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Biotechnological potential of a cysteine protease (CpCP3) from *Calotropis procera* latex for cheesemaking

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ABSTRACT

This article reports the characterization and evaluation of the biotechnological potential of a cysteine protease purified from *Calotropis procera* (CpCP3). This enzyme was highly stable to different metal ions and was able to hydrolyze κ-casein similarly to bovine chymosin. Atomic force microscopy showed that the process of casein micelle aggregation induced by CpCP3 was similar to that caused by chymosin. The cheeses made using CpCP3 showed higher moisture content than those made with chymosin, but protein, fat, and ash were similar. The sensory analysis showed that cheeses made with CpCP3 had high acceptance index (> 80%). In silico analysis predicted the presence of only two short allergenic peptides on the surface of CpCP3, which was highly susceptible to digestive enzymes and did not alter zebrafish embryos' morphology and development. Moreover, recombinant CpCP3 was expressed in *Escherichia coli*. All results support the biotechnological potential of CpCP3 as an alternative enzyme to chymosin.

1. Introduction

Cheese is among the most important milk-derived food products. It is an essential ingredient of many different types of dishes, as well as being rich in nutrients, such as proteins, vitamins, essential minerals, fat and calcium. Besides this, it has several health benefits, which include prevention of osteoporosis, protective effect against dental caries, weight reduction or anti-obese effects, and anti-hypertensive properties (Walther, Schmid, Sieber, & Wehrmüller, 2008). Cheesemaking is a complex procedure, involving many steps and biochemical transformations. Depending on the milk origin or coagulation process applied, a wide range of colors, textures, flavors, firmness levels and aromas can be obtained (Santiago-López et al., 2018). There are over 2,000 cheese varieties in the world. Some statistical studies have shown that the world cheese market was worth around 90 billion U.S. dollars in 2016, and it is forecast to reach to more than 100 billion by 2022 (https://www.statista.com/). For all these reasons, research for new enzymes able to produce cheeses with novel characteristics, aromas and flavors is still a very relevant topic, deserving continuous studies.

Milk coagulation is the foremost phase for producing cheese. This process can be attained by using coagulating enzymes, such as animal, microbial and plant-based proteases (Freitas et al., 2016; Meng et al., 2018). Chymosin (EC 3.4.23.4), extracted from calf stomachs, is the protease most extensively used for cheesemaking. Increasing demand for chymosin combined with limited availability of calf stomachs, as well as religious (Islam and Judaism) and dietary (vegetarianism) aspects are some factors that have stimulated studies to find alternative milk-clotting sources (Shah, Mir, & Paray, 2014). Although several plant proteases have been described with milk-clotting activity, many of them are unsuitable for cheesemaking because they do not meet most

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industrial requirements, such as cheese yield, processing time and taste (Shah et al., 2014).

Different parts of the *Calotropis procera* plant have been identified with milk-clotting activities (Aworh & Muller, 1987; Omotosho, Oboh, & Iweala, 2011). In addition, our group previously showed that proteases purified from *C. procera* latex were able to clot milk and produce cheeses with desirable characteristics for industrial production (Freitas et al., 2016). Those findings were important because we provided a perspective for using new plant extracts in cheesemaking. However, we used proteolytic fractions, which are constituted of several protease isoforms, besides other proteins (Freitas et al., 2007; Ramos et al., 2013). Thus, the main drawback of using a proteolytic fraction can be the difficulty of sample standardization and making cheese at the industrial level, besides the presence of possible toxic proteins in the extracts. In this respect, our hypothesis was that purified proteases from *C. procera* would exhibit the same milk-clotting potential of the total extract, besides not being toxic or allergenic. Here, we characterize a protease purified from *C. procera* with respect to its stability against different ions, specific hydrolysis of κ-casein and effects on the structure of casein micelles. We also describe its toxic and allergenic potentials, as well as its potential to produce cheese. The sensory analyses of cheeses made with CpCp3 was also assessed. Finally, we also report the recombinant expression in *Escherichia coli* cells to determine its biotechnological potential for use in cheese manufacturing.

2. Material and methods

2.1. Chemicals

Azocasein, κ-casein, papain, L-cysteine, pepsin, trypsin, dithiothreitol (DTT), isopropyl β-D-1-thiogalactopyranoside (IPTG), kanamycin sulfate, phenylmethylsulfonyl fluoride (PMSF), urea, sodium dodecyl sulfate (SDS), Triton X-100, and lysozyme were obtained from Sigma-Aldrich (São Paulo, SP, Brazil). HisTrap FF affinity column, CM-Sepharose fast-flow column, RESOURCE-S column, and molecular weight markers were purchased from GE Healthcare (São Paulo, SP, Brazil). Commercial bovine chymosin (Coalhopar®) was obtained at local markets (Fortaleza, CE, Brazil). All other chemicals were of analytical grade.

2.2. Latex processing

The latex of *C. procera* was collected from healthy and non-cultivated plants found growing in Fortaleza, Ceará, Brazil, as described by Freitas et al. (2007). Briefly, the fresh latex was collected in distilled water (1:1 ratio) and centrifuged at 10,000 × *g* for 10 min at 10 °C to eliminate rubber and other insoluble materials. Then the supernatant was dialyzed for three days against distilled water using 10 kDa cutoff membranes, and then centrifuged again, as described above. The new supernatant, named *Calotropis procera* latex proteins (CpLP), was lyophilized and used for purification of a cysteine protease (CpCP3).

2.3. Purification of Calotropis procera cysteine protease (CpCP3)

CpCP3 was purified according to Ramos et al. (2013). CpLP, when chromatographed in a CM-Sepharose fast-flow column equilibrated with 50 mM sodium acetate buffer (pH 5.0), was fractioned in three distinct protein peaks (PI, PII, and PIII). PII, eluted with 200 mM NaCl, was dialyzed against distilled water, lyophilized, and then submitted to ion-exchange chromatography using a Resource-S column, equilibrated in 20 mM sodium phosphate buffer (pH 6.0), coupled to an AKTA chromatography system (GE Healthcare). CpCP3 was eluted with a linear salt gradient from 0 to 500 mM NaCl during 30 min (1 mL/min flow rate) (Ramos et al., 2013).

2.4. Effect of different ions on proteolytic activity of CpCP3

The proteolytic activity was measured according to Freitas et al. (2007), using 1% azocasein as substrate at pH 6.5 (pH of milk). CpCP3 (20 μL, 2 mg/mL in 50 mM Tris-HCl buffer containing 1 mM L-cysteine, pH 6.5) was incubated with different ions at 10 mM (NaCl, MgCl, LiCl, KCl, or $CaCl_2$) for 30 min at 37 °C. Afterward, 1% azocasein substrate was added (200 μL) and the reaction was permitted to occur for 60 min at 37 °C. The reaction was stopped with 300 μL of 10% trichloroacetic acid and the reaction mixture was centrifuged at 10,000 × *g* for 10 min at 25 °C. Finally, 400 μL of the supernatants were mixed with 400 μL of 2 M NaOH. The absorbance was measured at 420 nm and one unit of specific proteolytic activity (SPA) was defined as the amount of enzyme (mg) required to increase the absorbance by 0.01 per minute.

2.5. In vitro hydrolysis of κ-casein

Hydrolysis of bovine κ-casein was evaluated by SDS-PAGE as described by Freitas et al. (2016). CpCP3 (50 μL, 0.1 mg/mL in 50 mM Tris-HCl buffer containing 1 mM L-cysteine, pH 6.5) was incubated with 450 μL of κ-casein (10 mg/mL in 50 mM Tris-HCl buffer, pH 6.5) for different time intervals (0, 1, 5, 10, 15, 20, 25, and 30 min) at 37 °C. The reactions were stopped by mixing each aliquot with SDS-PAGE sample buffer, followed by heating at 100 °C for 5 min. The extensiveness of the hydrolysis was observed by 15% SDS-PAGE. Bovine chymosin (Coalhopar®) was used as control (50 μL, 0.1 mg/mL in 50 mM Tris-HCl buffer, pH 6.5).

2.6. Milk-clotting activity

The milk-clotting assay was performed as described in Freitas et al. (2016). Different amounts of CpCP3 (2.5, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 μg), dissolved in 200 μL of 50 mM Tris-HCl buffer containing 1 mM L-cysteine (pH 6.5), were incubated with 2 mL of whole milk containing 50 mM $CaCl_2$ at 37 °C. The clotting time or milk clotting activity (MCA) was assigned after observation of discrete particles in the milk when the tubes were gently inclined. Commercial bovine chymosin (Coalhopar®) was used as control. The specific milk-clotting activity (SMCA) was defined as the amount of enzyme (mg) able to clot the milk in 40 min, according to the formula:

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

2.7. Atomic force microscopy (AFM)

The overview of casein micelles treated with CpCP3 was evaluated using AFM technology (MFP3D-BIO microscope from Asylum Research, Oxford Instruments, USA), as described by Freitas et al. (2019). CpCP3 (30 μg) or chymosin (30 μg), dissolved in 200 μL of 50 mM Tris-HCl buffer, were incubated with 2 mL of milk containing 50 mM $CaCl_2$ at 37 °C. The micelles' aggregation was studied using five different samples: the control (no enzyme, time = 0), and four samples observed after 15, 30, 45, and 60 min of CpCP3 or chymosin addition. After that, the samples were immediately diluted (1:20, v/v) in 50 mM Tris-HCl buffer (pH 6.5), spread on glass surfaces (10 μL), and dried at 25 °C. The experiments were repeated twice with independent samples. AFM was performed using tapping mode and Econo-LTESP-Au cantilevers from Oxford Instruments, with nominal spring constant of 5 N/m and frequency of 150 kHz.

2.8. Manufacture and partial characterization of cheese

CpCP3 was used to make cheese in laboratory scale, as described by Freitas et al. (2016). Briefly, 12.5 mg of CpCP3 was dissolved in 10 mL of distilled water containing 1 mM L-cysteine and incubated for 5 min at 25 °C. The enzymatic solution was added to 500 mL of pasteurized

whole milk containing 50 mM $CaCl_2$ and kept undisturbed for 40 min at 37 °C. After clotting, the curd was cut into pieces, the whey was removed, heated at 80 °C for 10 min, added again to the curds, and kept under constant stirring for 10 min. Finally, the curds were pressed during 6 h at 25 °C using a manually operated machine. Cheese samples were also made using bovine chymosin (Coalhopar®) and used as controls.

Texture profile analysis (TPA) was conducted according to Lashkari, Asl, Madadlou, and Alizadeh (2014) using a TA-XT2 texture analyzer (Stable Micro Systems, UK). A flat probe of 35 mm diameter was attached to the moving crosshead. Cylindrical cheese samples (12 × 10 mm), taken from a depth of 5 mm in the cheese block at 8 °C with a cork borer, were placed in air-tight plastic bags, kept refrigerated at 4 °C for 4 h to equilibrate and then set aside at 21 ± 1 °C for 45 min. Samples were compressed in two-cycle tests at a speed of 1.2 mm/s with 33% deformation from the initial height of the sample. Textural parameters such as hardness, adhesiveness, cohesiveness, gumminess, chewiness, and springiness were determined. Each sample was analyzed in triplicate. Statistical significance was calculated by the paired *t*-test ($p < 0.05$) using the GraphPad Prism 6 program.

Cheeses were also analyzed for moisture, protein, ash, fat, and carbohydrate. Crude protein content (N × 6.38) was estimated by the Macro-Kjeldahl method, using an automatic distillation and titration unit (model Pro-Nitro M Kjeldahl Steam Distillation System, Barcelona, Spain). Ash was determined by sample incineration (550 °C) and crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus. Total carbohydrates were calculated by difference according to the equation:

$$100 - (m_{moisture} + m_{proteins} + m_{fats} + m_{ashes})$$

2.9. Sensory analysis

Sensory analysis was performed with 109 untrained participants (74 women and 35 men) between 18 and 60 years old. Participants were teachers and students of the Federal Institute of Ceará's gastronomy course (Baturité, Ceará, Brazil). The samples were served in a monadic way, where each participant received two cubes (1.5 cm^3) of cheese made using CpCp3 and a glass of drinking water to cleanse the palate between the samples. The cheese was classified based on a 9-point hedonic scale (from 1 – "I really disliked it" to 9 – "I really liked it"), based on the following criteria: color, appearance, taste, texture, smell and general acceptance. The acceptance index (AI) of the samples was determined according to the formula: $AI = A \times 100/B$; where A = average grade obtained for the sample in a given attribute and B = maximum grade given to the sample in that attribute (Dutcosky, 2007). The protocols of the sensory tests were previously approved by the Institutional Ethics Committee for Human Testing of Federal University of Ceará, under protocol number 3.107.686, and by the Ethics Committee of Federal Institute of Ceará, Baturité Campus, under protocol number 3.200.402.

2.10. Molecular modeling and in silico evaluation of allergenic epitopes

The three-dimensional model of CpCP3 was obtained by molecular modeling using the X-ray crystal structure of two latex cysteine proteases: ervatamin B (PDB ID: 1IWD) and papain (PDB ID: 9PAP). The complete amino acid sequence of CpCP3 was obtained from the work of Kwon et al. (2015). The analyses were performed using the programs GalaxyWEB (http://galaxy.seoklab.org), M4T Server (http://manaslu.fiserlab.org/M4T/) and Phyre2 Server (http://www.sbg.bio.ic.ac.uk/phyre2/html/help.cgi?id=help/faq). The models were analyzed for their geometric and stereochemical quality using the ProSA, ERRAT2, Verify 3D and Molprobity programs, as described by Cruz et al. (2019). The PyMOL software (http://pymol.org/) was used to analyze and visualize the three-dimensional model generated.

The possible allergenicity of CpCP3 was estimated comparing its primary sequence with other allergenic protein sequences using the program Structural Database of Allergenic Proteins (SDAP) (http://fermi.utmb.edu/SDAP/). The CpCP3 sequence was searched for allergenic epitopes by matching six contiguous amino acid residues with all the sequences of allergenic proteins deposited in the SDAP database (Ivanciuc, Schein, & Braun, 2003). In order to compare CpCP3 with other proteases, the same study was performed using papain, which is a latex cysteine protease widely used in the food industry. The predicted allergenic epitopes were labeled in the three-dimensional structures of CpCP3 and papain using PyMOL program (http://pymol.org/).

2.11. Digestibility assay

The *in vitro* susceptibility to sequential digestion of CpCP3 was developed as described by Farias et al. (2015), with slight modifications. The sample was sequentially incubated in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) at a concentration of 1 mg/mL. CpCP3 was incubated in SGF [34 mM NaCl, 0.07% HCl (pH 2.0), and 1 mg/mL of pepsin] at 37 °C under stirring, and aliquots of 100 µL were collected at different incubation times and then transferred to appropriate stopping buffer (2% SDS, 10% glycerol, 6% β-mercaptoethanol, 0.01% bromophenol blue, 200 mM DTT and 500 mM Tris-HCl, pH 8.0) in a ratio of 1:1 (v/v). Sequentially, SIF was added [50 mM potassium phosphate (pH 8.0), and 1 mg/mL trypsin]. The mixture was incubated again at 37 °C under stirring, and aliquots of 100 µL were collected and then transferred to an appropriate stopping buffer (3% SDS, 17% glycerol, 8.5% β-mercaptoethanol, 0.01% bromophenol blue, 170 mM DTT, 6 mM PMSF, 200 mM Tris-HCl, pH 7.2). The aliquots were collected after 30 min, 1 and 2 h of incubation in SGF and SIF. The digestibility of CpCP3 was monitored by 15% SDS-PAGE. The cleavage sites by proteases pepsin and trypsin in the CpCP3 sequence were determined using the ExPASy PeptideCutter tool (https://web.expasy.org/peptide_cutter/).

2.12. In vivo toxicity

2.12.1. Zebrafish embryos

The zebrafish (*Danio rerio*) embryos were provided by the zebrafish facility established by Department of Molecular Biology, Federal University of Paraíba (João Pessoa, Brazil). Adult zebrafish (wild type strain) were kept at 26 ± 1 °C in a 14:10 h (light:dark) photoperiod. The water quality was maintained by activated-charcoal filtration, with conductivity at 750 ± 50 µS and dissolved oxygen above 95% saturation. The fishes were fed daily with commercial feed (Tropical Gran Discus, Sarandi, Brazil) and *Artemia* sp. nauplii, and were also monitored for abnormal behavior or disease development.

To obtain embryos, an egg trap was placed overnight in a tank containing male and female specimens (1:1 ratio) one day prior to testing. One hour after the beginning of the light cycle, eggs were collected with a Pasteur pipette and rinsed with E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$) for subsequent selection of embryos by using a stereomicroscope (80× magnification). Viable fertilized eggs were selected for further analysis by the fish embryo acute toxicity (FET) test (section 2.11.3). All experiments conducted with zebrafish in this study were approved by the Committee on Ethical Use of Animals in Research (CEUA) of Paraíba Federal University, Brazil, certified by number 6743030518.

2.12.2. Stability of CpCP3 to the conditions of the embryotoxicity test

CpCP3 was dissolved in E3 medium at 2 mg/mL. Then, the protein solution was transferred to a 24-well plate and left for 96 h in the same conditions as the FET test with zebrafish embryos. In this test, 8 wells were filled, each one with 2 mL of the protein solution. Every 24 h aliquots of 2 mL were collected from two wells and rapidly frozen at −20 °C. Just before the assays, the aliquots were thawed and prepared

for analysis of total proteolytic activity and SDS-PAGE. The proteolytic activity of CpCP3 in the exposure conditions of the FET test was determined by colorimetric measurement using azocasein as the substrate (Section 2.4). The autolysis of CpCP3 was evaluated by 15% SDS-PAGE.

2.12.3. Acute toxicity test using zebrafish embryos

The fish embryo acute toxicity (FET) test was conducted with CpCP3 according to OECD guideline number 236 (OECD, 2013). Twenty fertilized eggs were placed in 24-well plates (1 embryo per well) and exposed to 100 mg/L of CpCP3. This concentration is recommended by the FET test as the limiting concentration for embryotoxicity analysis of any chemical (OECD, 2013). Eggs exposed only to E3 medium were used as internal controls. Eggs up to 3 h after fertilization were exposed to CpCP3 for 96 h, and embryos were analyzed every 24 h for the apical endpoints: egg coagulation; lack of somite formation; lack of detachment of the tail-bud from the yolk sac, and lack of heartbeat. In the presence of any of these endpoints, the embryo/larva was considered dead. The exposure was under static conditions (without renovation of CpCP3 and E3 medium). Observations were performed with a stereomicroscope (80x magnification) and photographed (Zeiss camera). After 96 h, surviving larvae were euthanized with eugenol and discarded according to the CEUA of Paraíba Federal University.

2.13. Heterologous expression, purification, and proteolytic activity

The pET-SUMO expression vector (Supplementary Fig. 1), containing the CpCP3 sequence, was synthetized by GenOne Biotechnologies (http://www.geneone.com.br/) and used to transform *E. coli* SHuffe cells (New England Biolabs) by electroporation. To overcome the codon bias problem, synthetic gene of CpCP3 was optimized to contain the codons more frequently used in *E. coli*. To help in solubilization and purification of recombinant protein, the SUMO tag, containing six histidines (6xHis), was added to the *N*-terminal of CpCP3.

The transformed *E. coli* cells were cultivated at 37 °C for 16 h in solid Luria-Bertani (LB) medium containing kanamycin (50 μg/mL), and then an isolated colony was inoculated in 5 mL of liquid LB medium containing kanamycin and kept under constant stirring (200 rpm) at 37 °C for 16 h. Afterward, 5 mL of the medium was inoculated in 500 mL of another kanamycin-supplemented LB medium. When the OD_{600} was between 0.4 and 0.6, the expression was induced with different concentrations of IPTG (0.01, 0.1, 0.3, 0.5, 0.8 and 1 mM) at 180 rpm and 25 °C. Aliquots were taken at different incubation times (0, 6 and 18 h) and then centrifuged at 8,000 × *g* for 10 min at 4 °C. The cells were disrupted with 50 mM Tris-HCl buffer containing 300 mM NaCl, 100 μg lysozyme, 1 mM PMSF, 10% glycerol and 0.5% Triton X-100, for 30 min at 37 °C, followed by sonication for seven cycles of 30 s. The samples were centrifuged at 12,000 × *g* for 30 min. The precipitate (insoluble fraction) and the supernatant (the soluble fraction) were analyzed by 12.5% SDS-PAGE. The precipitates were solubilized in 50 mM Tris-HCl buffer (pH 8.0) containing 8 M urea for 30 min at 37 °C or in 50 mM Tris-HCl buffer, containing 500 mM NaCl and 1% SDS. Samples were then centrifuged at 12,000 × *g* for 30 min at 4 °C. Besides the SDS detergent, the sarkosyl and CTAB detergents were tested under the same conditions. The solutions were kept at 55 °C for 2 h and then centrifuged at 12,000 × *g* for 30 min at 25 °C. The supernatants and precipitates were analyzed by SDS-PAGE.

The recombinant protein (His_6-SUMO-CpCP3) was purified using a HisTrap FF affinity column coupled to an AKTA chromatography system (GE Healthcare). The column was previously equilibrated with 50 mM Tris-HCl (pH 8.0), containing 500 mM NaCl and 2 M urea and the recombinant His_6-SUMO-CpCP3 was eluted with a gradient of imidazole (0–500 mM) at a flow rate of 0.5 mL/min for 30 min. The chromatographic peaks were dialyzed (50 mM Tris-HCl buffer pH 8.0 containing 100 mM NaCl) and concentrated in Vivaspin ™ tubes with 10 kDa cutoff and then analyzed by 12.5% SDS-PAGE. To remove the His_6-SUMO fusion tag, His_6-SUMO-CpCP3 was incubated with Ubl-specific protease 1 (ULP1) from *Saccharomyces cerevisiae* for 4 h at 30 °C with constant stirring. The digested material was analyzed by 12.5% SDS-PAGE.

The proteolytic activity of recombinant CpCP3 was evaluated using zymogram according to Freitas et al. (2007). The recombinant protein without SUMO tag was separated by electrophoresis through 12.5% SDS-PAGE containing 0.1% gelatin at 25 °C. Then the gels were incubated in 2.5% Triton X-100 (renaturing solution) and gently shaken for 30 min at 25 °C to wash out SDS and allow protein rearrangement. The gels were then incubated in 50 mM Tris-HCl buffer (pH 6.5) containing 1 mM L-cysteine for 24 h at 37 °C and stained with 0.2% Coomassie brilliant blue R-350. Enzymatic activity was detected as transparent bands. Native CpCP3 was used as positive control for proteolytic activity.

3. Results and discussion

3.1. Effect of ions on CpCP3 activity

In a previous study, Freitas et al. (2016) showed that a proteolytic fraction obtained from *Calotropis procera* latex (CpLP) exhibited milk-clotting activity and thus had potential for cheesemaking. CpLP was already characterized and five cysteine proteases were described: procerain, procerain B, CpCP1, CpCP2, and CpCP3 (Dubey & Jagannadham, 2003; Singh, Shukla, Jagannadham, & Dubey, 2010; Ramos et al., 2013). As pointed out before, the use of a mixture of peptidases can have several disadvantages for cheesemaking, such as extensive hydrolysis of caseins causing low yield and bitter taste. To overcome these drawbacks, it is best to work with purified enzymes. In this respect, the most abundant protease (CpCP3) from *C. procera* latex fraction (CpLP) was purified, partially characterized, and its milk-clotting activity was assessed.

First, the effect of different metal ions on the proteolytic activity of CpCP3 was evaluated. None of the ions tested had effects on CpCP3 activity (Supplementary Table 1). Freitas et al. (2016) showed that 1 M NaCl or $CaCl_2$ had no inhibitory effect on proteolytic activity of CpLP fraction. In addition, similar results have been observed of other milk-clotting enzymes (Silva, Nascimento, Silva, Herculano, & Moreira, 2013). Studying the effect of NaCl and $CaCl_2$ on the proteolytic activity is crucial, since both salts are widely used during cheesemaking. NaCl is used to improve the stability of casein micelles (Guinee & Fox, 1993), whereas $CaCl_2$ is essential to the aggregation process of casein micelles and hence milk coagulation (Pires, Orellana, & Gatti, 1999). Regarding to lack of effect of the ions Li^+, K^+, and Mg^{2+} on CpCP3 activity, He et al. (2011) reported an equivalent result for Li^+ and Mg^{2+}, whereas K^+ exhibited only a slight inhibitory effect on the microbial coagulant protease.

3.2. Hydrolysis of κ-casein by CpCP3

Caseins (αs1-, αs2, β-, and κ-caseins) are the most abundant milk proteins and form well-ordered colloidal structures, which are named casein micelles (De Kruif, Huppertz, Urban, & Petukhov, 2012). Any event that destabilizes the casein micelle structure can result in milk coagulation. For instance, proteases coagulate milk because the hydrolysis of κ-casein reduces the steric and electrostatic repulsion between the micelles, promoting their aggregation (Dalgleish, 2011). However, extensive or unspecific hydrolysis of caseins can produce cheeses with undesirable characteristics. Therefore, it is very important to determine the specificity of the protease towards κ-casein (Egito et al., 2007). Here, CpCP3 was able to cleave κ-casein after one minute of incubation (Supplementary Fig. 2). The presence of a protein band of 16 kDa (*para*-κ-casein) suggests that CpCP3 cleaved κ-casein similarly to bovine chymosin. Accordingly, a protein band with the same molecular mass was observed after hydrolysis of κ-casein by other plant proteases (Egito et al., 2007). Moreover, after 5 min, other protein bands from κ-

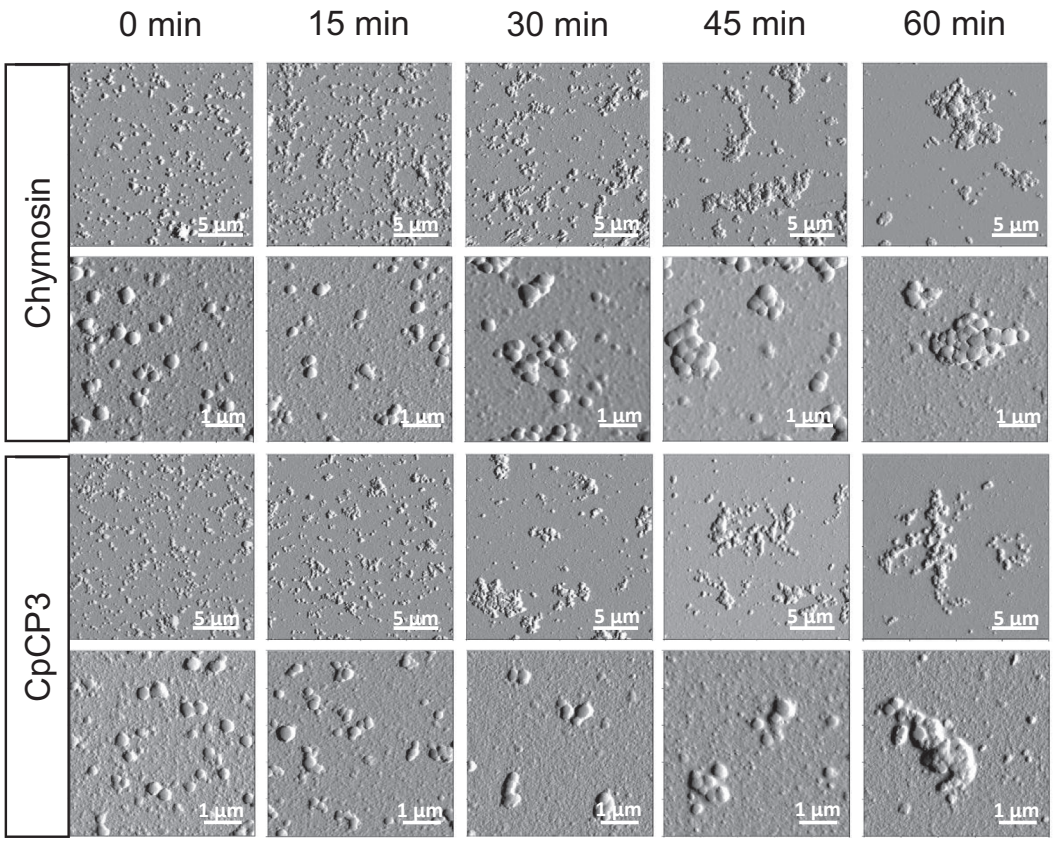


Fig. 1. Tapping mode AFM images of milk casein micelles after incubation with CpCP3 or bovine chymosin at different time points (0, 15, 30, 45 and 60 min) at 25 °C.

casein were observed after action of CpCP3 (Fig. 1). These results suggest that CpCP3 has a preference over the same chymosin cleavage site. However, after cleavage of all these bonds, CpCP3 can hydrolyze other ones, producing peptides of various sizes (Supplementary Fig. 2). Interestingly, the proteolytic fraction of *C. procera* (CpLP) also showed the same pattern of cleavage of κ-casein and even so the cheeses exhibited good taste and yield (Freitas et al., 2016). These results indicate that the time of coagulation and concentration of enzyme are parameters that must be controlled to avoid the extensive hydrolysis of caseins, which is the main drawback found in most plant proteases used for cheesemaking.

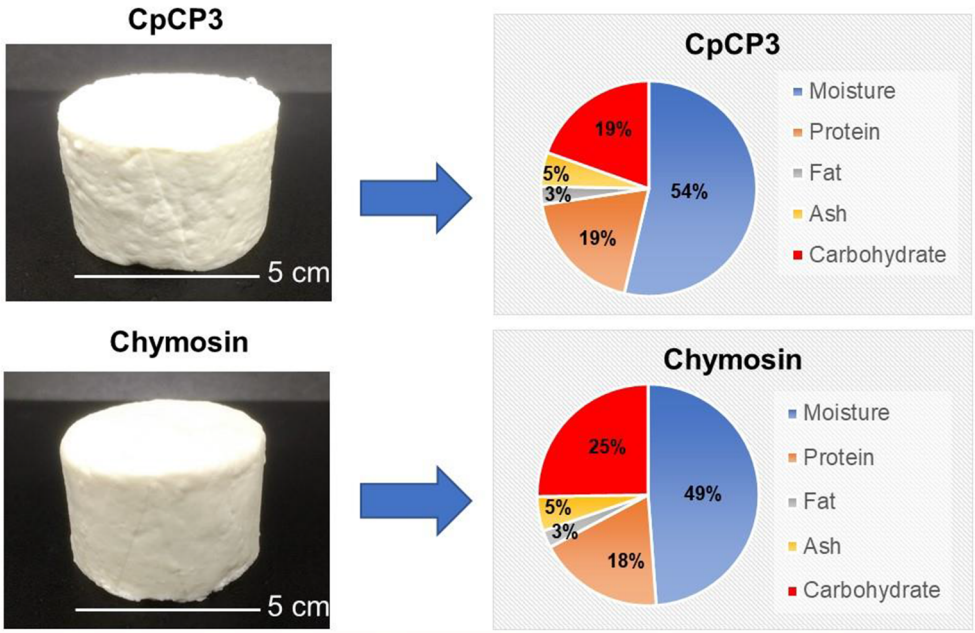
3.3. Milk-clotting activity

Supplementary Table 2 shows that 30 µg of CpCP3 was the lowest dose able to coagulate 2 mL of milk, in approximately 40–50 min (time recommended for enzymatic action during cheesemaking). The specific milk-clotting activity (SMCA) and proteolytic activity (SPA) of CpCP3 were 277.4 ± 72 and 33.7 ± 0.5, respectively. Although these two parameters are used to evaluate the potential of an enzyme for cheesemaking, the SMCA/SPA ratio is the most useful because a good coagulant should exhibit high SMCA and low SPA (Freitas et al., 2016). CpCP3 exhibited a SMCA/SPA ratio of around 8.4. In contrast, the SMCA/SPA ratio for bovine chymosin was 742.4, almost 90 times higher than CpCP3. This discrepancy is the result of low proteolytic activity of chymosin, since its SMCA was similar to that of CpCP3 (Supplementary Table 2). These results are in accordance with other studies, which have shown that bovine chymosin usually has higher SMCA/SPA ratio than other plant proteases (Mazorra-Manzano et al., 2013). This difference in SMCA/SPA ratio produced slight alterations in overall aspect of the curds, which were softer and less yellowish using CpCP3 than those made with chymosin (Supplementary Fig. 3).

3.4. Effect of CpCP3 on casein micelles

Recently, AFM was used as an alternative to study the milk-clotting process induced by chymosin at the casein micelle level (Freitas et al., 2019). According to the authors, this technology has several advantages compared to others, because it is fast, no additional dye, fluorophore or reagent is necessary, and its results are easy to interpret. Therefore, the action of CpCP3 on casein micelles during the coagulation process was evaluated using AFM and compared with bovine chymosin (Fig. 1). This technique showed that the entire aggregation process of the casein micelles induced by CpCP3 was very similar to that caused by chymosin. The casein micelles and their aggregates exhibited widths, heights, and areas very close to the corresponding values of chymosin (Supplementary Fig. 4). The widths of casein micelles were greater than the heights. These results are in agreement with those described by Freitas et al. (2019), who stated that this flattening occurs because the casein micelles are stabilized by non-covalent bonds, making them more susceptible to deformations during AFM experiments. This flattening of casein micelles during AFM analysis has also been described by other authors (Ouanezar, Guyomarc'h & Bouchoux, 2012).

After 30 min, casein micelle aggregates were observed in both samples (Fig. 1). This step occurs after the κ-casein hydrolysis because it is responsible for generating electrostatic repulsion among micelles (Dalgleish, 2011). Supplementary Fig. 2 corroborates these results, since the CpCP3 hydrolyzed κ-casein producing *para*-κ-casein peptides, similar to chymosin. After 45–60 min, casein micelles continued aggregating, forming structures like bunches of grapes, according to the Freitas et al. (2019). Finally, after 60 min, large clusters of casein micelles were formed (Fig. 1). Although it was not possible to visualize with AFM, the formation of a three-dimensional gel as the final step of milk coagulation is expected (Dalgleish, 2011).



Samples	Hardness (N)	Adhesiveness	Springiness	Cohesiveness	Gumminess (N)	Chewiness (N)
CpCP3	$3.5^b \pm 0.08$	$-0.84^a \pm 0.34$	$0.84^a \pm 0.01$	$0.50^b \pm 0.04$	$18.04^b \pm 2.02$	$16.19^b \pm 2.11$
Chymosin	$5.0^a \pm 0.85$	$-0.92^a \pm 0.33$	$0.82^a \pm 0.02$	$0.22^a \pm 0.07$	$6.52^a \pm 0.87$	$5.24^a \pm 0.60$

Fig. 2. Overview of cheeses made using CpCP3 and commercial chymosin (control), percentage of moisture, protein, fat, ash, and carbohydrate, and texture profile analysis. The values represent a mean of three replicates with their respective standard deviations. Different letters show statistical significance ($p < 0.05$). Statistical analysis was calculated by the paired *t*-test using the GraphPad Prism 6 program.

3.5. Characterization of cheeses made using CpCP3

Some characteristics of cheeses obtained using CpCP3 and chymosin are reported in Fig. 2. In general, cheeses exhibited similar yield, but those produced using CpCP3 were softer than those made with chymosin (49%) ($p < 0.05$), in line with the observations of overall aspect of curds (Supplementary Fig. 3). This was confirmed by higher moisture (54%) in the cheese made with CpCP3. In terms of composition, both cheeses presented equivalent protein, fat and ash percentages, which were 19, 3 and 5%, respectively (Fig. 2). However, the carbohydrate content was lower in cheeses made with CpCP3 (19% versus 25%) ($p < 0.05$), which can be related to softness of these cheeses. Likely the cheeses were softer because of the larger spaces between casein micelle aggregates, which form the three-dimensional gel. Thus, the process of curd pressing suggested a higher loss of small carbohydrates, such as lactose (Logan et al., 2014). All these results are in agreement with those found in experiments using total extracts of *C. procera*. Aworh et al. (1987) reported moisture and protein contents of 49% and 20%, in contrast to the 54% and 19% measured in this study. Accordingly, Omotosho et al. (2011) reported moisture of 50% and protein content of 25%.

Texture profile analysis of cheeses is also shown in Fig. 2. Cheese hardness was significantly lower using CpCP3 (30%) compared with chymosin (3.5 N versus 5.0 N) ($p < 0.05$). Mazorra-Manzano et al. (2013) reported that cheeses made with kiwi, ginger and melon proteases showed hardness values of 6.1, 4.1, and 1.9 N, respectively. These low values were associated with the high cheese moisture content (62–67%), in line with our results. The reduced hardness suggests the development of "weak" hydrophobic interactions among the caseins within the cheese matrix. Thus, the moisture content of cheese made using CpCP3 was accompanied by cheese softness through protein hydration, as suggest by Salek, Černíková, Maděrová, Lapčík, and Buňka (2016). The cohesiveness, gumminess, and chewiness were significantly higher for cheeses made with CpCP3, whereas adhesiveness and springiness values were not statistically different (Fig. 2). Hence, the results indicate that the cheese processed with CpCP3 were less fragile and susceptible to crumbling, and thus were more stable to chewing and disintegration in the mouth, producing a product with greater structural stability compared to chymosin.

As pointed out before, most cheeses produced with plant-derived proteases have a bitter taste. Therefore, to stress this aspect, new analysis of the cheeses made using CpCP3 was performed to evaluate some sensory attributes, such as color, appearance, smell, texture, flavor and global impression (Table 1). For this purpose, we used a hedonic scale (from 1 – "I really disliked it" to 9 – "I really liked it"), and the cheeses made with CpCP3 scored "I liked it slightly" (7), I liked it (8) or "I really liked it" (9). In all parameters, the acceptance index (IA) was higher than 80%, indicating good product acceptance (Table 1).

3.6. Evaluation of allergenicity, digestibility and toxicity of CpCP3

Only four potential allergenic epitopes (EKGALV; GSCWAFSAV; LISLSEQ and YWIVRNSWG) were identified in the CpCP3 sequence, which showed cross-reaction with allergens of insects, mites and foods (papaya, kiwi, pineapple) (Supplementary Table 3). In contrast, papain, a latex protease widely used industrially, exhibited several allergenic epitopes (Supplementary Table 4). Interestingly, the three-dimensional model of CpCP3 showed that only two allergenic peptides (EKGLV and LISLSEQ) were present on the surface of the molecule, which is, accessible to a possible antibody recognition and induction of allergy. On the other hand, almost all allergenic peptides of papain were displayed on its surface, covering around 70% of protein (Fig. 3). Although papain has a high allergenic potential, there are only a few reported cases of papain allergy (Quarre, Lecomte, Lauwers, Gilbert, & Thiriaux, 1995).

CpCP3 was highly susceptible to pepsin and trypsin digestion, after

Table 1
Average values and acceptance index (%) of the attributes verified in the sensory analysis of cheeses formulated with CpCP3.

Sensory Attributes					
Color	Appearance	Smell	Texture	Flavor	Global Impression
8.46 ± 0.74	8.46 ± 0.73	7.63 ± 1.38	7.93 ± 1.14	7.62 ± 1.59	8.02 ± 1.19
Acceptance Index (%)*					
93.99	93.99	84.1	88.07	84.71	89.09

* The acceptance index (IA) was calculated for each of the individual attribute according to Dutcosky (2007). Values greater than 70% indicate good product acceptance.

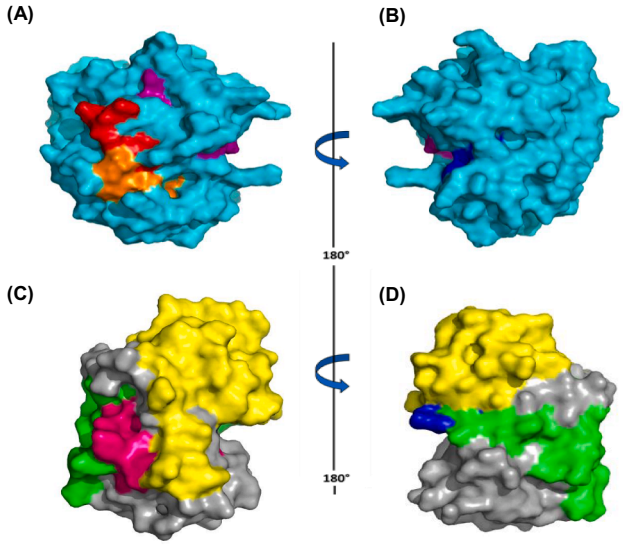


Fig. 3. Predicted allergenic peptides in the CpCP3 and papain sequences. Three-dimensional models of CpCP3 (A and B): putative allergenic peptides are marked in red (EKGALV), dark blue (GSCWAFSAV), orange (LISLSEQ), and purple (YWIVRNSWG). Papain (C and D): yellow (IPEYVDWRQKGAVTPVKQGSCGSCWAFSAVVTIEGIIKIRTGNLN), green (YSEQELLDCDRRSYGCNGGYPWSALQLVAQYGIHYRNTYPYEVQRYCRSREKGPYAAKTDGRQVQPYN), pink (GALLYSIANQPVSVVL). Dark blue represents the allergenic peptide (GSCWAFSAV) shared between CpCP3 and papain. Light blue (CpCP3) and grey (papain) represent the non-allergenic amino acid sequences. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

only 30 min (Fig. 4A). Thus, when this protein is ingested, fragments with high molecular mass are not expected to reach the final stage of digestion. Even if incomplete digestion occurred, small peptides would be degraded by other proteases such as chymotrypsin. These results corroborate the *in silico* digestion performed by pepsin and trypsin (Fig. 4B). Remarkably, the four allergenic peptides of CpCP3 (EKGALV, GSCWAFSAV, LISLSEQ and YWIVRNSWG) exhibit sites for trypsin and pepsin proteolysis. This high susceptibility to pepsin and trypsin digestion supports the hypothesis of the low allergenic and toxic potential of CpCP3 when ingested.

In the context of safety assessment of novel proteins, toxicity tests in animal models are often employed to gather information about the risks of these molecules to potential consumers. Although the gold standard for toxicity testing has traditionally been rodents, in recent years they have been gradually replaced by alternative animal models, for ethical and economic reasons. In this direction, zebrafish embryos are considered an emerging alternative to the use of mammals in several toxicological studies, such as for investigation of acute toxicity of diverse substances (Falcão, Souza, Dolabella, Guimarães, & Walker, 2018). The use of zebrafish embryos in toxicology has many advantages, such as low cost of production, availability in large numbers, optical transparency, rapid development, easy manipulation and the possibility of obtaining a large amount of toxicological information (e.g., teratogenicity, lethality) in a single experiment. More importantly, the results obtained in toxicity tests performed on zebrafish embryos are equivalent to those found in mammalian models, demonstrating the reliability of zebrafish toxicity studies (Falcão et al., 2018).

The acute toxicity of CpCP3 was assessed in dechorionated zebrafish embryos at a concentration of 100 mg/L (OECD FET's limit test). Before performing the test, the protein sample was tested for its stability, including proteolytic activity and autolysis. CpCP3 retained all its proteolytic activity as well as maintained the same protein profile in the electrophoresis even after 96 h of incubation (data not shown). This ensured that the zebrafish embryos were exposed to a fully active protease and ruled out the need for daily renewal of the test solution during the assays. Newly fertilized zebrafish eggs were exposed to activated CpCP3 for 96 h. Even under those exposure conditions, CpCP3 caused no mortality or any other morphological or developmental alteration in the zebrafish embryos/larvae (Supplementary Fig. 5), similar to the negative control.

Our group has shown that the proteolytic fraction of *C. procera* (CpLP) is not allergenic or toxic in other animal models. CpLP was totally digested by digestive proteases and was not detected in fecal material of rats even after 35 consecutive days of consumption (Ramos et al., 2006). Besides this, no death or toxic effects were described. In another study, CpLP did not induce allergy in animals by the oral route (Ramos et al., 2007). More recently, *in vivo* toxicity and oral immunological tolerance of CpLP were reported (Bezerra et al., 2017). Animals receiving a high oral dose of CpLP (5000 mg/kg) exhibited only slight behavioral changes and alteration of monocytes. No death was observed after 14 days.

All the results presented here with purified CpCP3 and those with CpLP suggest that CpCP3 has a very low or no acute toxic and allergenic effect on vertebrates. With respect to environmental risk assessment, the results also show that CpCP3 does not represent a potential risk to aquatic vertebrates. Moreover, the cheeses made with proteolytic extract of *C. procera* latex (CpLP) did not show proteolytic activity or residual latex proteins (Freitas et al., 2016). This is important because it means the risk of toxicity is very low and there will be no hydrolysis of milk proteins during the cheese ripening process, avoiding changes in texture, taste, and flavor.

3.7. Heterologous expression, purification, and proteolytic activity

The best conditions for expression of recombinant CpCP3 (His_6-SUMO-CpCP3) were 0.1 mM IPTG after 6 h of induction at 25 °C (Fig. 5A). However, recombinant CpCP3 was expressed mainly in inclusion bodies (Fig. 5B), a recurrent factor in several heterologous proteins. Similarly, two cysteine proteases from *C. procera* latex were also expressed in inclusion bodies, even when using different vectors and host cells (Singh, Yadav, & Dubey, 2013; Kwon et al., 2015). Urea and SDS detergent were the best agents to solubilize the inclusion bodies compared to the other chemicals used (Fig. 5C). Other studies have also reported success in these methods for solubilization of insoluble proteins (He & Ohnishi, 2017). Because SDS inhibits the SUMO

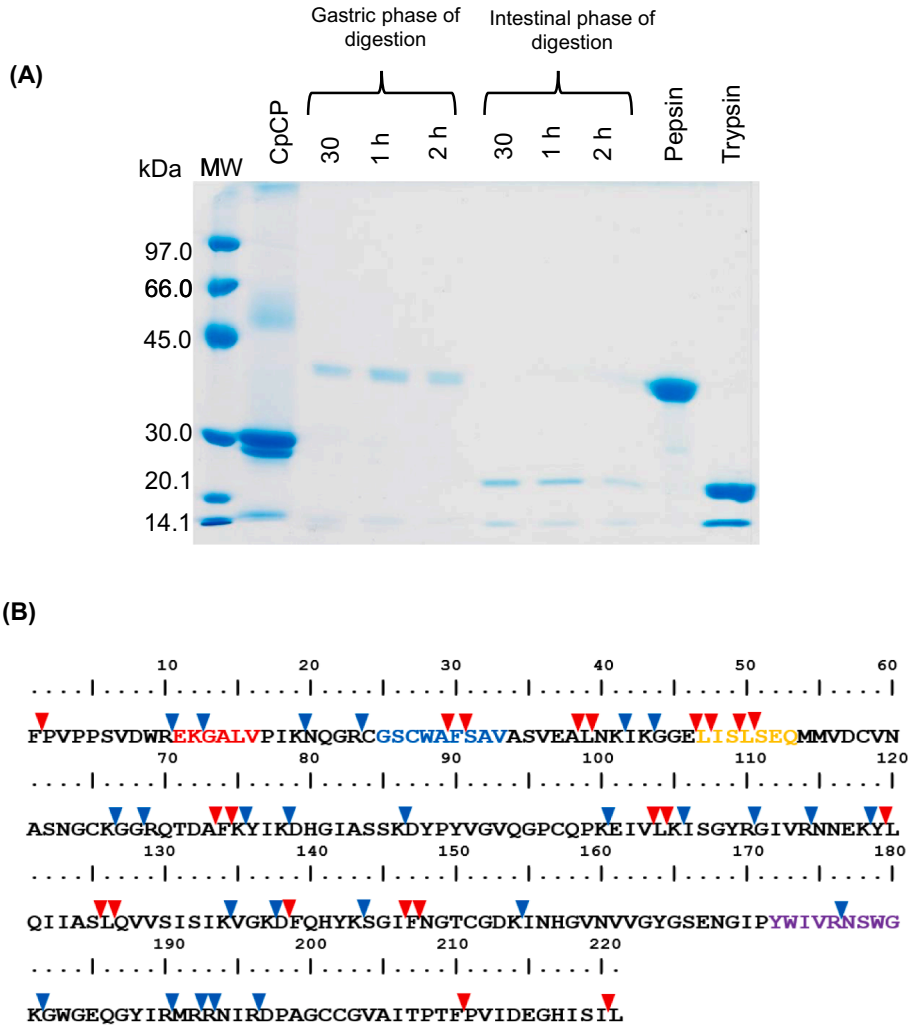


Fig. 4. (A) SDS-PAGE (12.5%) of CpCP3 after incubation with simulated gastric fluid and simulated intestinal fluid after 30 min, 1 and 2 h. MW: molecular weight markers. Controls: CpCP3, pepsin and trypsin. (B) Predicted cleavage sites of CpCP3 by pepsin (▼) and trypsin (▼) using the PeptideCutter program. Allergenic peptides predicted are highlighted in red (EKGALV), blue (GSCWAFSAV), orange (LISLSEQ), and purple (YWIVRNSWG). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

protease activity, the material solubilized with urea was used in further assays for purification of protein. Using a HisTrap FF affinity column, His_6-SUMO-CpCP3 was purified after elution with 300 mM imidazole, containing 0.5 M NaCl (Fig. 5D). Afterwards, His_6-SUMO-CpCP3 was digested with SUMO protease to remove the His_6-SUMO tag (Fig. 5E). Unfortunately, the recombinant CpCP3 was not active, as revealed by zymogram (Fig. 5F). Similarly, even using three different *E. coli* strains (Origami, C41, C43) and a pelB leader sequence to induce proper folding, Kwon and collaborators (2015) were not able to express an active cysteine protease from *C. procera*. Likewise, Singh et al. (2013) tried to express active procerain B (cysteine protease from *C. procera*) in BL21 (DE3) *E. coli*. They also used other modified *E. coli* strains [Rosetta-gami and Rosetta 2-(DE3)], but the protein was expressed again in inclusion bodies and without proteolytic activity.

A particular characteristic of *C. procera* is intense production of latex, which flows out abundantly when its tissues, especially its green parts, are injured (Freitas et al., 2007). In Brazil, it grows widely in the Northeast, especially in the state of Ceará, where it can easily be found on roadsides, sidewalks and just inland from beaches, among other places. This means the plant does not have large requirements for water or fertile soil, good advantages for further agronomic studies and large-scale latex extraction. It is easy to collect a liter of latex within one hour, which would yield approximately 12 g of enzymatic fraction (Freitas et al., 2007). Moreover, the latex collection process does not compromise plant health. After two weeks, they are fully recovered and can be used for new collection (personal observations). From this fraction, CpCP3 can be easily purified with yield of 20% (Ramos et al., 2013). We found that 12.5 mg of CpCP3 was required to produce 50 g of cheese. Thus, 12.5 g of CpCP3 could yield up to 50 kg of cheese. The cost of obtaining CpCP3 has not been estimated yet. However, we believe that it is low because the entire process requires simple equipment. All these characteristics show the strong potential of CpCP3, not as a substitute of chymosin, but as an alternative coagulant to produce cheeses with new sensory characteristics. Besides this, the cheese can be consumed by lacto-vegetarians.

Although CpCP3 production is economically possible, we also studied its expression in a heterologous system because this system is used widely for commercial production of many proteins. The major advantages are the high level of recombinant protein expression, rapid cell multiplication and simple media requirement. A practical example is chymosin itself, which can be obtained from calf stomachs but has been also produced in heterologous systems (Ulusu, Şentürk, Kuduğ, & Gökçe, 2016). There are several types of host organisms, but bacteria, including *Escherichia coli*, have been the most widely used, because they grow rapidly and in inexpensive growth media (Kaur, Kumar, & Kaur, 2018). However, the major drawback is the formation of aggregates or inclusion bodies, as observed here. To overcome this problem, CpCP3 was constructed containing the SUMO-tag, which has been used to promote the solubilization of several proteins in *E. coli* (Kaur et al., 2018). Moreover, the vector pET-SUMO was inserted into *E. coli* SHuffle, which is an expression system dedicated to correctly form disulfide bonds in proteins (Lobstein et al., 2012). Despite all the efforts, recombinant CpCP3 was not recovered in its active form. The lack of proteolytic activity can be attributed to protein misfolding because

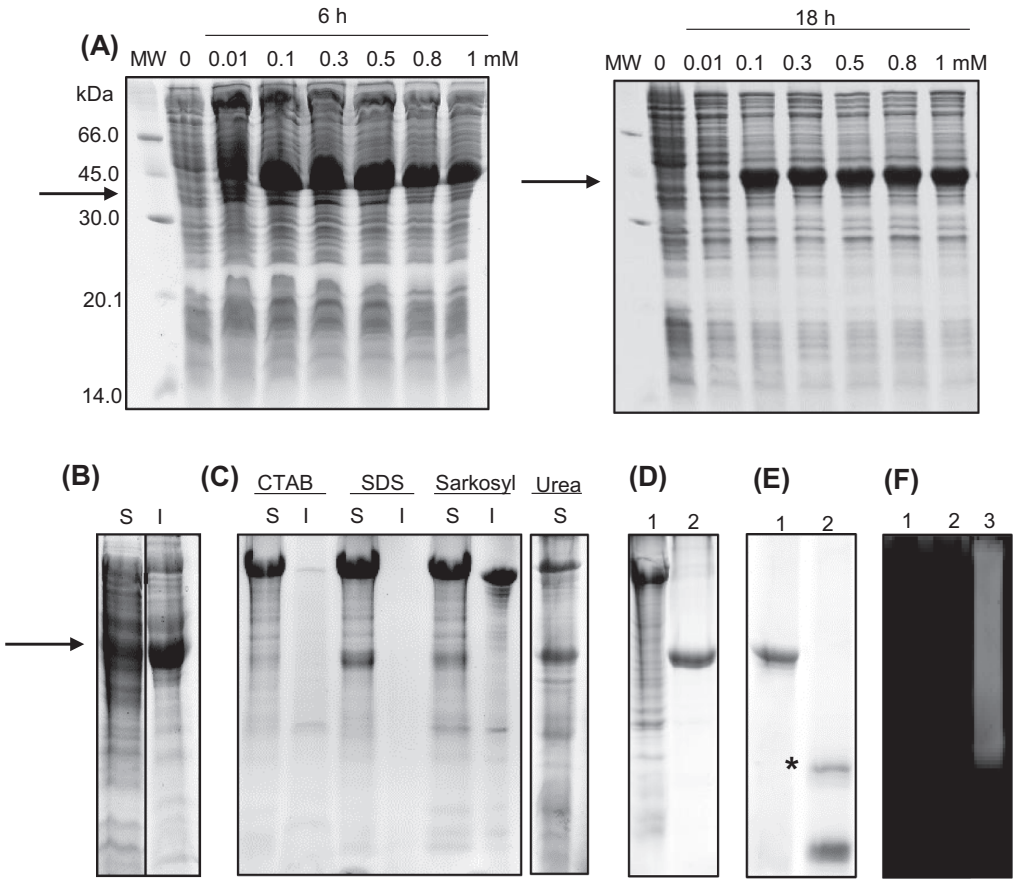


Fig. 5. Expression, solubilization, purification, and proteolytic activity of recombinant CpCP3 (rCpCP3). (A) Effect of IPTG concentration (0.01 – 1 mM) and induction time (6 or 18 h) on rCpCP3 expression. (B) Protein profile of soluble (S) and insoluble (I) fractions of total cell lysates after 6 h and using 0.1 mM IPTG. (C) Effect of different detergents (CTAB, SDS and Sarkosyl, all at 1%) and urea (8 M) on solubility of the insoluble fraction (rCpCP3). The lanes show the soluble (S) and insoluble (I) proteins after detergent addition. (D) Purification of rCpCP3 using a HisTrap affinity column. Lane 1: non-retained proteins; lane 2: purified rCpCP3. (E) Digestion with UPL1 SUMO protease. Lane 1: rCpCP3 non-digested. Lane 2: rCpCP3 after digestion (*). (F) Zymogram for proteolytic activity. Lane 1: rCpCP3 non-digested with UPL1 SUMO protease. Lane 2: rCpCP3 after digestion with UPL1 SUMO protease. Line 3: native CpCP3 (Positive control). Legend: MW, molecular weight markers. Arrows represent recombinant CpCP3 (His_6-SUMO-CpCP3) and asterisks show recombinant CpCP3 without His_6-SUMO tag.

the presence of a pro-peptide region is essential for the correct folding of proteases (Singh et al., 2013; Kwon et al., 2015).

4. Conclusion

The results of this study support our hypothesis of the biotechnological potential of using CpCP3 for cheesemaking, since it has several characteristics that are advantageous for industrial application: (1) it is stable to various metal ions; (2) it hydrolyzes κ-casein and induces casein micelle aggregation similarly to chymosin; (3) it produces cheeses with yield, protein, fat and ash contents equivalent to counterparts made with chymosin; (4) the sensory analysis showed that cheeses made with CpCP3 had high acceptance index for the parameters color, appearance, smell, texture, flavor and global impression; (5) it has a very low allergenic and toxic potential; (6) it is fully active even after 96 h at 25 °C and does not suffer autolysis; and (7) it can be successfully expressed in *E. coli* cells, so it can be produced in high quantity. Further assays should be performed to enhance the refolding of CpCP3 and reach its proteolytic activity.

5. Contributions

MZRS, SRS, and JPBO performed purification of CpCP3, proteolytic activity and milk-clotting assays as well the hydrolysis of k-casein. AFBS and JSS performed the AFM assays. DFF, CAS and JACR performed all assays of digestibility and toxicity using zebrafish. RAZ and ACS conducted the tests for characterization of cheeses. MSV and SRS performed the sensory analysis. GPF and TBG helped in heterologous expression of CpCP3. CDTF and MVR are the main researches of the project, supporting, designing and analyzing all assays and results. All authors contributed to data analysis, discussion and writing of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.125574.

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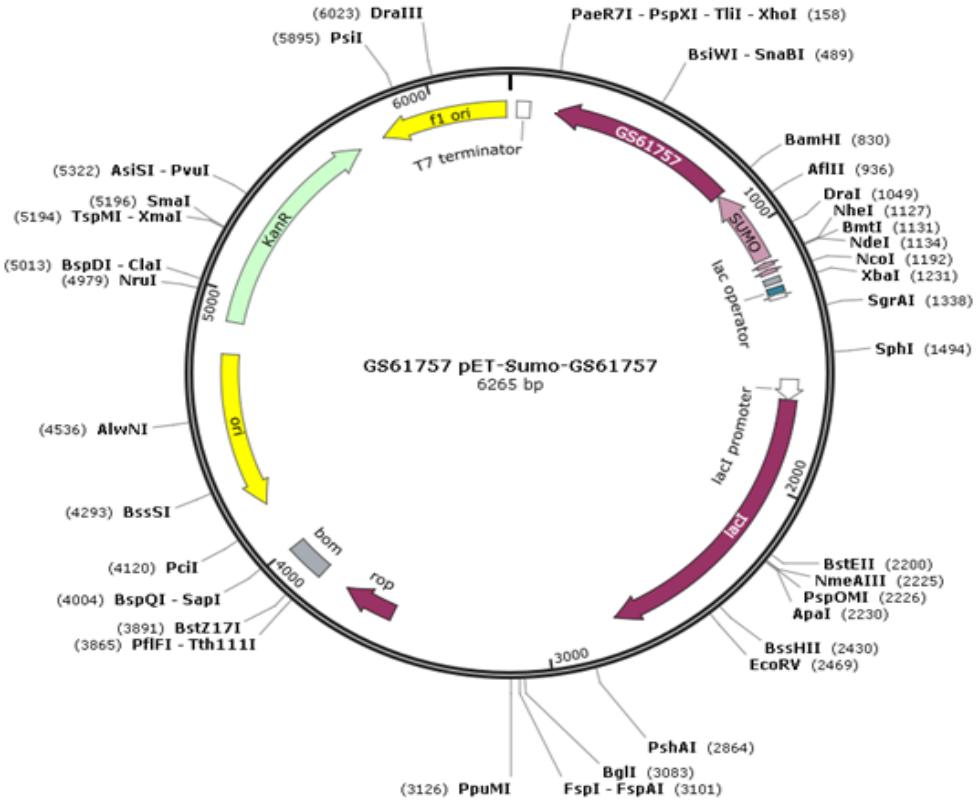
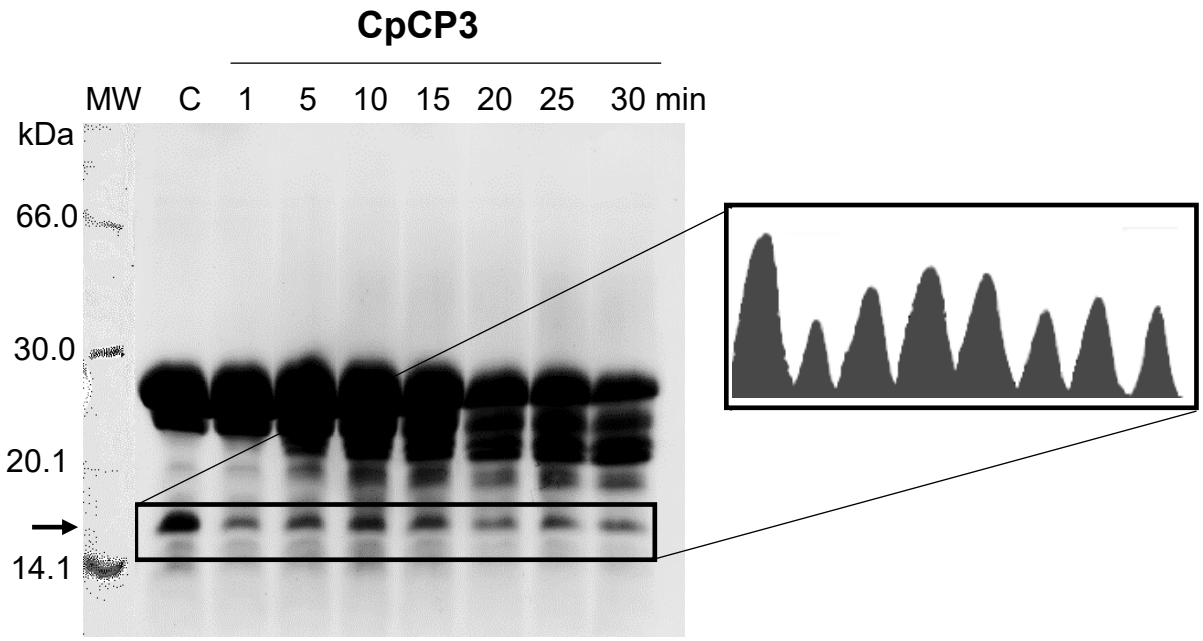
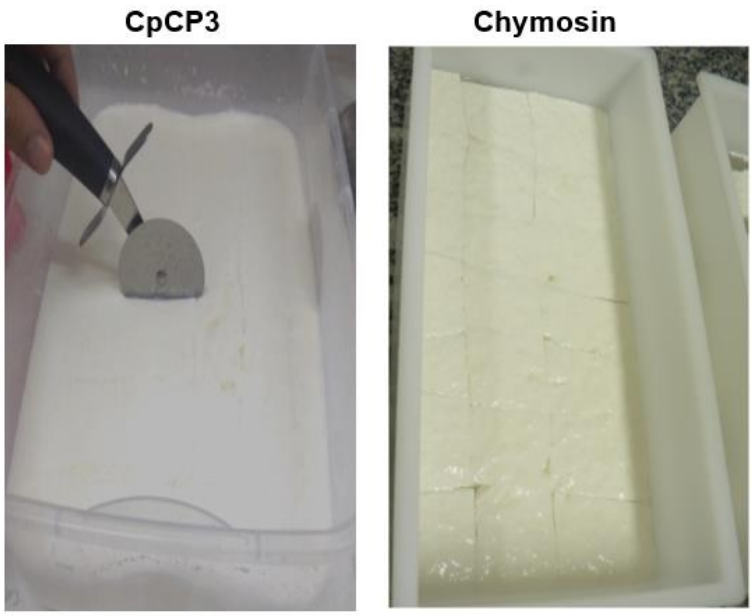


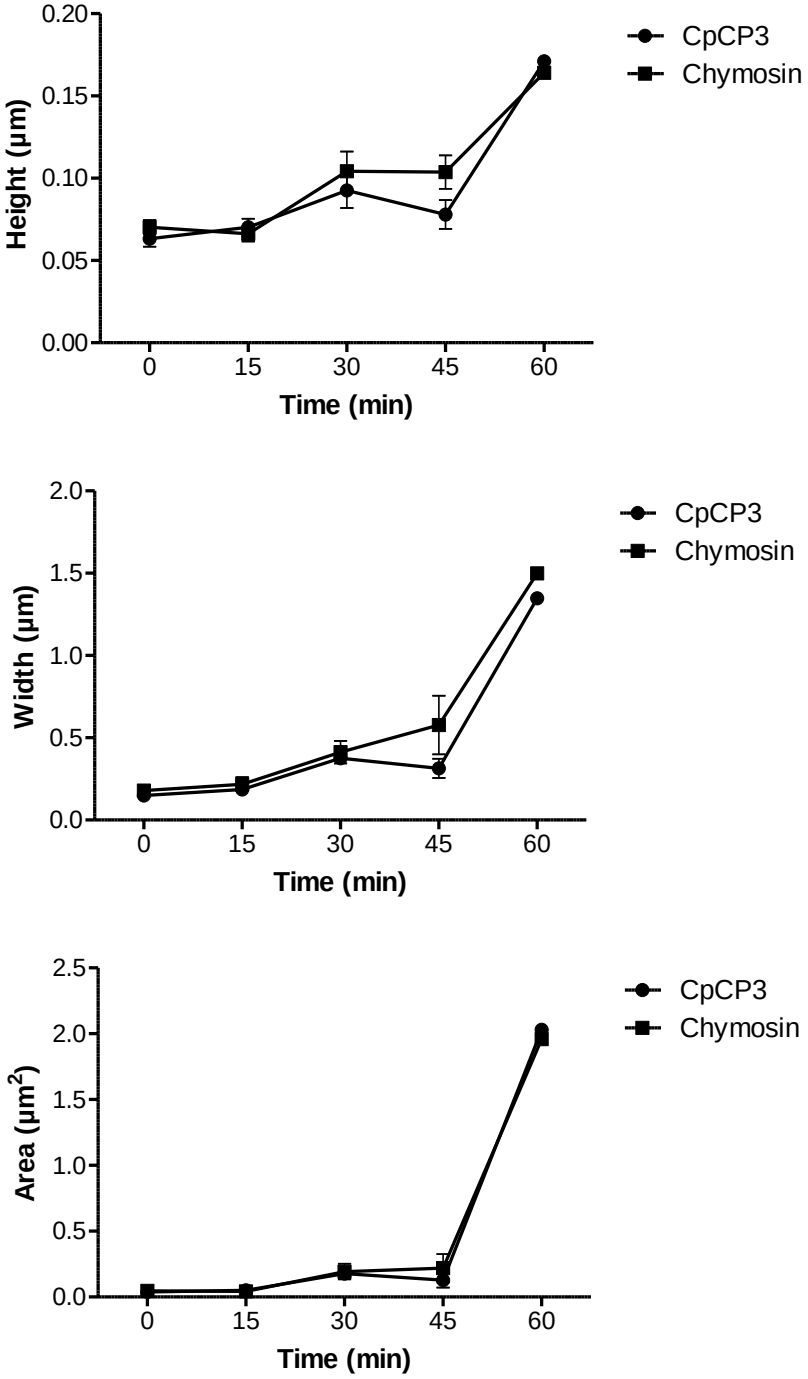
Supplementary Fig. 1. pET-SUMO vector used for CpCP3 expression. The pET-SUMO vector was designed to contain an N-terminal 6xHis and SUMO fusion protein in frame with CpCP3 (represented as GS61757).



Supplementary Fig. 2. SDS-PAGE (15%) of κ-casein hydrolyzed by CpCP3 at different time points (1, 5, 10, 15, 20, 25 and 30 min). The assay was performed at 37 °C and pH 6.5 using an enzyme:substrate ratio of 1:900 (mass:mass). Legend: C (Control): κ-casein hydrolyzed by bovine chymosin (Coalhopar®). MW: molecular weight markers. The arrow shows the para-κ-casein peptide produced after κ-casein hydrolysis and panel represents its densitometry during the test.



Supplementary Fig. 3. Overall aspect of the curds produced by CpCP3 and commercial chymosin. The photos were taken 30 min after the addition of each enzyme, at 25 °C.



Supplementary Fig 4. Height, width and area of casein micelles measured by AFM during milk-clotting process. The height was obtained from the micelle center and width was considered as the longest path between two points of the micelles. The data were obtained from three independent analyses. There was no statistical difference between casein micelles incubated with CpCP3 and bovine chymosin ($p > 0.05$).

Supplementary Fig. 5. Lateral view of zebrafish larvae after exposure to 100 mg/L CpCP3 (A) and E3 medium (B) for 96 h. No morphological or developmental alterations were observed in embryos and larvae during 96 h of exposure.

Supplementary Table 1. Effect of different ions on the proteolytic activity of CpCP3.

Ions	**Concentration**	**Residual activity* (%)**
NaCl	10 mM	103.14 ± 0.6
$CaCl_2$	10 mM	107.93 ± 6.8
LiCl	10 mM	99.02 ± 2.6
KCl	10 mM	98.64 ± 4.7
$MgCl_2$	10 mM	96.58 ± 2.3

* Each value represents the mean ± SD of three replicates. The assays were performed using azocasein as substrate (pH 6.5) at 37 °C.

Supplementary Table 2. Milk-clotting and proteolytic activity of CpCP3 and chymosin.

	MAE[1] (µg)	**MCT** [2] (min)	**SMCA**[3] (AU/mg)	**SPA**[4] (AU/mg)	**Ratio** (SMCA/SPA)
CpCP3	30.0	48.8 ± 1.7[a]	277.4 ± 72.1[a]	33.70 ± 0.50[a]	8.4
Commercial Chymosin	30.0	54.0 ± 1.1[b]	245.4 ± 32.8[a]	0.33 ± 0.03[b]	742.4

Each value represents the mean ± SD of three replicates. [1] MAE: Minimum amount of enzyme able to clot 2 mL of milk. [2] MCT: milk-clotting time. [3] SMCA: specific milk-clotting activity. [4] SPA: specific proteolytic activity using azocasein as substrate, pH 6.5, at 37 °C. Different letters show statistical difference ($p<0.05$). Statistical analysis was calculated by the paired t-test using the GraphPad Prism 6 program.

Supplementary Table 3. Allergenic peptides predicted in the CpCP3 sequence using Structural Database of Allergenic Proteins.

Allergens	**Species**	**Peptides**	**Source**
Api m 1	*Apis mellifera*	EKGALV	Bee
Der p 1, Car p 1	*Dermatophagoides pteronyssinus, Carica papaya*	GSCWAF	Mite, Plant
Der p 1, Eur m	*D. pteronyssinus, Euroglyphus maynei*	SCWAFS	Mite
Car p 1, Act d 1	*C. papaya, Actinidia deliciosa*	CWAFSA	Plant
Car p	*C. papaya*	WAFSAV	Plant
Act d 1	*A. deliciosa*	LISLSE	Plant
Act d 1	*A. deliciosa*	ISLSEQ	Plant
Eur m, Der p, Der f, Ana c 2	*E. maynei, D.pteronyssinus, D. farinae, Ananas comosus*	YWIVRN	Mite, Plant
Eur m, Der p, Ana c 2, Der f	*E. maynei, D. pteronyssinus, A. comosus, D. farinae*	IVRNSW	Mite, Plant
Ana c 2	*A. comosus*	VRNSWG	Plant

Supplementary Table 4. Allergenic peptides predicted in papain sequence using Structural Database of Allergenic Proteins.

Allergens	Specie	Peptides	Source
Car p papain	*Carica papaya*	IPEYVD	Food
Car p papain	*C. papaya*	PEYVDW	Food
Car p papain	*C. papaya*	EYVDWR	Food
Car p papain	*C. papaya*	YVDWRQ	Food
Car p papain	*C. papaya*	VDWRQK	Food
Car p papain	*C. papaya*	DWRQKG	Food
Car p papain	*C. papaya*	WRQKGA	Food
Car p papain	*C. papaya*	RQKGAV	Food
Car p papain	*C. papaya*	QKGAVT	Food
Car p papain	*C. papaya*	KGAVTP	Food
Car p papain	*C. papaya*	GAVTPV	Food
Car p papain	*C. papaya*	AVTPVK	Food
Car p papain	*C. papaya*	VTPVKN	Food
Car p papain	*C. papaya*	TPVKNQ	Food
Car p papain	*C. papaya*	PVKNQG	Food
Car p papain	*C. papaya*	VKNQGS	Food
Car p papain	*C. papaya*	KNQGSC	Food
Car p papain	*C. papaya*	NQGSCG	Food
Blo t 1, Car p papain	*Blomia tropicalis*	GSCGSC	Mite
car p papain	*C. papaya*	QGSCGS	Food
Blo t 1, car p papain	*B. tropicalis, C. papaya*	SCGSCW	Mite, Food
Der p 1, Eur m 1 Der f 1, Blo t 1, car p papain	*Dermatophagoides pteronyssinus, Euroglyphus maynei, D. farinae, B. tropicalis, C. papaya*	GSCWAF	Mite, Food

Der p 1, Eur m 1, Der f, Car p papain	*D. pteronyssinus, E. maynei, D. farinae, C. papaya*	SCWAFS	Mite, Food
Car p papain, Act d 1	*C. papaya, A. deliciosa*	CWAFSA	Food
Car p papain	*C. papaya*	WAFSAV	Food
Car p papain	*C. papaya*	AFSAVV	Food
Car p papain	*C. papaya*	FSAVVT	Food
Car p papain	*C. papaya*	SAVVTI	Food
Car p papain	*C. papaya*	AVVTIE	Food
Car p papain	*C. papaya*	VVTIEG	Food
Car p papain	*C. papaya*	VTIEGI	Food
Car p papain	*C. papaya*	TIEGII	Food
Car p papain	*C. papaya*	IEGIIK	Food
Car p papain	*C. papaya*	EGIIKI	Food
Car p papain	*C. papaya*	GIIKIR	Food
Car p papain	*C. papaya*	IIKIRT	Food
Car p papain	*C. papaya*	IKIRTG	Food
Car p papain	*C. papaya*	KIRTGN	Food
Car p papain	*C. papaya*	IRTGNL	Food
Car p papain	*C. papaya*	RTGNLN	Food
Car p papain	*C. papaya*	YSEQEL	Food
Blo t 1,Car p papain	*B. tropicalis, C. papaya*	SEQELL	Mite, Food
Blo t 1, Car p papain	*B. tropicalis, C. papaya*	EQELLD	Mite, Food
Blo t 1, Car p papain	*B. tropicalis, C. papaya*	QELLDC	Mite, Food
Car p papain	*C. papaya*	ELLDCD	Food
Car p papain	*C. papaya*	LLDCDR	Food
Car p papain	*C. papaya*	LDCDRR	Food

Car p papain	*C. papaya*	DCDRRS	Food
Car p papain	*C. papaya*	CDRRSY	Food
Car p papain	*C. papaya*	DRRSYG	Food
Car p papain	*C. papaya*	RRSYGC	Food
Car p papain	*C. papaya*	RSYGCN	Food
Car p papain	*C. papaya*	SYGCNG	Food
Car p papain	*C. papaya*	YGCNGG	Food
Car p papain, Act d 1	*C. papaya, Actinidia deliciosa*	GCNGGY	Food
Car p papain	*C. papaya*	CNGGYP	Food
Car p papain	*C. papaya*	NGGYPW	Food
Car p papain	*C. papaya*	GGYPWS	Food
Car p papain	*C. papaya*	GYPWSA	Food
Car p papain	*C. papaya*	PWSALQ	Food
Car p papain	*C. papaya*	WSALQL	Food
Car p papain	*C. papaya*	SALQLV	Food
Car p papain	*C. papaya*	QLVAQY	Food
Car p papain	*C. papaya*	VAQYGI	Food
Car p papain	*C. papaya*	QYGIHY	Food
Car p papain	*C. papaya*	YGIHYR	Food
Car p papain	*C. papaya*	GIHYRN	Food
Car p papain	*C. papaya*	IHYRNT	Food
Car p papain	*C. papaya*	HYRNTY	Food
Car p papain	*C. papaya*	YRNTYP	Food
Car p papain	*C. papaya*	RNTYPY	Food
Car p papain	*C. papaya*	NTYPYE	Food
Car p papain	*C. papaya*	TYPYEG	Food
Car p papain	*C. papaya*	YPYEGV	Food
Car p papain	*C. papaya*	PYEGVQ	Food
Car p papain	*C. papaya*	YEGVQR	Food

Car p papain	*C. papaya*	EGVQRY	Food
Car p papain	*C. papaya*	GVQRYC	Food
Car p papain	*C. papaya*	VQRYCR	Food
Car p papain	*C. papaya*	QRYCRS	Food
Car p papain	*C. papaya*	RYCRSR	Food
Car p papain	*C. papaya*	YCRSRE	Food
Car p papain	*C. papaya*	RSREKG	Food
Car p papain	*C. papaya*	SREKGP	Food
Car p papain	*C. papaya*	REKGPY	Food
Car p papain	*C. papaya*	EKGPYA	Food
Car p papain	*C. papaya*	KGPYAA	Food
Car p papain	*Carica papaya*	GPYAAK	Food
Car p papain	*C. papaya*	PYAAKT	Food
Car p papain	*C. papaya*	YAAKTD	Food
Car p papain	*C. papaya*	AAKTDG	Food
Car p papain	*C. papaya*	AKTDGV	Food
Car p papain	*C. papaya*	KTDGVR	Food
Car p papain	*C. papaya*	TDGVRQ	Food
Car p papain	*C. papaya*	DGVRQV	Food
Car p papain	*C. papaya*	GVRQVQ	Food
Car p papain	*C. papaya*	VRQVQP	Food
Car p papain	*C. papaya*	RQVQPY	Food
Car p papain	*C. papaya*	QVQPYN	Food
Car p papain	*C. papaya*	GALLYS	Food
Car p papain	*C. papaya*	ALLYSI	Food
Car p papain	*C. papaya*	LLYSIA	Food
Car p papain	*C. papaya*	LYSIAN	Food
Car p papain	*C. papaya*	YSIANQ	Food
Car p papain	*C. papaya*	SIANQP	Food

Car p papain	*C. papaya*	IANQPV	Food
Car p papain	*C. papaya*	ANQPVS	Food
Car p papain	*C. papaya*	NQPVSV	Food
Car p papain	*C. papaya*	QPVSVV	Food
Car p papain	*C. papaya*	PVSVVL	Food
Car p papain	*C. papaya*	AAGKDF	Food
Car p papain	*C. papaya*	AGKDFQ	Food
Car p papain	*C. papaya*	GKDFQL	Food
Car p papain	*C. papaya*	KDFQLY	Food
Car p papain	*C. papaya*	DFQLYR	Food
Car p papain	*C. papaya*	FQLYRG	Food
Car p papain	*C. papaya*	QLYRGG	Food
Car p papain	*C. papaya*	LYRGGI	Food
Car p papain	*C. papaya*	YRGGIF	Food
Car p papain	*C. papaya*	RGGIFV	Food
Car p papain	*C. papaya*	GGIFVG	Food
Car p papain	*Carica papaya*	GIFVGP	Food
Car p papain	*C. papaya*	IFVGPC	Food
Car p papain	*C. papaya*	FVGPCG	Food
Car p papain	*C. papaya*	VGPCGN	Food
Car p papain	*C. papaya*	GPCGNK	Food
Car p papain	*C. papaya*	PCGNKV	Food
Car p papain	*C. papaya*	CGNKVD	Food
Car p papain	*C. papaya*	GNKVDH	Food
Car p papain	*C. papaya*	NKVDHA	Food
Car p papai	*C. papaya*	KVDHAV	Food
Car p papain	*C. papaya*	VDHAVA	Food
Car p papain	*C. papaya*	DHAVAA	Food
Car p papain	*C. papaya*	HAVAAV	Food

Tri a TAI, Hor v 1, Carica p papain	*Triticum aestivum, Hordeum vulgare, C. papaya*	AVAAVG	Food
Car p papain	*C. papaya*	VAAVGY	Food
Car p papain	*C. papaya*	AAVGYG	Food
Car p papain	*C. papaya*	AVGYGP	Food
Car p papain	*C. papaya*	VGYGPN	Food
Car p papain	*C. papaya*	GYGPNY	Food
Car p papain	*C. papaya*	YGPNYI	Food
Car p papain	*C. papaya*	GPNYIL	Food
Car p papain	*C. papaya*	NYILIK	Food
Car p papain	*C. papaya*	YILIKN	Food
Car p papain	*C. papaya*	ILIKNS	Food
Car p papain	*C. papaya*	LIKNSW	Food
Car p papain	*C. papaya*	IKNSWG	Food
Car p papain	*C. papaya*	KNSWGT	Food
Blo t 1, Carica p papain	*Blomia tropicalis, C. papaya*	NSWGTG	mite, Food
Car p papain	*C. papaya*	GTGWGE	Food
Car p papain	*C. papaya*	TGWGEN	Food
Car p papain	*C. papaya*	GWGENG	Food
Car p papain	*C. papaya*	WGENGY	Food
Car p papain	*C. papaya*	GENGYI	Food
Car p papain	*C. papaya*	ENGYIR	Food
Car p papain	*C. papaya*	NGYIRI	Food
Car p papain	*C. papaya*	GYIRIK	Food
Car p papain	*C. papaya*	YIRIKR	Food
Car p papain	*C. papaya*	IRIKRG	Food
Car p papain	*C. papaya*	RIKRGT	Food
Car p papain	*C. papaya*	IKRGTG	Food
Car p papain	*C. papaya*	KRGTGN	Food

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Car p papain	*C. papaya*	TGNSYG	Food
Car p papain	*C. papaya*	GNSYGV	Food
Car p papain	*C. papaya*	NSYGVC	Food
Car p papain	*C. papaya*	SYGVCG	Food
Car p papain	*C. papaya*	YGVCGL	Food
Car p papain	*C. papaya*	GVCGLY	Food
Car p papain	*C. papaya*	VCGLYT	Food
Car p papain	*C. papaya*	CGLYTS	Food
Car p papain	*C. papaya*	GLYTSS	Food
Car p papain	*C. papaya*	LYTSSF	Food
Car p papain	*C. papaya*	YTSSFY	Food
Car p papain	*C. papaya*	TSSFYP	Food
Car p papain	*C. papaya*	SSFYPV	Food
Car p papain	*C. papaya*	SFYPVK	Food
Car p papain	*C. papaya*	FYPVKN	Food