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Potential of quixaba (*Sideroxylon obtusifolium*) latex as a milk-clotting agent

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Abstract

There are several obstacles to the use of chymosin in cheese production. Consequently, plant proteases have been studied as possible rennet substitutes, but most of these enzymes are unsuitable for the manufacture of cheese. The aim of this study was to evaluate the potential of latex from *Sideroxylon obtusifolium* as a source of milk-clotting proteases and to partially characterize the enzyme. The enzyme extract showed high protease and coagulant activities, with an optimal pH of 8.0 and temperature of 55 °C. The enzyme was stable in wide ranges of temperature and pH. Its activity was not affected by any metal ions tested; but was inhibited by phenylmethanesulfonyl fluoride and pepstatin. For the coagulant activity, the optimal concentration of CaCl₂ was 10 µmol L⁻¹. Polyacrylamide gel electrophoresis showed four bands, with molecular weights between 17 and 64 kDa. These results indicate that the enzyme can be applied to the cheese industry.

Keywords: proteolytic activity; plant proteases; rennet.

1 Introduction

Milk-clotting can occur by two ways, lactic and enzymatic. The enzymatic coagulation of milk is the result of the hydrolysis of peptide bonds of κ -casein, in the case of chymosin is between very specific amino acids, phenylalanine-105 and methionine 106 in bovine milk. Thus, the hydrolysate of κ -casein is converted into paracasein and glycomacropeptides that precipitate in the presence of calcium ions and are transferred for the aqueous phase, allowing the soluble calcium has access to internal caseins forming a net which entraps fat and water to make the curd. This process is dependent on temperature, pH and calcium content in milk (PICÓN; MEDINA; NUÑEZ, 1995; PERRY, 2004; KETHIREDDIPALLI; HILL; DALGLEISH, 2010).

Chymosin is the main enzymatic component of calf rennet. It has been used over the years in cheese production due to its high specificity for κ -casein and its low proteolytic activity (VISHWANATHA; APPU RAO; SINGH, 2010). However, increases in cheese consumption and production, the high price and reduced supply of rennets, and the ethical issues associated with the use of the enzyme have led to the search for alternative or additional rennet substitutes (AHMED et al., 2009). Recently, plant coagulants have been an area of interest (CHAZARRA et al., 2007).

Plant enzymes are widely studied as potential coagulants in cheese production, including extracts of *Cynara scolymus* L. (CHAZARRA et al., 2007), *Helianthus annuus* and *Albizia lebbek* (EGITO et al., 2007), *Centaurea calcitrea* (RAPOSO; DOMINGOS, 2008), the flowers of *Silybum marianum* (CAVALLI et al., 2008) and *Jacaratia corumbensis* O. Kuntze (DUARTE et al., 2009). A single extract obtained from *Cynara cardunculus* (L.) is particularly popular (GALAN et al.,

2008) and has been used for centuries in traditional artisanal cheeses, such as those made with sheep milk (PRADOS; PINO; FERNÁNDEZ-SALGUERO, 2007).

Studies were conducted on the milk-clotting activity of proteases in plants from the Brazilian semi-arid region to determine their usefulness in cheese production (DUARTE et al., 2009). *Sideroxylon obtusifolium*, also known as quixaba, is a lactescent tree native to the Caatinga Northeast and occurs in the floodplain of wetlands and river banks of savanna vegetation in the Northeast, sandbank on the coasts of Ceará and Rio Grande do Sul, in the Pantanal of Mato Grosso, and also very frequent in the São Francisco Valley. Its occurrence is given preferably in clay soils rich in calcium, the distribution is predominantly discontinuous along the area it occupies (LORENZI; MATOS, 2002; SILVA et al., 2012), and is considered a species with medicinal properties (FERRAZ; ALBUQUERQUE; MEUNIER, 2006). The object of this study is to evaluate the potential of quixaba latex as a milk-clotting agent.

2 Materials and methods

2.1 Materials

The enzyme was extracted from the latex of the plant *Sideroxylon obtusifolium*, from the city of Gouveia Delmiro, Alagoas State, Brazil. The latex was collected from quixaba stems after the removal of fruit; the latex was dripped into microfuge tubes that were then transported to the CENLAG (UFRPE/UAG). All reagents used in this study were of analytical grade unless otherwise stated.

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2.2 Methods

Preparation of the enzyme extract

The latex was solubilized in 0.1 M phosphate buffer, pH 6.0, at a ratio of 1 g of latex to 100 mL of solution. This mixture was stirred for one hour at 24 °C to accelerate solubilization, then filtered through filter paper and frozen at -20 °C.

Determination of the total protein content in the enzyme extract

The Bradford method (1976) was used to quantify the total protein content, which was modified to incorporate Brilliant Coomassie Blue. The calibration curve was generated from stock solutions of bovine serum albumin (BSA) in a concentration range of 0-600 µg mL⁻¹. Solutions were subjected to an absorbance reading at 595 nm in a spectrophotometer (Biochrom Libra S6*, Cambridge, UK).

Milk-clotting activity

Milk-clotting activity assay was performed using the method described by Arima, Yu and Iwasaki (1970), with 1.0 mL of skimmed milk powder 12% (w / v) solution containing 10 µM CaCl₂, this milk solution was preheated for 10 minutes in a water bath at 37 °C, and then 100 µL of the enzyme extract was added to begin the reaction. The time count was completed when the first milk clots appeared. To visualize the clot forming, the tube was rotated manually from the start time until the first particles arose. One milk coagulating unit per milliliter (U mL⁻¹) is defined as 400 t⁻¹; the amount of enzyme that coagulates the milk in one minute has 400 milk coagulating units. The variable *t* is the time required for the first clots of milk to form.

Protease activity

The proteolytic activity assay was developed according to the method described by Alencar et al. (2003), adding 100 mL of azocasein (1.0% in Tris-HCl buffer) to 60 µL of enzyme extract. The reaction mixture was incubated for one hour at room temperature. After incubation, the reaction was stopped by adding 480 µL of trichloroacetic acid (TCA) to 10% (w / v) of the reaction mixture, followed by centrifugation for 5 minutes at 8000xg and a temperature of 4 °C. After centrifugation, 320 µL was removed from the supernatant and added to 560 µL of sodium hydroxide (1 M). One unit of activity is equivalent to a change in optical density of 0.01 nm per minutes at 440 nm.

Effect of pH on enzyme activity and stability

The optimum pH for the enzymatic activity of quixaba protease was determined using different buffers at 0.1 M: phosphate buffer (pH 5.8-8.0), Tris-HCl buffer (pH 7.0-9.0) and carbonate-bicarbonate buffer (pH 9.5-10.7). The determination of the enzyme activity was performed as described above. The pH stability was measured by subjecting the extracts to the same buffers before starting the enzymatic reaction. Aliquots were taken at 30, 60, 90 and 120 minutes to determine the protease activity at each time point.

Effect of temperature on enzyme activity and stability

The optimum temperature was determined by measuring the protease and milk-clotting activities of the crude extract at different temperatures: 35-80 °C for protease activity and 30-80 °C for milk-clotting activity. To determine the thermal stability, the enzyme was subjected to temperatures of 35-70 °C. Aliquots were taken at time points of 0, 30, 60, 90, 120 and 180 minutes to determine the specific activities. The samples at each time point were subjected to analytical determinations.

The effect of the activation or inhibition of metal ions on the protease activity

The ions Mg²⁺, Zn²⁺, K⁺, Mn²⁺, Na⁺ and Fe²⁺ were tested for their influence on the activity of the enzyme. Solutions of azocasein (1.0% in Tris-HCl buffer) were prepared, each containing 10 mm L⁻¹ of one ion, and the enzymatic activity was determined as described by Alencar et al. (2003). Calcium was used as a control ion.

Inhibitors

Inhibition of the enzyme activity was studied by adding the following protease inhibitors to the crude enzyme extract: 100 mM EDTA (ethylenediaminetetraacetic acid), 100 mM PMSF (phenylmethanesulfonyl fluoride), 100 mM iodoacetic acid and 1 mM pepstatin A. Each inhibitor was added to the extract in a 1:1 (v / v) ratio for 30 minutes at 28 °C, followed by the addition of azocasein as previously described to determine the proteolytic activity.

The Effect of the Ca²⁺ concentration on milk-clotting activity

To evaluate the effect of the Ca²⁺ concentration on the milk-clotting activity, the assay was performed according to Arima, Yu and Iwasaki (1970). However, different concentrations of CaCl₂ were used (1.0, 5.0, 10.0 and 15.0 mM).

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970), using gel concentrations of 4.0% and 15.0% to achieve separation. The gels were stained for two hours in a solution of Coomassie brilliant blue R-250 and destained with a 0.25% solution of 45.0% methanol and 10.0% acetic acid until the bands could be visualized. The molecular mass of the bands was determined by the software LabImage 1D (Loccus Biotechnology), using phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and trypsin inhibitor (20.1 kDa) as standard proteins (GE healthcare).

Statistical Analysis

All experiments were performed in triplicate. The results are expressed as mean ± standard deviation (SD).

3 Results and discussion

The enzyme of the quixaba latex strongly coagulated skimmed milk solution within four minutes (which corresponds

to a specific clotting activity of 917 U mg⁻¹), forming a white and firm curd.

Hashim et al. (2011) provided values for coagulant and protease activities, as well as the relationship between the clotting activity study and the protease activity, which is presented in Table 1. The coagulants described by Hashim had a milk coagulation activity lower than that of the quixaba latex. The quixaba protease also had a coagulant activity higher than other plant-derived coagulants and even chymosin, which has long been considered the best clotting enzyme.

The ratio of milk-clotting activity to proteolytic activity (25.38 U mg⁻¹) of quixaba latex was determined. These results show that the ratio of coagulant activity to protease activity in the quixaba latex (5731) is sufficiently high for the commercial production of cheese. This result suggests that the enzyme in the latex of quixaba has a specific clotting activity because there was no excessive hydrolysis of other proteins not related to the coagulation of milk, which could reduce cheese yield and product quality (VASCONCELOS; ARAÚJO; VERRUMA-BERNARDI, 2004; NEVES-SOUZA; SILVA, 2005; JAROS; SEITLER; RAHM, 2008).

When different pH values were tested, the enzyme showed the highest protease activity in 0.1 M Tris-HCl at a pH of 8.0 and the lowest activity at a pH of 5.8, in 0.1 M phosphate buffer. This higher activity at alkaline pH suggests that the enzyme belongs to a group of proteases that are often used commercially (GUPTA; BEG; LORENZ, 2002). Similar results were observed for the protease in the latex of *Streblus asper*, which had an optimum pH of 9.0, keeping it in the alkaline range (TRIPATHI; TOMAR; JAGANNADHAM, 2011). However, a protease from *Ficus racemosa* showed higher activity at an acidic pH, with 80% of its activity occurring at a pH of 4.0 (DEVARAJ; GOWDA; PRAKASH, 2008). A protease from *Jacaratia corumbensis* O. Kuntze showed greater activity in a nearly neutral pH range—pH 6.5 for the crude extract of the enzyme and pH 7.0 for the purified enzyme (DUARTE et al., 2009).

The optimum temperature for protease activity was 55 °C, and the residual activity was above 50% for all temperatures tested. These results corroborate Duarte et al. (2009) when testing a protease from the latex of *Jacaratia corumbensis* O. Kuntze, which had improved protease activity at 55 °C. For a protease from *Streblus asper*, the optimum temperature was 65 °C (TRIPATHI; TOMAR; JAGANNADHAM, 2011), and 60 °C was ideal for a protease from *Ficus racemosa* (DEVARAJ;

GOWDA; PRAKASH, 2008). An enzyme from *Capparis spinosa*, proteases from *Euphorbia amygdaloides* (DEMIR et al., 2005) and the enzyme from *Synadenium grantii* (MENON et al., 2002) all had an optimum temperature of 60 °C. The protease extracted from *Calotropis procera* showed improved activity between 55 and 60 °C (DUBEY; JAGANNADHAM, 2003).

The clotting activity was found to be directly proportional to the temperature, which reached 100% of residual activity at 80 °C. To study the influence of temperature on the milk-clotting activity of extracts from dried artichoke flowers, Chazarra et al. (2007) selected a temperature range of 20–60 °C, and observed that the coagulation time decreased with increasing temperature. A progressive reduction in the clotting times with temperatures increasing from 20 to 40 °C was reported by other authors (NÁJERA; DE RENOBLES; BARRON, 2003). Nevertheless, other studies have shown that high temperatures hinder the clotting process (DYBOWSKA; FUJIO, 1996). Although changes in salt concentrations are also involved, the primary factor responsible for decreasing the clotting time of milk is a warmer temperature of the complex formed between κ -casein, β -lactoglobulin and lactalbumin (BALCONES; OLANO; CALVO, 1996). In the case of “rennet” made using the latex of quixaba, increasing the coagulation temperature from 30 to 80 °C resulted in a progressive reduction of the clotting time, which was even more pronounced between 50 and 80 °C.

When the enzyme stability relative to pH was tested, the phosphate buffer led the enzyme residual activity to remain below 100% for 180 minutes at all tested pH values (5.8, 6.0 and 7.0). The same result was observed in the other two buffers tested (Tris-HCl and carbonate-bicarbonate). A progressive decline in residual activity was observed over time for all buffers. Figure 1 illustrates the pH stability of the enzyme. For the 180 minute reaction, the enzyme remained stable in the pH range of 5.8 to 10.7, demonstrating that despite the decline observed in the activity, the enzyme could last 180 minutes with a residual activity exceeding 30%. According to Demir et al. (2008), pH-stable enzymes are advantageous in food production. Papain and bromelain are traditional plant proteases reported to be stable between pH 5.0 and 9.0 (RAO et al., 1998). Coagulants

Table 1. Ratio of milk-clotting activity/proteolytic activity of quixaba protease and other coagulants.

Enzyme	SCA ^a (U mg ⁻¹)	PA ^b (U mg ⁻¹)	Ratio SCA/O.D. PA ^c
Chymosin	269	0.08	3363
Rennet from <i>Mucor</i>	438	0.16	2738
Papain	208	0.51	408
Protease of Ginger	314	0.19	1653
Protease of Quixaba	917	0.16	5731

^aSCA: Specific Clotting Activity, ^bPA: Proteolytic Activity, ^cO.D.: Optical Density at 440 nm.

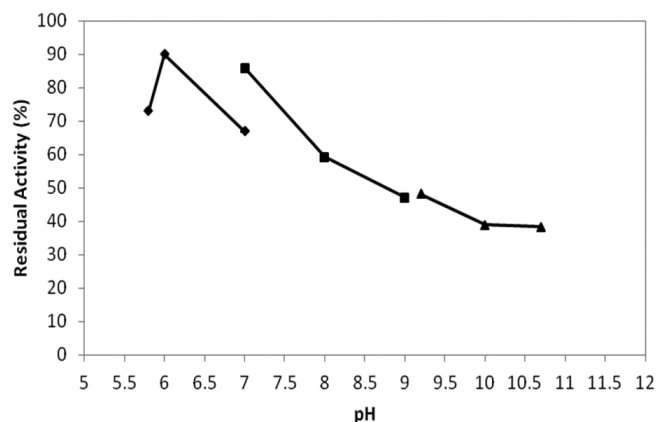


Figure 1. Protease stability curve for pH over a 180-minute test. Buffers: 0.1 M phosphate (♦), 0.1 M Tris-HCl (■) and 0.1 M carbonate-bicarbonate (▲).

should not be sensitive to variations in the pH of milk because this sensitivity can decrease the yield of cheese or cause defective cheese formation owing to clots too soft for cutting (HARBOE; BUDTZ, 1999).

During the temperature stability test, the protease activity declined over time, showing that the enzyme loses activity when exposed to the tested temperatures for longer periods of time. The enzyme residual activity was higher at temperatures of 35 to 40 °C and had a residual activity of 115.57% after 60 minutes at 40 °C. However, the enzyme lost 81.05% activity by the end of the 180 minute test period at 60 °C.

Figure 2 shows the decrease in activity observed with increasing temperatures over time. These results are similar to those obtained by Duarte et al. (2009) with the protease of *Jacaratia corumbensis* O. Kuntze, which also lost activity over time at the temperatures tested and was completely inactivated after 90 minutes at 75 °C. These results can also be compared with other studies that did not report a loss of coagulant activity,

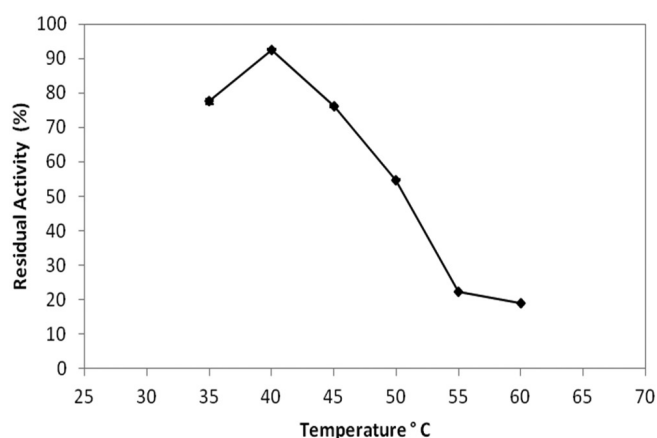


Figure 2. Protease stability curve for temperature over a 180 minute test.

Table 2. Effect of metal ions on protease activity.

Ions	RPA (%)
Ca ²⁺ +	100 ± 0.151
Zn ²⁺	109.26 ± 0.264
K ⁺	127.9 ± 0.851
Mn ²⁺	106.0 ± 1.094
Na ⁺	106.3 ± 0.236
Fe ²⁺	115.5 ± 0.460
Mg ²⁺	98.1 ± 0.247

(*): Control; RPA: Residual Protease Activity (%) ± SD.

Table 3. Enzyme inhibition in the presence of the main protease inhibitors.

Inhibitors	Target Protease	Concentration (mM)	Residual Activity (%) ± SD	Inhibition (%)
Control	-----	-----	100 ± 0.0	-----
PMSF	Serine	100 mM	11.67 ± 0.035	88.33
EDTA	Metallo	100 mM	115.14 ± 0.023	0
Pepstatin A	Aspartic	1 Mm	4.73 ± 0.023	95.27
Iodoacetic Acid	Cystein	100 mM	2.52 ± 0.011	97.48

such as the crude extract obtained from *Solanum dobium* that lasted 10 minutes at 40 and 50 °C (YOUSIF; McMAHON; SHAMMET, 1996). A protease from lettuce was not significantly affected by incubation at 50 °C, as it retained 80% of its initial protease activity (LO PIERO; PUGLISI; PETRONE, 2002). These results suggest that proteases derived from quixaba latex can be a potential source of enzymes useful in dairy applications.

The metal cations used had no significant effect on protease activity (Table 2). The highest level of enzyme inhibition was 1.9% by Mg²⁺, while K⁺ increased the enzyme activity by 27.9% of the maximum. Many studies have investigated the effect of ions on the physicochemical characteristics of milk-clotting by adding NaCl or CaCl₂ (VAN HOOYDONK; HAGEDOORN; BOERRIGTER, 1986; FOX, 1997), but few have demonstrated that ions had a significant influence (FAMELART; LE GRAET; RAULOT, 1999). These results agree with Moreira et al. (2003), who observed no significant inhibition of the activity of a protease from *Nocardiopsis sp.* owing to metal ions but determined that the protease activity can be increased in the presence of the K⁺.

For the concentrations of CaCl₂ tested, a concentration of 10 µm produced the highest clotting activity (882.10 U mg⁻¹). This is the same concentration suggested by Arima, Yu and Iwasaki (1970) in its methodology for clotting activity. The coagulation stage of milk requires the presence of Ca²⁺, although the coagulation also depends greatly upon the temperature (ORDÓÑEZ et al., 2005).

The effect of protease inhibitors (Table 3) on enzyme activity was examined. The enzyme was inhibited by PMSF, pepstatin and iodoacetic acid. The lowest activities were observed when iodoacetic acid was included, suggesting the presence of a cysteine protease in the enzyme of the crude extract. The protease of *Jacaratia corumbensis* O. Kuntze is also a cysteine protease because its activity was inhibited in the presence of iodoacetic acid (DUARTE et al., 2009). The protease of quixaba latex was not inhibited by EDTA, indicating that this protease is not a metalloprotease. In fact, EDTA induced enzyme activation, and the residual protease activity increased to 115%.

The enzyme derived from quixaba latex was analyzed on polyacrylamide gel, revealing four bands with molecular weights of 64, 53, 20 and 17 kDa (Figure 3), one of which may be one of the protease of interest. These values were compared to the molecular weights of milk-clotting proteases of plant origin provided by other studies. A study on ginger root obtained an enzyme of 36 kDa (HASHIM et al., 2011), and the *Jacaratia corumbensis* O. Kuntze protease was 33 kDa (DUARTE et al., 2009). However, it is important to note that these proteases were

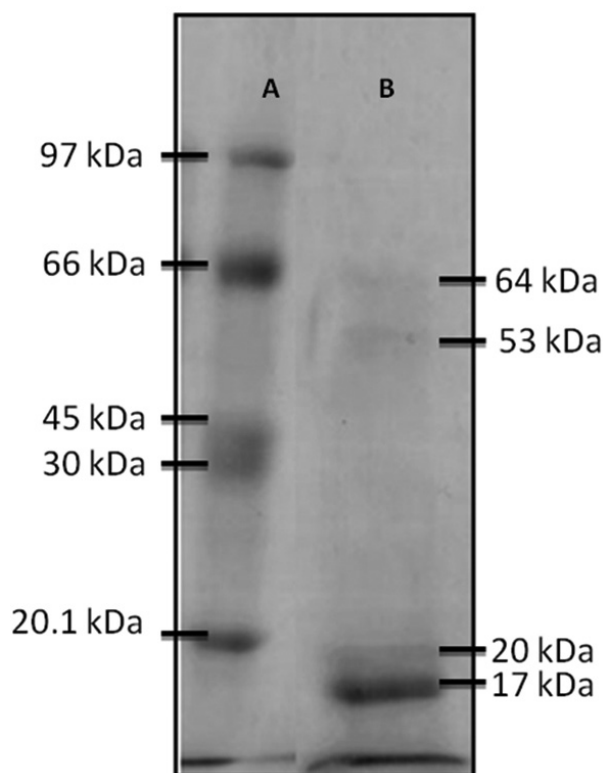


Figure 3. Electrophoretic profile of the enzyme obtained from the latex of quixaba. "A" = bands formed by protein standards and "B" = bands formed by proteins from the quixaba latex.

purified in these other studies, and purified proteases likely differ greatly from those derived from crude plant extracts such as the quixaba latex used in this research.

4 Conclusions

The latex of the quixaba plant is a potential source for a milk-clotting protease. The best performance as a milk-clotting agent was achieved at alkaline pH and temperatures above 40 °C. The protease was stable at room temperature and at various tested pH values, evidencing that the quixaba latex is a possible alternative to animal rennet for the production of cheese. Future studies can further explore this new research avenue.

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