the latex peptidases were also determined, as described above.

2.6. Casein hydrolysis analysis

2.6.1. Preparation of bovine whole casein

Whole casein was obtained by isoelectric precipitation as described by Egito et al. (2007), with slight modifications. Pasteurized bovine milk obtained from the local market was skimmed by centrifugation (2100×g at 30 °C for 30 min). The supernatant was acidified up to pH 4.6 with HCl, centrifuged in the conditions described above, and the precipitate (casein fraction) was washed twice with distilled water, and centrifuged again. The casein fraction was dialyzed against distilled water at 8 °C for two days using a dialysis membrane with a cut off of 8000 Da and then freeze-dried.

2.6.2. Analysis of casein hydrolysis by SDS-PAGE

Hydrolysis of the whole casein fraction (α-, β-, and k-caseins) and purified k-casein were performed as described by Ahmed et al. (2010), with slight modifications. The reaction mixtures were constituted of 50 μL proteolytic fractions (0.1 mg/mL, dissolved in 50 mM Tris-HCl buffer pH 6.5 containing 1 mM L-cysteine) and 450 μL of whole casein fraction or purified k-casein (10 mg/mL). After 1, 5, 10, 15, 20, 25, and 30 min at 37 °C, an aliquot of 50 μL of each reaction was mixed with the Tris-HCl buffer (pH 6.8), containing 0.1% SDS and 5% mercaptoethanol, and boiled at 100 °C for 5 min. Samples were applied on a 15% SDS-PAGE (8 × 8 cm), as described by Laemmli (1970). Identical assays were performed using commercial chymosin (Coalhopar®) as the control.

2.6.3. Analysis of k-casein hydrolysis by RP-HPLC and mass spectrometry

The specific hydrolysis of the k-casein by latex peptidases of *C. procera, C. grandiflora*, and commercial chymosin (Coalhopar®) were determined by reversed-phase high performance liquid chromatography (RP-HPLC) and mass spectrometry. After hydrolysis of the k-casein by peptidases (described in Section 2.6.2.), aliquots of each reaction (500 μL) were centrifuged at 10000×g for 10 min at 10 °C and filtered through 0.22 μm filters. Volumes of 50 μL were loaded onto the C₂/C₁₈ column (μRPC C₂/C₁₈ ST, bed length 100 mm, i.d. 4.6 mm, 3 μm particle size, 12 nm porosity, Amersham Bioscience), coupled to a HPLC Jasco CO-2060 Plus. The peptides were eluted using a linear gradient from 5% to 40% acetonitrile for 30 min, containing 0.1% trifluoroacetic acid (TFA), at 0.5 mL/min flow rate. Proteins were detected at 230 nm.

In another assay, the k-casein peptides were separated by 15% SDS-PAGE (8 × 8 cm). The 16 kDa peptides (corresponding to para-k-casein peptide) were excised from the gels and electro-eluted using an electro-eluter Model 422 (Bio-Rad Laboratories, Brazil) at 200 V for 3 h at 25 °C, following the manufacturer's instructions. The peptides (16 kDa) were dialyzed against distilled water (using membranes with a cut-off of 3 kDa) and freeze-dried. The molecular masses of the para-k-casein peptides were determined using a Synapt HDMS mass spectrometer

(Waters, Manchester, UK) coupled to a 2D NanoUPLC-ESI system as describes by Freitas et al. (2015).

2.7. "Coalho" cheese manufacture

For the activation of the cysteine peptidases, the proteolytic fractions from *C. procera* and *C. grandiflora* (in different concentrations), were dissolved in 10 mL of distilled water (containing 1 mM L-cysteine) and incubated for 5 min at 25 °C. Separately, the proteolytic fractions were added to 500 mL of pasteurized bovine milk containing 50 mM of $CaCl_2$. After 30–40 min at 25 °C, the coagulum formed was manually cut into cubes, making two phases: curd and whey. Part of the whey (250 mL) was removed, heated at 80 °C for 10 min, and added again to the curd. After constant stirring for 10 min, the whey was totally drained from the curd by pressurizing for 12 h at 25 °C using a handmade machine. The pressed materials represented the "*Coalho*" cheeses.

2.8. Proteolytic activity in cheeses

Cheeses manufactured (1 g) using *C. procera* and *C. grandiflora* peptidases were lyophilized and proteins were extracted with 10 mL of 50 mM Tris-HCl buffer (pH 6.5) for 10 min at 8 °C. The suspension was centrifuged at $5000 \times g$ for 10 min at 5 °C, and different aliquots of the supernatants (50, 100, and 200 μL) were incubated with 1% azocasein. The proteolytic activity was determined as described in Section 2.3. Identical assays were performed with cheeses manufactured using commercial chymosin (Coalhopar®). Extracts obtained in the same way were used to detect residual latex proteins in cheeses by ELISA assays.

2.9. Detection of latex proteins in cheeses

Immunological assays were performed to detect the remaining latex proteins in the cheeses. *C. procera* (CpLP) and *C. grandiflora* (CgLP) Latex Peptidases (1 mg) were dissolved in a 0.5 mL saline solution, and mixed with 0.5 mL of Freund's complete adjuvant as described by Freitas et al. (2015). Separately, each suspension (1 mL) was injected intramuscularly into two adult New Zealand white male rabbits followed by booster injections (21, 35, and 42 days) of the same dose with Freund's incomplete adjuvant. Serum samples were collected weekly from day 21, and mixed as a pool. The anti-CpLP and anti-CgLP IgG pools were maintained at −20 °C until use. Experimental procedures and animal handling were performed in accordance with the guidelines approved by the Institutional Animal Ethics Committee of the Federal University of Ceara, Brazil.

ELISA assays were performed using anti-CpLP and anti-CgLP IgG as the primary antibodies (1:10000 dilution) and goat anti-rabbit IgG conjugated to alkaline phosphatase (1:5000 dilution) as the secondary antibodies. Experiments were developed in 96-well microplates using the substrate *p*-nitrophenyl phosphate disodium (5 mg/mL). Initially, each well of the microplates was filled with 150 μL of the cheese protein extracts (obtained as described in Section 2.8). After incubation at 25 °C for 12 h, the wells were blocked with 200 μL of BSA (5 mg/mL) for 2 h at 25 °C, and washed with 150 μL of PBS buffer. Primary antibodies (150 μL) were added to each well and, after 2 h at 25 °C, the plates were washed again with 150 μL of PBS buffer. Secondary antibodies were added (150 μL), incubated for 2 h at 25 °C and washed with 150 μL of PBS buffer. The substrate was then added, and incubated for 1 h in the dark. The reaction was stopped by the addition of 3 M NaOH (50 μL) and the reaction was quantified by absorbance at 405 nm using an automated microplate reader (ELx800 Absorbance Microplate Reader, BioTek Customer Care).

2.10. Statistical analyses

The statistical analyses were performed using GraphPad Prism Software (Version 5.0, San Diego, CA), obtained by variance analysis (ANOVA, n = 3), followed by Tukey's test for multiples comparison. P < 0.05 was considered as statistically significant.

3. Results and discussion

3.1. Protein profile and proteolytic activity

Protein profiles of all studied latex fractions are shown in Supplementary Fig. 1A. The results obtained were in concordance with those originally described by Freitas et al. (2007, 2010), where the latex fractions were also characterized by two dimensional gel electrophoresis and mass spectrometry. The latex fractions exhibited protein profiles with molecular masses ranging from 12 kDa up to 100 kDa (Supplementary Fig. 1A). These dissimilarities in protein contents of latex fluids were recently discussed by Freitas et al. (2016), where the protein profile of the *Thevetia peruviana* latex was studied by two dimensional gel electrophoresis. The most interesting is that independent of qualitative or quantitative protein content, the latex fluids, in general, exhibit proteolytic activity (Domsalla & Melzing, 2008). The latex fractions of *C. procera* and *C. grandiflora* exhibited high proteolytic activity *in vitro*, similar to *C. papaya* latex fraction and papain (cysteine peptidases used as controls) (Supplementary Fig. 2A). These results were confirmed by zymogram, where, at least, three different bands with intense proteolytic activity were detected in each sample (Supplementary Fig. 1B). The proteolytic activity *in vitro* of the *P. rubra* latex fraction was negligible (Supplementary Fig. 2A), and the zymogram showed the presence of two bands with proteolytic activity (Supplementary Fig. 1B). The latex fraction of *H. drasticus* did not exhibit proteolytic activity at all (*in vitro* and by zymogram). Latex fluids have been described as rich sources of cysteine and serine peptidases (Domsalla & Melzing, 2008). In the latex fluids of *C. papaya* (Azarkan, El Moussaoui, van Wuytswinkel, Dehon, & Looze, 2003), *C. procera* and *C. grandiflora* only cysteine peptidases have been described, whereas in *P. rubra* latex there is a mixture of serine and cysteine peptidases (Freitas et al., 2010; Ramos, Araújo, Jucá, Monteiro-Moreira, & Vasconcelos, 2013).

The presence of cysteine peptidases was confirmed by proteolytic enzyme activation of the *C. procera* latex fraction with the reducing agents dithiothreitol (DTT) and L-cysteine (Supplementary Fig.2B). In another study, Oseni and Ekperigin (2013) showed the activation effect of L-cysteine on proteolytic enzymes of *C. procera* latex. Dubey and Jagannadham (2003) showed also that procerain, a cysteine peptidase from *C. procera* latex, was activated with DTT (2–3 mM), and at high concentrations of DTT (10 mM) its activity was loss, which may be due to reduction of internal disulfide bonds of the enzyme. Ascorbic acid was not able to activate the peptidases from *C. procera* (Supplementary Fig. 2B). The *C. grandiflora* and *C. papaya* latex peptidases, and purified papain were activated by DTT and L-cysteine, similar to *C. procera* peptidases. *P. rubra* latex peptidases were not activated by any reducing agent assayed (data not shown). Because L-cysteine is a non-toxic essential amino acid and exhibited activity similar to DTT, it was used further in all milk-clotting assays. Therefore, besides its action as an activator, L-cysteine can be used as a nutritional supplement in cheeses manufactured with latex cysteine peptidases. So far, the use of L-cysteine in cheese is unique, which open new perspectives in the cheese market.

3.2. Milk-clotting activity

Peptidases from *C. procera* and *C. grandiflora* exhibited dose-dependent milk-clotting activity up to 50–60 μg to 2 mL of milk (Fig. 1A). These results were very similar to *C. papaya,* and purified papain, used
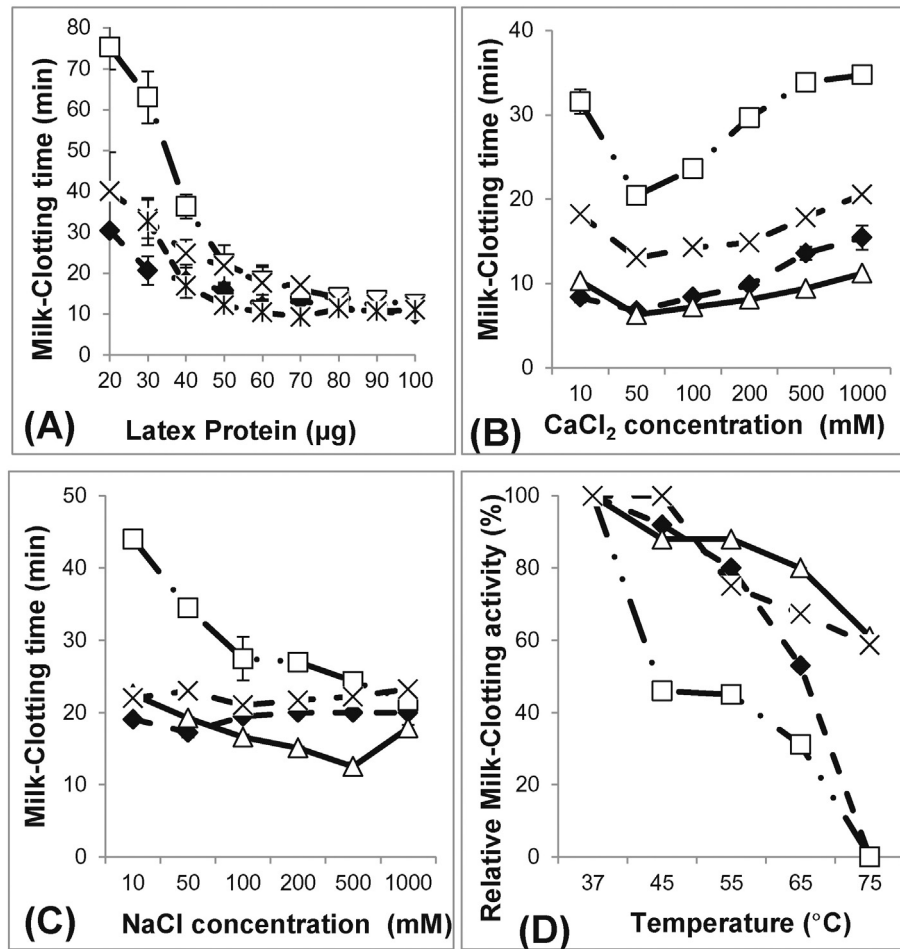
Fig. 1. (A) Effect of the protein concentration, (B) CaCl₂, (C) NaCl (D) and temperature on milk-clotting activities of the latex peptidases. (♦) *C. procera*; (□) *C. grandiflora*; (×) *C. papaya*; (△) Papain. All samples were dissolved in 50 mM Tris-HCl buffer at pH 6.5, containing 1 mM L-cysteine. It was used 60 μg of latex peptidases in the assays (B), (C) and (D). Each value represents the mean of three independent experiments and error bars indicate standard error of the mean.

as control cysteine peptidases. Doses higher than 60 μg were not effective to improve substantially the milk-clotting activity. The proteolytic activities of the latex peptidases exhibited a similar kinetic, with maximum activity using 60 μg (Supplementary Fig. 2A). Therefore, the dose used of each latex fraction in all further assays was 60 μg. Our data were similar to those showed by Silva and Malcata (2005), which correlated the clotting time with the peptidase concentration of *Cynara cardunculus*. No difference was observed in milk-clotting activity when using whole or skimmed milk (data not shown). Interestingly, the overall aspects of the formed curds were different between some enzymatic preparations assayed, which can indicate, at least in part, the specific activity of each peptidase (Fig. 2). *C. papaya* and purified papain (cysteine peptidase controls) made curds similar to Brazilian commercial chymosins (Coalhopar® and Halamix®). Likewise, proteolytic extracts of *Moringa oleifera* and *Solanum dubium,* and a purified cysteine peptidase of *Euphorbia microsciadia* coagulated the skimmed milk and formed a white and firm curd (Ahmed et al., 2010; Pontual et al., 2012; Rezanejad, Karbalaei-Heidari, Rezaei, & Yousefi, 2015). *P. rubra* and *H. drausticus* were not able to coagulate the milk even at the highest tested dosage (100 μg).

Specific milk-clotting (SMCA) and proteolytic activities (SPA) of the latex fractions, papain and two commercial chymosins (Halamix® and Coalhopar®) are shown in Table 1. The ratio of milk-clotting activity to proteolytic activity (SMCA/SPA) is an important indicator used to determine the potential of the peptidase as coagulants for cheese manufacture (Arima et al., 1970). This means that good coagulants present high milk-clotting activity and low proteolytic activity, resulting in

less hydrolysis of the caseins. Among all samples, the best results were obtained for papain (1880.0 SMCA/SPA) and *C. procera* (1788.3 SMCA/SPA) latex peptidases. Similar results were obtained with a ginger rhizome cysteine peptidase (1653.0 SMCA/SPA) (Hashim, Mingsheng,
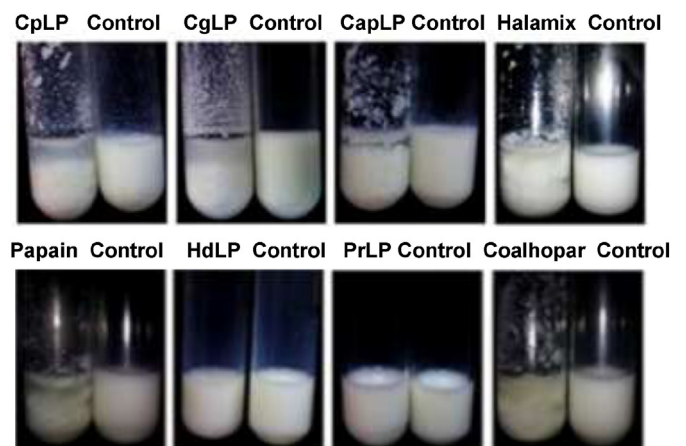

Fig. 2. Overall aspect of the milk-clotting activities of the latex peptidases and Brazilian commercial chymosins (Coalhopar® and Halamix®). The samples (60 μg) were dissolved in 50 mM Tris-HCl buffer (pH 6.5), containing 1 mM L-cysteine and added to 2 mL of skimmed milk (10%). The photos were taken after 30 min at 25 °C. CpLP: *C. procera*; CgLP: *C. grandiflora*; PrLP: *P. rubra*; CapLP: *C. papaya*; HdLP: *H. drasticus*. Control: Tris-HCl buffer.

Table 1
Milk-clotting and proteolytic activities of the latex peptidases and commercial coagulants.

Enzymes	SMCA[a] (AU/mg)	SPA[b] (AU/mg)	Ratio (SMCA/SPA)
Latex peptidases			
Calotropis procera	566.3 ± 86.9	0.31 ± 0.04	1788.3
Carica papaya	394.3 ± 11.9	0.29 ± 0.02	1359.6
Cryptostegia grandiflora	404.2 ± 10.6	0.35 ± 0.09	1154.8
Himatantus drasticus	Nd[*]	Nd[*]	–
Plumeria rubra	Nd[*]	0.03 ± 0.01	–
Purified peptidase			
Papain	720.7 ± 47.3	0.38 ± 0.06	1880.0
Commercial rennets			
CoalhoPar® rennet	415.3 ± 30.6	0.24 ± 0.04	1730.4
Halamix® rennet	226.3 ± 41.2	0.21 ± 0.03	1077.6

Each value represents the mean of three independent experiments and error bars indicate standard error of the mean.
Nd[*] = not detected at pH 6.5.
[a] SMCA: specific milk-coagulating activity.
[b] SPA: specific proteolytic activity.

Iqbal, & Xiaohong, 2011). Other plant cysteine peptidases such as those from kiwi fruit (Actinidin), *E. microsciadia* and *Vasconcellea quercifolia* latex also showed potential as milk-clotting agents (Grozdanovic, Burazer, & Gavrovic-Jankulovic, 2013; Rezanejad et al., 2015; Torres et al., 2010).

The concentration of ions such as calcium and sodium represents an important element because both can be used during cheese manufacture and some milk-clotting enzymes can be inactivated by them. $CaCl_2$ is usually added to milk to improve the milk coagulation. However, it is also documented that the addition of high concentrations of calcium chloride can reduce the pH of milk, resulting in a decreased aggregation rate and possible proteolytic inactivation (Wolfschoon-Pombo, 1997). Therefore, the effect of $CaCl_2$ concentrations on milk-clotting activity of the latex peptidases was evaluated (Fig. 1B). All samples presented maximum milk-clotting activity at a final concentration of 50 mM $CaCl_2$, a similar result found for other clotting plant enzymes (Bruno et al., 2010; Pontual et al., 2012). At higher concentrations of $CaCl_2$ (100–1000 mM), there was a reduction in the milk-clotting activity, when compared with 50 mM $CaCl_2$. However, even at 1 M $CaCl_2$, the milk-clotting activity was similar to that displayed when using 10 mM $CaCl_2$ for all latex peptidases (Fig. 1B). These results were interesting because the addition of calcium chloride in milk was reported to increase milk-clotting time by calf rennet (Famelart, Le Graet, & Raulot, 1999) and crude enzyme extracts of *Withania* sp. and *S. dubium* (Ahmed et al., 2010; Naz, Masud, & Nawaz, 2009).

NaCl can be added to milk for many functions such as taste, stability of endogenous protein and syneresis of the curd. It also influences cheese ripening, principally through its effect on water activity (Guinee & Fox, 2004). Besides, traditionally, in Domiati and Gibna cheeses, high concentrations of NaCl are added to milk to control the growth of microorganisms (Guinee & Fox, 2004). The NaCl concentration increased the milk-clotting activity of *C. grandiflora* peptidases and papain. At the highest concentration assayed (1 M NaCl), the relative activity for *C. grandiflora* and papain were 152% and 122%, respectively, when compared at 10 mM NaCl (100%) (Fig. 1C). Similar results were exhibited by *S. dubium* seed peptidases, where 2 M NaCl was a powerful stimulator of the enzyme activity (Ahmed et al., 2010). On the other hand, the milk-clotting activity of *C. procera* peptidases did not change even at 1 M NaCl (Fig. 1C). These results reinforce again that latex peptidases from *C. procera* and *C. grandiflora* can be good candidates for cheese manufacture, because it is also known that NaCl can decrease the milk-clotting activity of plant coagulants (Guiama et al., 2010).

The milk-clotting activities of the latex peptidases of *C. procera* and *C. grandiflora* were maximum at 37 °C and decreased 20% and 55%, respectively after pre-heating at 55 °C for 10 min (Fig. 1D). Papain started its

denaturation at 45–55 °C, according to thermal denaturation studies that used differential scanning calorimetry and spectrophotometric methods (Sumner et al., 1993). Excluding *C. papaya* peptidases and papain, all samples studied here lost milk-clotting activities after pre-heating at 75 °C for 10 min (Fig. 1D). *M. oleifera* flower peptidases were also totally inactivated at 60–70 °C (Pontual et al., 2012). In contrast, milk-clotting enzymes from different plants such as *Solanum esculentum, Solanum melongena* and *Solanum macrocarpon* were stable proteins, remaining active after heating to 70 °C (Guiama et al., 2010) and a milk-clotting peptidase named religiosin B, purified from *Ficus religiosa* latex, showed milk-clotting activity at 60 °C (Kumari, Sharma, & Jagannadham, 2012). High thermal and pH stability are important biochemical properties used for the choice of enzymes to be used in several biotechnological applications. However, a coagulant with high pH and thermal stabilities can produce an extensive hydrolysis of the milk proteins, resulting in cheeses with undesirable flavors. A step used in the production of the "Coalho" cheese in Brazil is the removal of part of the whey from the curd and its heating up to 80 °C, and further addition to mixture to cook the curd. In this case, inactivation of the peptidases at 80 °C can avoid an extensive hydrolysis of milk caseins during cheese maturation, which can modify properties of the cheese such as texture and taste.

3.3. Autolysis

In general, peptidases can undergo inactivation or autolysis, depending on several factors, including the storage time and temperature (Chen, Shun, Zhang, & Gao, 2003). The proteolytic activity of the *C. procera* and *C. grandiflora* latex fractions remained stable (100%) at 37 °C for 8 h at 2 mg/mL. Only after 24 and 48 h, there was a considerable loss of activity (20–80%), depending on the latex fraction (Supplementary Fig. 3A). Similarly, milk-clotting latex peptidases of *Ficus carica* were stable up to 24 h at 37 °C (Zare et al., 2013). In contrast, a milk-clotting cysteine peptidase of *Euphorbia nivulia* latex exhibited low stability, retaining only 20% of its residual activity after 10 min of incubation at 40 °C (Badgujar & Mahajan, 2014). The autolysis of *C. procera* and *C. grandiflora* proteolytic fractions was evaluated by SDS-PAGE (Supplementary Fig. 3B). Both fractions did not exhibit changes in their protein contents (autodigestion) even after 48 h of incubation at 37 °C, reinforcing their high stabilities and potentials use in food industry.

3.4. Casein hydrolysis by latex peptidases

The degree of α-, β-, and k-casein hydrolysis by latex peptidases was analyzed by SDS-PAGE (Supplementary Fig. 4). The latex peptidases from *C. procera*, *C. grandiflora*, and *C. papaya* exhibited unspecific hydrolysis on all caseins after 5 min of incubation. In contrast, the commercial coagulants (Coalhopar® and Halamix®) were specific, cleaving only the k-casein electrophoretic band. Peptidases from *P. rubra* and *H. drasticus* were not able to cleave the caseins. In order to quantify the casein hydrolysis by peptidases, the gels were scanned and the densitometries of the casein bands were plotted as a percentage of the control residual casein (time 0). The densitograms revealed that *C. procera* peptidases (CpLP) were more specific in k-casein hydrolysis than *C. grandiflora* (CgLP) and *C. papaya* (CapLP) peptidases (Fig. 3). Similar results were exhibited by *S. dubium* seed and *Bromelia hieronymi* fruit peptidases, which were able to hydrolyse the three casein components, but with a higher specificity to k-casein (Ahmed et al., 2010; Bruno et al., 2010).

The qualities of the cheese, which include taste, flavor and texture, can be reflected by the specific hydrolysis of the k-casein. Chymosin cleaves specifically between Phe[105] and Met[106] bond of the k-casein, producing the para-k-casein and glycomacropeptide (Egito et al., 2007). Therefore, assays were performed by incubating purified bovine k-casein with *C. procera* and *C. grandiflora* peptidases for different times. The action of commercial chymosin on k-casein produced two major
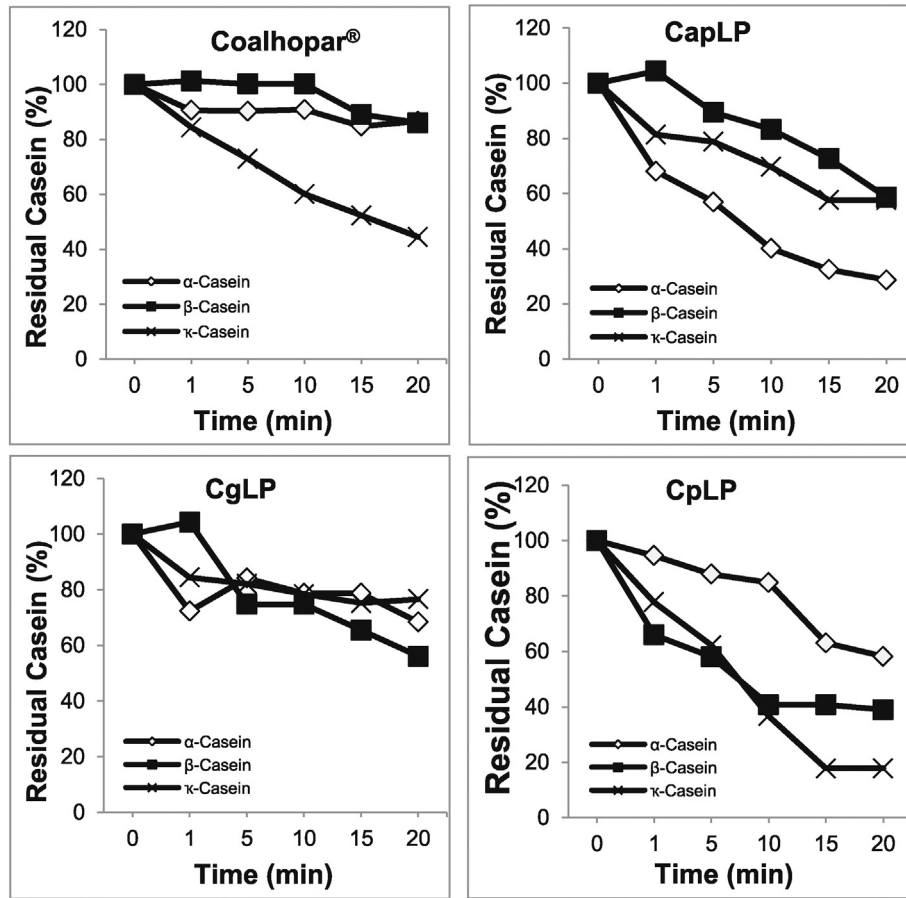
Fig. 3. Degradation of caseins by latex peptidases. Values come from the corresponding densitograms of SDS-PAGE (Supplementary Fig. 4). Samples (0.1 mg/mL) were incubated with casein fractions (10 mg/mL) for different times at 37 °C and pH 6.5. CpLP: *C. procera*; CgLP: *C. grandiflora*; CapLP: *C. papaya*. Coalhopar®: commercial chymosin.

peaks, as seen in RP-HPLC analysis (Fig. 4). These two peaks probably correspond to para-k-casein and glycomacropeptide (Egito et al., 2007). Peaks, eluted in same position, were also observed after k-casein hydrolysis by *C. procera* or *C. grandiflora* peptidases, as early as 5 min (Fig. 4). These results suggested, at least in part, that both, *C. procera*

and *C. grandiflora* peptidases hydrolyse k-casein in same position that commercial animal coagulant (Coalhopar). In the same way, Cardosin A and B, two aspartic peptidases from *C. cardunculus*, and sunflower peptidases hydrolyzed k-casein at the $Phe_{105}-Met_{106}$ bond, as does chymosin (Egito et al., 2007; Silva & Malcata, 2005). It appears that
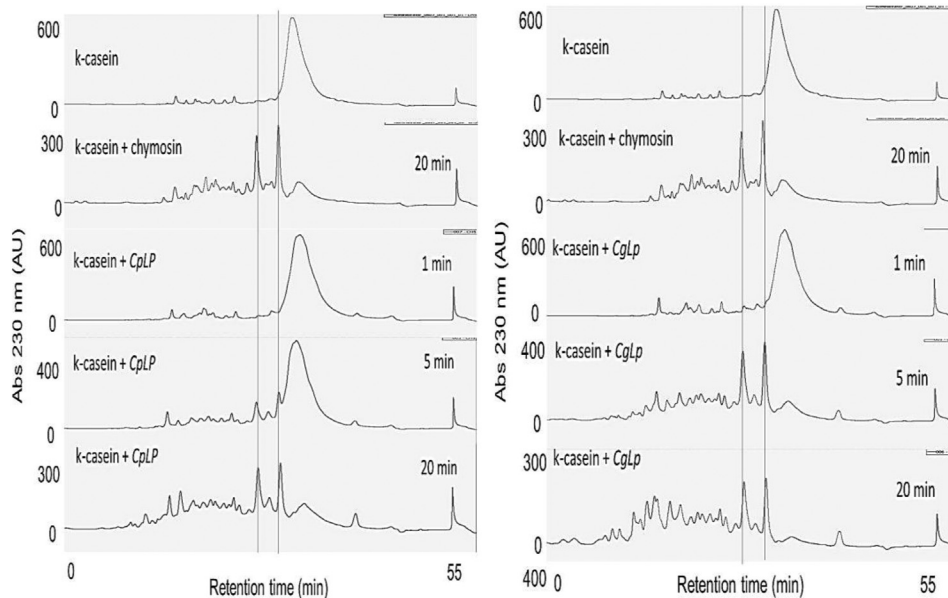

Fig. 4. Reversed-phase high performance liquid chromatography (RP-HPLC) (C_2/C_{18} column) of bovine k-casein peptides generated by *C. procera* (CpLP), *C. grandiflora* (CgLP) latex peptidases and commercial chymosin (Coalhopar®) at pH 6.5, 37 °C for different times.

other peptides, besides para-k-casein and glycomacropeptide, were produced with action of *C. procera* and *C. grandiflora* peptidases, indicating that latex peptidases can cleave k-casein in other sites (Fig. 4). This thinking was also supported by electrophoresis (Fig. 5). Milk-clotting peptidases belonging to albizia and ginger cleaved k-casein in other sites, such as Lys_{116}–Thr_{117}, Ala_{90}–Glu_{91} and His_{102}–Leu_{103}, respectively (Egito et al., 2007; Huang, Chen, Luo, Guo, & Ren, 2011). It has also been described that these slight differences in cleavage sites do not affect milk-clotting activity and taste of the cheeses (Hang et al., 2016).

The action of *C. procera* and *C. grandiflora* peptidases on k-casein was also performed by SDS-PAGE (Fig. 5). The reduction in intensity of the k-casein band by *C. procera* and *C. grandiflora* peptidases was accompanied by an increase in the intensity of a 16 kDa peptide band, which probably corresponded to para-k-casein peptide (insert Fig. 5A). A similar protein band was observed when k-casein was hydrolyzed with the commercial chymosin (Coalhopar®). In addition, the k-casein hydrolysis by *C. procera* peptidase also produced other peptides with molecular masses between 29 and 20 kDa, which was not observed when *C. grandiflora* and commercial chymosin were assayed (Fig. 5A). The para-k-casein peptides (16 kDa bands) produced by action of the two latex peptidases and by commercial chymosin were excised from the SDS-PAGE gels, electro-eluted, and submitted to LC/ESI-MS analysis (Fig. 5B). The three 16 kDa protein bands produced by action of *C. procera* and *C. grandiflora* peptidases and by commercial chymosin (Coalhopar) on k-casein exhibited molecular masses of 8602 Da (Fig. 5B). Previous study reported para-k-casein with molecular mass of 12,270 Da when digested with purified chymosin (Egito et al., 2007). The lower para-k-casein observed here may be result of additional hydrolysis performed by the proteolytic activity of both, *C. procera* and *C. grandiflora* samples. Examining the protein content of commercial chymosin (Coalhopar) by MS/MS analysis after electrophoresis it was identified the peptidase trypsin, in

addition of chymosin (data not shown). Thus, this contaminating activity could account for further hydrolysis of para-k-casein, resulting in the shorter peptide.

3.5. Cheese manufacture and partial characterization

Some characteristics of the cheeses made with *C. procera* and *C. grandiflora* peptidases are presented in Table 2. The best results of coagulation time with 500 mL of milk were obtained with 9 and 40 mg of the *C. procera* and *C. grandiflora* proteolytic fractions, respectively. The time of coagulation was between 7 and 17 min at 25 °C. In these concentrations, the yield, dry mass and soluble proteins' values and appearances of cheeses made with latex peptidases were similar to those prepared with commercial chymosin (Table 2 and Fig. 6). The complete sensory analysis of the two cheeses will be performed and described in a future work.

The cheeses made with latex peptidases did not show residual proteolytic activity, similar to cheeses manufactured with commercial chymosin (data not shown). This result was important because there will be a low or even no hydrolysis of the caseins during the cheese ripening process, avoiding possible changes in texture, taste, and flavor of the product. These results corroborated with those from Fig. 1D where *C. procera* and *C. grandiflora* peptidases were inactivated after heating at 75 °C for 10 min. During the manufacture of the "Coalho" cheeses, one step consists of the heating of the whey up to 80 °C to cook the curd. Thus, in this step the latex peptidases can be inactivated. Llorente, Obregón, Avilés, Caffini, and Vairo-Cavalli (2014) showed that flower proteolytic extracts from *Cynara scolymus* coagulated the milk and made cheeses similar to those achieved with animal chymosin. However, they showed that the proteolytic fractions were active even during the cheese maturation for 45 days, with an accompanying bitter
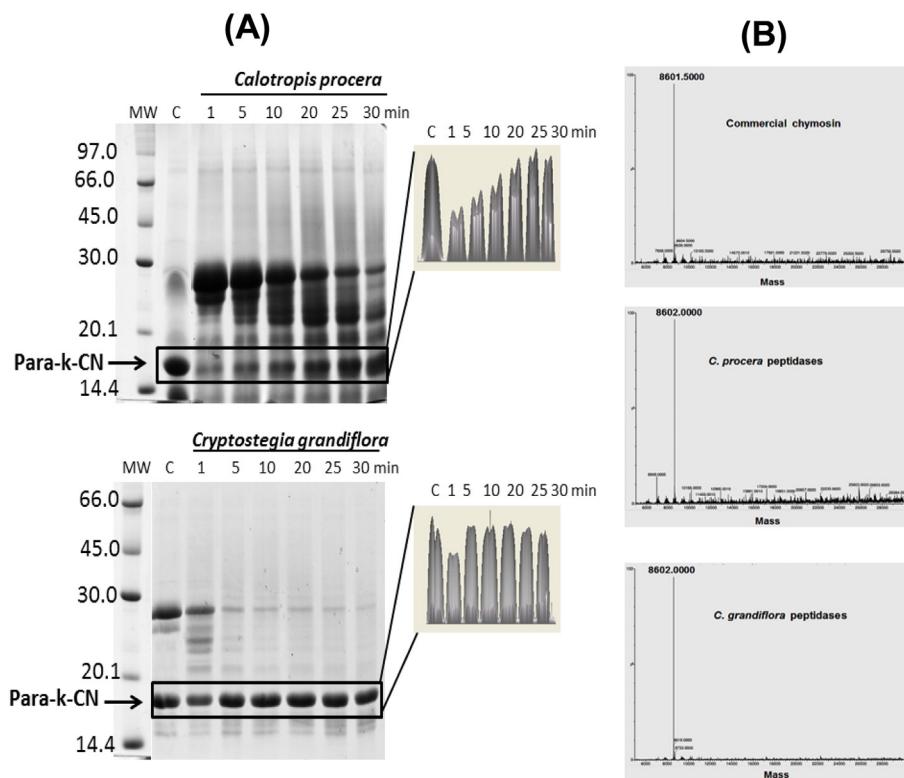

Fig. 5. (A) SDS-PAGE (15%) pattern of hydrolysis of the bovine k-casein by latex peptidases of *C. procera* and *C. grandiflora*. Line C: k-casein incubated with commercial chymosin for 20 min (Coalhopar®); Lines 1–30, k-casein (10 mg/mL) incubated with latex peptidases (0.1 mg/mL) for 1, 5, 10, 20 and 30 min, at 37 °C. Insert: 3D view of para-k-casein bands were obtained using ImageMaster 2D Platinum 6.0 Software (Amersham Biosciences). (B) Mass spectra of para-k-caseins (16 kDa bands eluted from SDS-PAGE) generated by the digestion of the purified k-casein with commercial chymosin, *C. procera* and *C. grandiflora* peptidases.

Table 2
Some characteristics of the cheeses manufactured with *Calotropis procera* and *Cryptostegia grandiflora* peptidases in comparison with a commercial animal coagulant (Coalhopar®).

Coagulant	Sample (mg)/500 mL milk	Fresh cheese (g)	Dry cheese[1] (g)	Soluble proteins[2] (mg)/g cheese	Milk-clotting time
C. procera	8	–	–	–	Nd
	9	59.3 ± 2.2[a]	27.7 ± 1.8[a]	14.7 ± 2.9[a]	17′05″ ± 2′
	10	64.7 ± 5.6[b]	25.2 ± 5.9[a]	15.7 ± 2.3[a]	18′56″ ± 5′
	11	59.1 ± 1.1[a]	24.4 ± 3.7[a]	11.7 ± 3.6[a]	10′53″ ± 2′
C. grandiflora	30	–	–	–	Nd
	40	53.3 ± 2.8[a]	29.2 ± 1.7[a]	14.0 ± 3.0[a]	7′18″ ± 30″
	50	51.8 ± 4.3[a]	27.8 ± 2.6[a]	12.4 ± 7.6[a]	4′00″ ± 50″
Coalhopar®	10	52.1 ± 2.5[a]	28.0 ± 1.1[a]	11.1 ± 5.1[a]	16′24″ ± 40″

Same uppercase letters, in the same column, do not indicate significant difference at P < 0.05, when compared with Coalhopar®. Each value represents the mean of three independent experiments and error bars indicate standard error of the mean.
Nd: non detected milk-clotting activity.
[1] The fresh cheeses were freeze-dried and the dry masses were recorded.
[2] Soluble proteins of the cheeses were extracted with 50 mM Tris-HCl buffer (pH 6.5) and determined by Bradford (1976).

taste. Likewise, cheeses made using *C. cardunculus* flower peptidases, a traditional coagulant used in goats' cheese, demonstrated higher proteolytic activity than those made with chymosin during the ripening period (Pino, Prados, Galán, McSweeney, & Fernández-Salguero, 2009). Therefore, the residual proteolytic activity in cheese can be listed as the main drawback of very active plant peptidases.

The residual *C. procera* and *C. grandiflora* latex proteins were not detected in cheeses made with these fractions by ELISA assays (Supplementary Fig. 5). This result can be explained because during the manufacture of the cheeses, the curds were pressed for 12 h to drain all whey. In this step, the latex proteins can be totally eliminated together with other soluble proteins of the whey. Previously, Ramos et al. (2006) showed that latex proteins of *C. procera* were digested by the action of digestive peptidases and they were not detected in fecal material of rats after 35 consecutive days of consumption. No death or toxic effects were observed. In another work, the same protein fraction of *C. procera* did not induce antibodies production when animals received latex proteins by oral route and therefore, did not develop allergy (Ramos et al., 2007). Accordingly, these results suggest that a possible

toxic or allergenic reaction of cheeses produced with latex proteins of *C. procera* should be insignificant or null.

Other works showed the potential use of *C. procera* leaves in the manufacture of cheeses (Aworh & Muller, 1987; Adetunji & Salawu, 2008). In these studies, a crude juice made from *C. procera* leaves was used. The use of this total extract can be limited because it is known that latex fluids are rich sources of very toxic protein and secondary metabolites (Konno, 2011). Consequently, the quality, taste and food safety of the cheese can be very difficult to determine. Here, a purified fraction from the latex was used, which is rich in proteolytic enzymes and free of secondary metabolites and low molecular mass molecules (Freitas et al., 2007). Other works showed that cysteine peptidases from *C. procera* exhibited milk-clotting activity (Badgujar & Mahajan, 2012; Oseni & Ekperigin, 2013; Singh & Dubey, 2011). However, their results were very preliminary. Here, we characterized the milk-clotting activity of *C. procera* latex peptidases which was not described before wherever such as salt effects, temperature, specific hydrolysis of k-casein by SDS-PAGE, HPLC and mass spectrometry, protein autolysis, immunological detection, and residual proteolytic activity in the cheeses.

Calotropis procera (CpLP)

Cryptostegia grandiflora (CgLP)

Fig. 6. Overall aspect of the cheeses manufactured with latex peptidases of *C. procera* (CpLP) and *C. grandiflora* (CgLP), using 9 mg (CpLP) or 40 mg (CgLP) of the latex fractions to 500 mL of bovine milk. Bars: 5 cm.

C. grandiflora is a plant poorly studied. There is only two works that describes the characterization, purification and biological activity of cysteine peptidases from its latex (Freitas et al., 2010; Ramos et al., 2014). There is no study concerning the application of *C. grandiflora* peptidases in cheese manufacture.

4. Conclusion

It was studied the potential use of latex fractions from four plant species as milk coagulants. However, only *C. procera* and *C. grandiflora* peptidases showed potential as alternatives to the use of commercial animal chymosin. Both purified proteolytic fractions were not affected by high concentrations of NaCl and CaCl₂, and were inactivated after heating at 75 °C and the hydrolysis partners on k-casein were similar to commercial chymosin. Cheeses made with *C. procera* and *C. grandiflora* peptidases presented a good yield, appearance, and did not present residual proteolytic activity or latex proteins.

Conflict of interest

The authors confirm that this article content has no conflicts of interest.

Contributions

CDTF, HBL, JPBO, JLA, ASE and SVC performed the main research work in this study, including latex processing, protein purification, proteolytic activity, HPLC analysis, production of polyclonal antibodies and milk-clotting assays. MDPL and ACOMM performed the analysis of casein hydrolysis by SDS-PAGE and mass spectrometry. CDTF, SVC and MVR contributed to data analyses and discussion and wrote the manuscript.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2016.06.020.

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