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CHEMICAL SCIENCES

Proteolytic characterization of a novel enzymatic extract from *Bromelia serra* leaves

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Abstract: Bromelia serra leaves collected from Corrientes, Argentina, were assessed to analyze and characterize the proteolytic system and to evaluate its potential use as an industrial catalyst. The specific activity of the enzymatic extract (EE), which was prepared using acetone as a precipitating agent of the crude extract (CE), increased 2-3 folds with different substrates (hemoglobin, azocasein and casein). The proteins present in the EE have isoelectric points between 4.55-8.15 and they were significant inhibited by pepstatin A (50%) and E-64 (15%). Proteolytic activity in EE presented high activity in acidic pH (2.7-4), and low activity in neutral alkaline pH (6-11.75). The EE optimum activity was reached at 60ºC, and referring to the thermal stability, it retained over 97% of the proteolytic activity after incubation at a temperature range of 37–60 ºC for 60 min. The effect of reducing agents and ionic strength were also measured, and it showed that the EE had its maximum activity with 5mM of cysteine, and it was inactivated with 2.5 M of NaCl. The chromatography procedures presented two purified enzymes of 21 and 54 KDa with proteolytic activity. The characteristics of the EE suggest that it is a potential candidate as an industrial catalyst.

Key words: Acidic protease, *Bromelia serra* leaves, enzymatic extract, size-exclusion chromatography, thermal stability.

INTRODUCTION

Enzymes are biocatalysts that are employed in several industrial commercial applications. Proteolytic enzymes or proteases are an important group of hydrolases that catalyze the hydrolysis of peptide bonds present within protein substrates and are employed in various industrial processes as catalysts (Husain 2018, Adrio & Demain 2014).

Proteases constitute about 60% of world enzyme market; the latter being represented by producers such as Genencor International, Gist-Brocades, Miles Laboratories, and Novo Industries. Proteases are widely used in industrial applications such as food biotechnology, textile and leather processing, pharmaceuticals, detergents, proteomics research, synthesis of useful compounds, among others. Today, the proteolytic enzyme market is increasing due to the growing awareness of the population about the need to protect the environment from the impact caused by the chemical industry (Arshad et al. 2014, Husain 2018).

The plant kingdom offers a wide variety of species that have huge amounts of proteolytic enzymes that could be useful in different application areas. In fact, there are vegetable peptidases that are currently used in different industries. Bromelain is a crude preparation obtained from pineapple (*Ananas comosus,* Bromeliaceae) that contains at least four good-studied peptidases named stem bromelain,

fruit bromelain, comosain and ananain (Tochi et al. 2008, Arshad et. 2014). This mix of enzymes is being employed in many and varied applications such as tenderization in foods, beverage, baking industry, cosmetic products, protein hydrolysate preparation, animal feed, tooth whitening, and textile and pharmaceutical industries, among others (Arshad et al. 2014, Manzoor et al. 2016). Numerous studies were published about bromelain antimicrobial capacity, both its capacity as an antibiotic for medicinal use and the possibility of controlling pathogens that affect agricultural species. Dutta & Bhattacharyya (2013) worked with an extract of pineapple crown leaf establishing that the main protein present in it was the fruit bromelain protease determined by proteomic studies and this extract had a considerable antimicrobial effect and did not induce any toxicity in rats after oral administration (acute and subacute doses). A study carried out by López-García et al. (2012) demonstrated that bromelain from pineapple stem provokes 90% growth inhibition of *Fusarium verticillioides* exhibiting a potent antifungal activity and suggesting its potential use as an effective agent for crop protection, as an alternative to the use of chemical fungicides.

The enzymes present in pineapple are abundant in stem and fruit of this plant, but they can also be recovered from parts considered as waste, such as core, leaves, peel and crown, the latter being the waste tissue that yields the highest proteolytic activity. Bromelain extraction from waste tissues is a promising option from both the environmental and economical point of view (Arshad et al. 2014, Manzoor et al. 2016, Martins et al. 2014) Numerous studies about purification of pineapple proteases from crude extracts have been carried out using a diverse set of techniques such as extraction, ultrafiltration, precipitation, and chromatography. Precipitation of bromelain with 30–70% of ethanol achieved a purification factor of 2.07-fold and yielded over than 98% of enzyme recovery (de Lencastre Novaes et al. 2016, Martins et al. 2014).

Bromelia serra is a native plant of northern Argentina which grows as terrestrial understory plant in the semiarid Chaco region and propagates by both sexual and asexual reproduction. It presents numerous narrow, spiny-edged leaves 1.5 m long and 4 cm wide, which form a rosette up to 40 cm high. The infructescence has a globose shape, containing dozens of yellow tricarpelar berries (Caffini et al. 1988, Montero et al. 2017). Acetone powder from fruits was prepared and showed the presence of cysteine peptidases, which presented good thermal stability and an optimum pH range from 6.0 to 7.8 (Caffini et al. 1988).

The majority of proteases studied in Bromeliaceae family come from their fruits. Nevertheless, the presence of these fruits depends on the climate or season to bloom. The availability of leaves is constant and in the case of *B. serra* is abundant. The aim of the present work was to prepare an extract from leaves of *B. serra*, analyze and characterize the proteolytic system present in this plant material. The knowledge about the enzymatic extract characteristics may be useful as it can be applied as a catalyst in different industrial areas.

MATERIALS AND METHODS

Plant source

Leaves were collected from wild species of *B. serra* (BS) in May 2018 and 2019 in San Cosme, Corrientes, Argentina. The vegetal material was placed in plastic bags and kept at –20 °C until use.

Crude and enzymatic extracts preparation

Crude extracts (CE) were prepared by homogenizing the BS leaves using a domestic blender (SC 2031, Smart-tek, Buenos Aires, Argentina) with cold buffer 0.1 M sodium phosphate, pH 7.0 (1:1 w/v ratio) containing 5 mM EDTA and 5 mM cysteine as protective agents. The homogenate was filtered through a twice-folded piece of gauze to remove plant debris and centrifuged at 12,000×*g* for 15 min at 4ºC (Sorvall, ST 8R, ThermoScientific, Germany). Several supernatants of the CE were combined, filtered when necessary, and immediately frozen at -20 °C until analysis. All procedures were done at 0–4 °C.

This extract was treated with four volumes of cold (-20ºC) acetone (1:4, respectively) with gentle agitation and left to settle for 20 min at -20° C before centrifugation at 4,000×*g* for 20 min. Pellets were dried in a vacuum desiccator for removal of acetone and suspended in the same extraction buffer (4 mL). This dissolved acetone precipitate was designated as Enzymatic Extract (EE).

Protein quantification

Protein concentration in CE and EE was measured by the Bradford' method (1976), using bovine serum albumin as standard. A portion of the extracts was mixed with Bradford reagent and incubated for 10 min at ambient temperature for color development, and absorbance was registered at 595 nm using UV spectrophotometry (V-630, JASCO Germany GmbH, Gross-Umstadt, Germany).

Proteolytic activity

Proteolytic activity in CE and EE was evaluated using different substrates: hemoglobin, azocasein, and casein.

Hemoglobin (Sigma-Aldrich) was used as a substrate for acidic proteolytic activity determination, according to Barrett (1970), with slight modifications. The reaction mixture contained 0.1 mL of enzyme extract (0,16 mg of protein content) and 1.46 mL of hemoglobin (2.5g in 100 mL of 0.1 M sodium formate-formic acid buffer at pH 3.3). After incubation at 37 °C for 30 min, the reaction was stopped by addition of 1 mL of 10% (w/v) trichloroacetic acid (TCA). The mixture was centrifuged at 4,000 *xg* for 15 min, and then the absorbance of the supernatant was measured at 280 nm. Assays were run in triplicate including appropriate blanks, which were prepared by combining the TCA with the enzyme and then adding the hemoglobin substrate. Enzyme units per mL (EU/mL) and enzyme units per mg (EU/mg) were calculated as follows:

$$EU/mL=((AS_{280}-AB_{280}) \times 2.66 \times DF) / 0,1$$

$$EU/mg\ protein=(EU/mL) / (mg\ protein/mL\ enzyme)$$

where $AS_{280\ and}$ AB_{280} are Absorbance values of sample and blank, respectively; 2.66, total volume (mL) of assay, DF, sample dilution factor, and 0,1, volume (mL) of enzymatic extract

Moreover, proteolytic activity was assayed using 1% azocasein as substrate. The generation of TCA soluble peptides was followed under alkaline conditions at 440 nm after 30 minutes of incubation at 37ºC. An assay without enzyme served as the negative control. One unit of caseinolytic activity, on azocasein, was defined as the amount of enzyme required to produce an absorbance change of 1.0 in a 1 cm cuvette under the assay conditions (Sarath et al. 1989).

Caseinolytic activity assays were carried out according to Bruno et al. (2006). For this determination, the reaction mixture contained 1.1 mL of 1% (w/v) casein in 0.1 M sodium phosphate buffer (pH 7) with 5 mM cysteine and 0.1 mL of enzyme solution. The assay was

incubated at 37 °C for 30 minutes and after the reaction was stopped with the addition of 1.8 mL of 5% (w/v) TCA. Sample blanks were prepared by adding TCA to the enzyme, then adding the substrate. The samples were centrifuged at 7,000 *xg* for 20 min and then the absorbance of supernatants at 280 nm was measured. An arbitrary enzyme unit, named Ucas (Caseinolytic Unit) was defined as the amount of enzyme that produces an increase of one absorbance unit (1-cm light path) per minute under the assay conditions (López et al. 2000).

Characterization of the enzymatic extract

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Gels were run in mini slabs (Bio-Rad Mini Protean Tetra Cell Model). SDS-PAGE was performed, according to Laemmli's (1970), using a separating and a stacking gel (12% w/v and 4% w/v acrylamide, respectively). Samples to be run, under reducing conditions, were boiled for 1 min in sample buffer containing 5% v/v of 2-mercaptoethanol (2-ME) and centrifuged (10,000 ×*g*, 20 min, 25 °C). Electrophoresis was performed at a constant current of 30 mA per gel for approximately 45 min. The following molecular weight standards were used to estimate the molecular masses of proteins: phosphorylase b (94 kDa); bovine serum albumin (67 kDa); ovalbumin (45 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (20.1 kDa); a-lactalbumin (14.4 kDa), (Pharmacia Hepar Inc., Franklin, OH, USA). Gels were fixed and stained with Coomassie Brilliant Blue dye solution (2 g/L) in water/methanol/acetic acid (5:5:2) overnight and destained with aqueous solution of 25% v/v methanol and 10% v/v acetic acid. The molecular weights of protein bands were estimated with ImageJ analysis software (Schneider et al. 2012).

Zymogram analysis of gelatinolytic activity

Zymography was performed, described by Hames (1981) with slight modifications, to verify the gelatinolytic activity. Briefly, samples from the enzymatic extract were diluted in SDS sample buffer without reducing agents and electrophoresed on 12% SDS-polyacrylamide gels copolymerized with 1.5 mg/mL of gelatin. Next, the gels were washed twice for 30 min in 2.5% Triton X-100 to remove SDS and then incubated in a zymography buffer (0.1 M sodium phosphate pH 7.0 with 15 mM cysteine) at 37 ºC for 18 h. After incubation, gels were stained with Coomassie blue for two hours and then distained. The presence of gelatinolytic activity was verified as clear bands on a uniform blue background.

Isoelectric focusing

Isoelectric focusing (IEF) was developed on 5% polyacrylamide gels containing broad pH range ampholytes (Biolyte 3–10, Bio-Rad) in a Mini IEF Cell (Model 111, Bio-Rad). Samples were precipitated with four volumes of cold (-20 ºC) acetone and centrifuged. The protein sediments were redissolved and precipitated once again with acetone. Finally, they were redissolved in a volume of deionized water enough to contain 1–10 µg of protein. Focusing was carried out under constant voltage conditions in a stepped procedure: 100 V 15 min, 200 V 15 min, and 450 V 60 min. Gels were fixed and then stained with Coomassie Brilliant Blue R-250.

Effects of the pH on the enzyme activity

The effect of pH on the enzymatic activity of the EE was studied over a pH range of 2.7–10 in different 0.1 M buffers: formate-formic (pH 2.7-4.7), acetate (pH 5-6), phosphate (pH 7), Tris (pH 8) and carbonate (pH 9-10) with hemoglobin and 6–11.75 using Good's buffers: MES, MOPS, TAPS,

AMPSO, and CAPS (Sigma Chem. Co., St. Louis, MO), with casein as a substrate (Bruno et al. 2006).

Effects of the temperature on the enzyme activity and thermal stability

The influence of temperature on proteolytic activity was assayed in thermostatic water baths (model MASSON, Vicking , Buenos Aires, Argentina) from 20 ºC to 80ºC. Then the reaction was stopped, and residual activity was measured. Samples and substrates were pre-incubated for 5 min at the defined temperature before the reaction.

Thermal stability was determined by incubating the EE for 0, 5, 10, 20, 40, and 60 min at 37, 45, 60, 75, 85, and 98 ºC in thermostatic water baths. After the end of the incubation period, the samples were kept in an ice bath until measured residual caseinolytic activity. The nonheated enzyme extract was considered as control (100% of activity).

Effect of NaCl on the enzyme activity

The proteolytic activity of EE was assayed in the presence of sodium chloride at final concentrations of 0, 0.4, 0.8, 1.2 and 2.5 M. The results are reported as a percent relative to the proteolytic activity observed in the absence of NaCl (100% of activity).

Effect of cysteine on the enzyme activity

To determine the optimal concentration of cysteine in the reaction mixture, so that the enzymes show their maximum activity, a caseinolytic activity test was carried out by preparing tubes with different concentrations of cysteine (0, 5, 15, 25, 35, and 50 mM).

Effect of specific inhibitors on enzyme activity

Enzyme extracts were incubated with different specific protease inhibitors such as E-64 (1 mM), pepstatin A (1 mM), EDTA (10 mM) and PMSF (10 mM). An equal volume of each inhibitor solution was mixed with the enzyme extract and preincubated for 30 min at 37 ºC. After incubation, caseinolytic activity was measured. The inhibitors PMSF and pepstatin A were dissolve in ethanol while EDTA and E-64 in distilled water. Blanks contained the enzyme extract and the inhibitor solvent to be used as controls. Residual activity is reported in percent (%) by considering 100% activity when the EE was in the absence of inhibitors.

Purification of the enzymatic extract

FPLC Anion-Exchange Chromatography

Exchange chromatography was performed onto a column (Pharmacia XK 16/40, with AK16 adaptors) packed with 33 mL of Q-Sepharose High Performance (Pharmacia) and equilibrated with 50 mM Tris–HCI buffer (pH 8.5) at 20 ºC in an ÄKTA-Purifier chromatograph (GE, Uppsala, Sweden). After washing the column with the same buffer, the enzymatic extract was loaded onto the column (0.5 mL) and proteins were eluted with a saline gradient: 165 mL of 0.00–0.1 M sodium chloride. The elution was analyzed for absorbance at 280 nm and essayed for proteolytic activity with casein.

Size-exclusion Chromatography

A glass column (1 cm × 85 cm) was packed with Sephadex G-75 (Sigma Chemicals, St. Louis, USA) and equilibrated with 400 mL of 0.1 M phosphate buffer at pH 7.0. A flow rate of 0.250 mL/min was maintained. The enzymatic extract (0.5 mL) was then applied to the Sephadex G-75 gel bed and protein was eluted with the same buffer. The chromatographic fractions were monitored at 280 nm and essayed for proteolytic activity with azocasein.

Statistical analysis

Data represent the mean ± standard deviation (SD) of at least four replications. Statistical significance was tested by one-way ANOVA and Tukey (HSD) and p-values inferior to 0.05 were considered significant. All statistical analyses were carried out with InfoStat software (Di Rienzo et al. 2013).

RESULTS AND DISCUSSION

Protein content and enzyme activity of crude and enzyme extracts

The total protein content from the CE of BS leaves (Table I) was between those reported for *Bromelia balansae* (Pardo et al. 2000) and *Bromelia antiacantha* (Vallés & Cantera 2018). The proteins from the CE were precipitated with cold acetone and later resuspend in 4 mL buffer. The proteolytic activity of the BS extracts was performed using different substrates: azocasein, casein, and hemoglobin. The purification folds in terms of activity increased between 2 to 3 folds for all substrates. The specific activity using casein as substrate was lower compared with other Bromeliaceae fruits (Pardo et al. 2000, Bruno et al. 2008, Payrol et al. 2008), while activity with hemoglobin was similar than those reported by Moreno-Hernández et al. (2017) in the extracts of *Bromelia pinguin*. As for azocasein the specific activity in this study was higher than those reported from waste peel of pineapple (Bresolin et al. 2013).

Characterization of the enzymatic extract

Protein profile and zymogram analysis

The protein profile of BS extract is shown in Figure 1a. The results of SDS-PAGE show a clear band between 20 and 30 KDa in the CE (lane 2). Protein bands of similar molecular weight have been reported for proteases from the Bromeliaceae family and according to Moreno-Hernández et al. (2017) they are associated with cysteine proteases. However, EE samples (lane 3) under non-reducing conditions (without 2-mercaptoethanol) show major proteins, above 67 kDa, and smaller proteins with approximate molecular weights of 50 and 40 kDa but did not show clear bands between 20 and 30 KDa (lane 3). In reducing conditions (with 2-mercaptoethanol) the EE exhibited proteins bands of 60, 45 and 22 kDa (lane 4). There were no clear protein bands between 20 and 14 kDa in non-reducing and reducing conditions. Proteins bands disappearance with 2-mercaptoethanol (lane 4) may be because they were reduced to a low molecular weight by the reducing agent. The presence of a reducing agent, such as 2-mercaptoethanol, breaks the disulfide bonds of the protein structure, making it less stable and losing functional and/or structurally important elements of the domain tertiary structure (Walsh 2002). Most proteases from Bromeliaceae fruits found so far are ranged from 20 to 23 kDa (Natalucci et al. 1995, Bruno et al. 2006, Payrol et al. 2008). The gelatinolytic activity is observed in

Table I. Protein content and specific activity in crude and enzymatic extract from *B. serra* leaves using hemoglobin, azocasein and casein as substrates.

	Volume (mL)	Total protein (mg)	Protein (mg/mL)	Specific Activity Hemoglobin (U/mg)	Specific Activity - Azocasein (U/mg)	Specific Activity - casein (Ucas/mg)
Crude Extract (CE)	60	13.81±0.65	0.23±0.02	1.1±0.02	3.57±0.52	0.014±0.001
Enzimatic Extract (EE)	4	6.43±0.32	1.6±0.22	3.5±0.07	10.65±1.42	0.032±0.002

Figure 1b. The zymography of EE shows a clear or white zone, between 94 and 67 kDa, indicating gelatin degradation caused by hydrolytic activity (on the gray background). Previous studies from *Bromelia karatas* fruits also found hydrolytic activity in zymograms from proteases in this range (Meza-Espinoza et al. 2018) and Villanueva-Alonzo et al. (2019) presented 40 clear zones in a two-dimensional-zymogram with apparent molecular weights between 27.3 and 290 kDa, potentially representing proteases.

The isoelectric focusing of the EE showed proteins with isoelectric points (IP) between 4.55 and 8.15 and a predominant band of 5.85 (Figure 1c). On the basis of these results, ion exchange chromatography was selected for further purification steps. Caffini et al. (1988) isolate an endopeptidase with an IP above 8 in BS fruits. In both *Bromelia hieronymi* and *Bromelia balansae*, the isoelectric points varied from 5.45 to 9.3 (Pardo et al. 2000, Bruno et al. 2002), suggesting that the proteins found in this study are more acidic than those described in Bromeliaceae fruits.

Effect of inhibitors on EE activity

Proteolytic enzymes can be classified according to their mechanistic class. The inhibitors used were EDTA, E-64, PMSF and pepstatin A for metalloproteases, cysteine protease, serine proteases and aspartic proteases respectively (Figure 2). Compared to control, the EE was

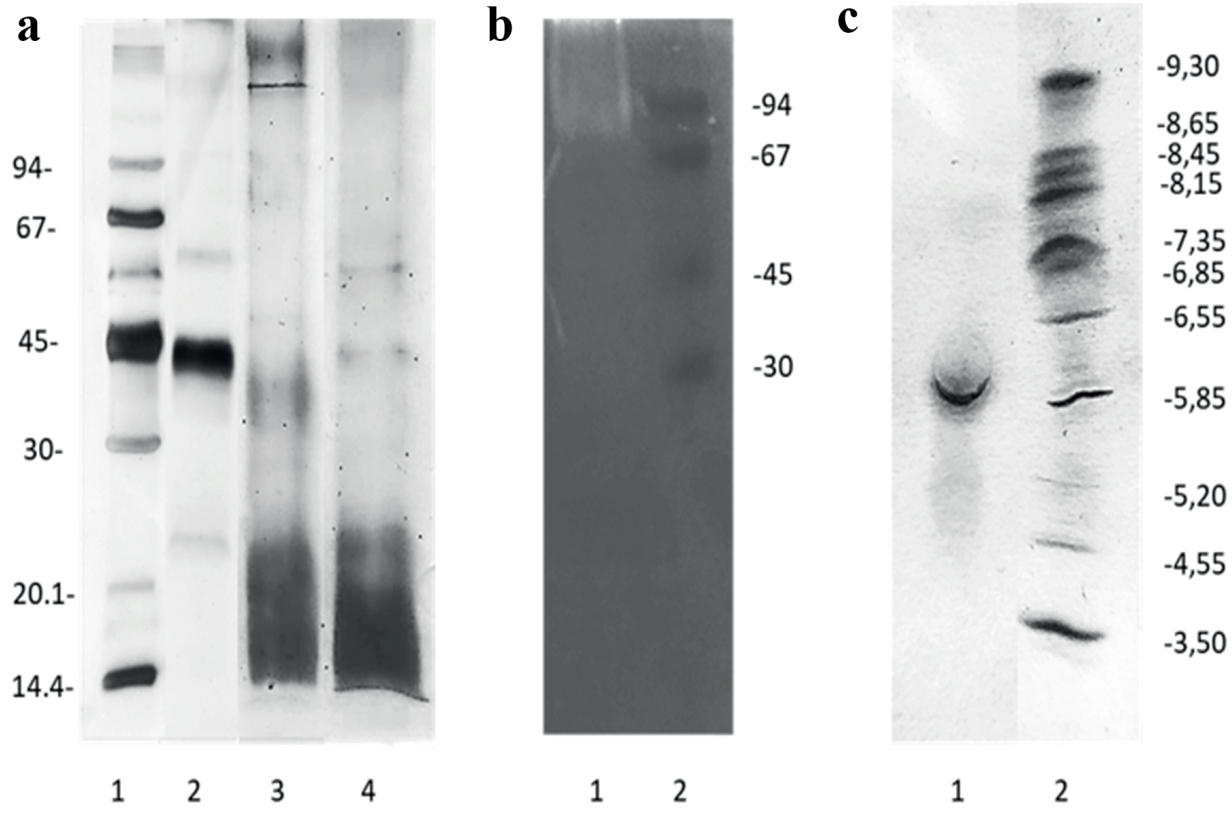

Figure 1. SDS-polyacrylamide gel electrophoresis (PAGE). Lane 1: standard protein markers; lane 2: crude extract; lane 3: enzymatic extract; lane 4: enzymatic extract with 2-mercaptoethanol (a). Zymography. Lane 1: enzymatic extract; Lane 2: standard protein markers (b). IEF. Lane 1, enzymatic extract of BS; lane 2, pI markers: amyloglucosidase (pI 3.50), trypsin inhibitor (pI 4.55), b-lactoglobulin A (pI 5.20), carbonic anhydrase II (pI 5.85), carbonic anhydrase I (pI 6.55), myoglobin (pI 6.85 and 7.35), lectins from *Lens culinaris* (pI 8.15, 8.45 and 8.65), and trypsinogen (pI 9.30) (c).

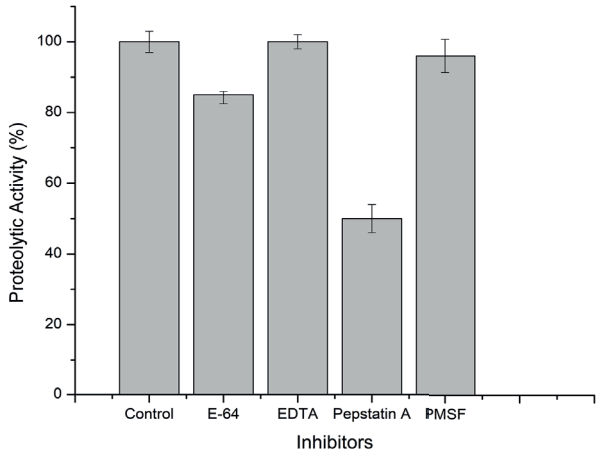

Figure 2. Effect of specific inhibitors on enzyme activity. Bars represent standard deviation (n=4).

significantly inhibited by pepstatin A (50%) and E-64 (15%). However, EDTA and PMSF did not inhibit protease activity compared to the control ($p<0.05$). These results suggested the presence of aspartic and cysteine proteases. Moreno-Hernández et al. (2017) also detected these two types of proteases in *B. pinguin* extracts. Dutta & Bhattacharyya (2013) similarly found multiple proteases in leaf extract of *Ananas comosus*. The cysteine proteases from Bromeliaceae have molecular weights from 20 to 25 kDa, so the protein band of 23 kDa from the CE of this study could belong to the cysteine protease family (Figure 1a, lane 2). Although Moreno-Hernández et al. (2017) detected the presence of aspartic proteases in *B. pinguin* by inhibiting the activity in 50 % with pepstatin A, it is not common to find them in Bromeliaceae species. The aspartic proteases are widely investigated for milk clotting properties, especially in Asteraceae for the activity found in their flowers (Vioque et al. 2000). There are also reports of IP from aspartic proteases that range from 3 to 4,5, suggesting that the acidic proteins found in this work from IEF could be associated with this type of proteases (Yegin & Dekker 2013). Aspartic proteases from *Solanum tuberosum* showed antimicrobial activity against potato pathogens and cytotoxic activity against plant and sperm pathogens. These proteins are capable of binding to the surface of microbial cells and causing changes in the flow of ions from the plasma membrane, provoking the cell death (Mendieta et al. 2006).

Effect of pH on proteolytic activity

The effect of pH on EE is shown in Figure 3a in a range of pH 2.7 to 10 using hemoglobin and in 3b in a range of 6 to 12 with casein as a substrate. In the first pH range (3a), the highest activity was found between pH 3.7 to 4.1 with 5.6 U/mL (3.5 U/mg). As the pH increased, the activity decreased significantly to 1.3 U/mL (0.8 U/mg) at pH 9, and at pH 10, there was no remaining activity. It is noteworthy to mention that with a rise of only 0.6 units of pH (4.1 to 4.7) the remaining activity was less than 50% compared to the highest. Moreno-Hernández et al. (2017) found the highest activity at pH 2 with 3.2 U/mg in *B. pinguin* fruits. In the second pH range (3b), the maximum enzymatic activity was in pH 6 (0.2 Ucas) and as pH increased, the activity diminished significatively ($p<0.05$). At pH 7.5 the EE only maintained 50% of the maximum activity and in pH 12 there was a total loss of activity. The pH dependence of proteolytic activity could be suggesting the presence of aspartic proteases in acidic medium and cysteine proteases in the neutral medium. The same pH pattern of activity was presented in *B. pinguin* fruits (Moreno-Hernández et al. 2017). Caffini et al. (1988) evaluated the pH dependence of activity in BS fruits, in a range of 6 to 11, finding the highest proteolytic activity at pH 6. The high proteolytic activity in acidic conditions can be useful as a possible antibacterial property for acidophilic bacteria such as *Alicyclobacillus* spp. that are related to deterioration of industrial products such as citrus juices and beverages and are not inactivated with heat treatments (dos Anjos et al. 2016).

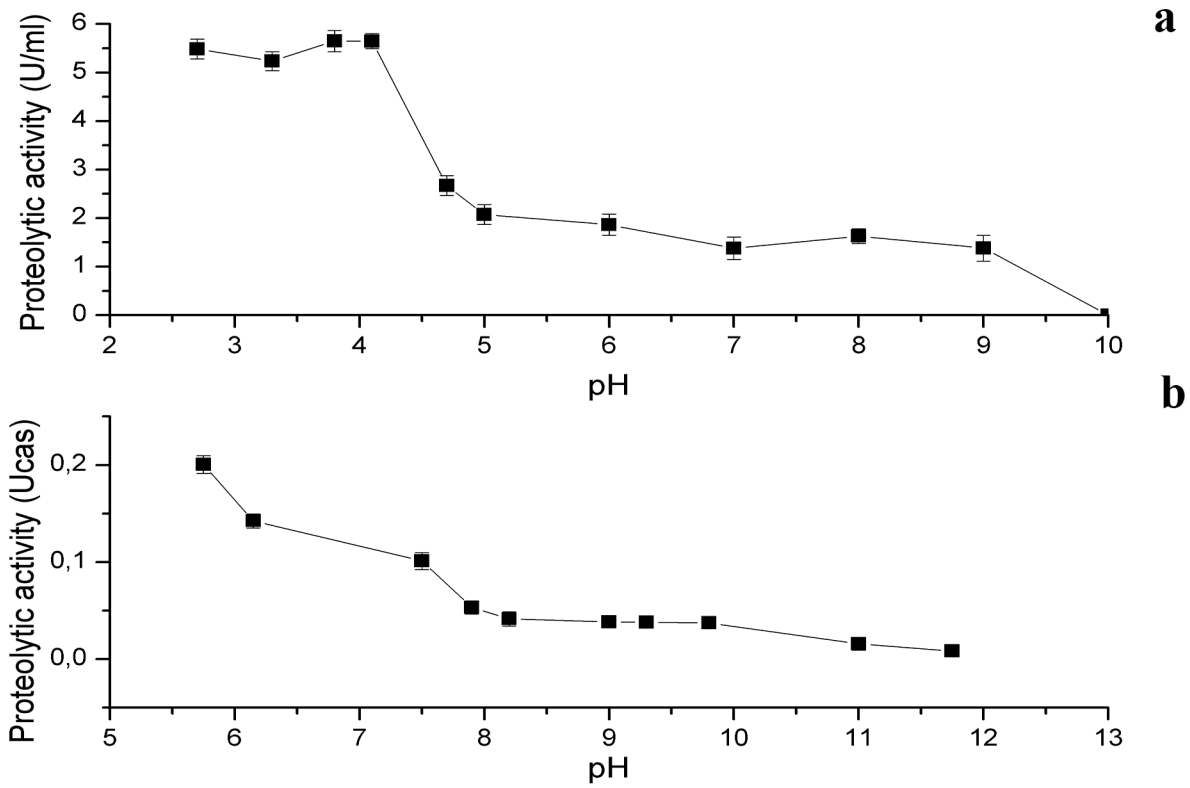

Figure 3. Effect of pH on proteolytic enzymatic extract with hemoglobin as substrate (a) Effect of neutral alkaline pH on proteolytic enzymatic extract with casein (b). Bars represent standard deviation with hemoglobin and casein as substrate (n=4).

Temperature effect on proteolytic activity and stability

As can be seen in Figure 4a, the optimum activity for the EE was at 60 ºC, which is in accordance with the findings from Caffini et al. (1988) fruit extracts from the same species. A significant decrease was found at higher temperatures (80ºC), presenting only 55% of residual activity. The effect of the temperature on stability of the EE is shown in Figure 4b. The extract retained over 97% of the proteolytic activity after incubation at temperature range of 37–60 ºC for 60 min. Temperature influences virtually every aspect of a biochemical reaction, including pH dependent ionization of enzyme and substrate, enzyme conformational changes, protein oligomerization, hydrogen bonding, hydrophobic interactions, transition states, etc. (Purich 2010). The conformational changes in protein tridimensional conformation may lead to an increase on the enzyme activity. Proteases found in *B. pinguin* (Payrol et al. 2005) also conserved high proteolytic activity (80 %) at 55 ºC for the same time of incubation. As the temperature increased, the activity loss was significant, reaching 47% of residual proteolytic activity at 98 ºC. This behavior could be due to the presence of other proteins in the extract that perform a protective role at high temperatures (Priolo et al. 2000) and/or enzymes with structural characteristics such as disulfide bond that makes them thermostable (Sharma et al. 2019). Moreover, the SDS-PAGE in reducing and non-reducing conditions demonstrated the presence of proteins with disulfide bonds (Figure 1a). On the other hand, Caffini et al. (1988) found that the proteases in fruit extracts

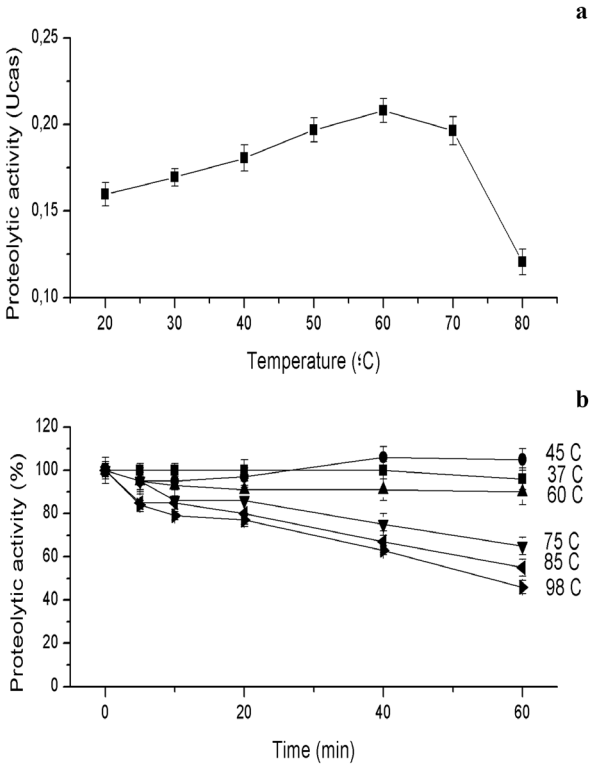

Figure 4. Effect of temperature on proteolytic enzyme extract (a) and enzyme stability (b). Bars represent standard deviation (n=4).

of BS had 10% of residual activity by incubating at 65 ºC for 60 min. According to Dumorne et al. (2017), applications for thermostable proteases (60–80 ºC) are useful on**:** the detergents industries, partial hydrolysis in food technology (feed, brewing and baking), pharmaceutic and biotechnology.

Effect of NaCl concentration on proteolytic activity

The stability of proteolytic activity in a saline medium is shown in Figure 5a. In the presence of 0.4 M of NaCl, the enzymatic extract preserves 65% of the activity. As the molarity rises, the activity decreased to less than 40% and is inactivated with 2.5 M NaCl. On the other hand, Vallés et al. (2007) found that low sodium chloride concentrations (0.4 M) did not affect caseinolytic activity but diminish with the increase of salt concentration in fruits from *B. antiacantha* (28% of residual activity at 2.5M NaCl). The ionic strength of the enzymatic extract and its ability to be inactivated with low values of salt is of great importance in the food industry due to the possibility of being used as a meat tenderizer or as milk clotting agent.

Effect of cysteine concentration on proteolytic activity

The addition of 5 mM of cysteine notably increased by 80% of the proteolytic activity (Figure 5b). However, as the concentration of cysteine rises, the activity varied randomly. On the other hand, Payrol et al. (2005) added increasing concentrations of cysteine in *B. pinguin* extracts, and the activity rose proportionally. Cysteine is used as a reducing agent to favor proteolytic activity and cysteine proteases are concentration-dependent of this reducing agent, meaning that increasing concentrations of the cysteine will increase the proteolytic activity. These results suggest that the BS extract is not mainly composed of cysteine proteases and that the lower cysteine concentration used (5mM) to rise the activity of extract on 80%.

PURIFICATION OF THE ENZYMATIC EXTRACT

FPLC anion exchange chromatography

The Elution profile of the Anion exchange chromatography (Q-Sepharose High Performance) of EE (Figure 6) displays three peaks: peak 1 (0,016 Ucas/mL), peak 2 (0,0029 Ucas/mL) and peak 3(0,0029 Ucas/mL). The SDS Page from the elution peaks of the Q-Sepharose HP column showed several bands on the three peaks; indicating no clear purification. Bruno et al. (2008) also obtained three active fractions from *B. hieronymi* fruits in anion exchange chromatography.

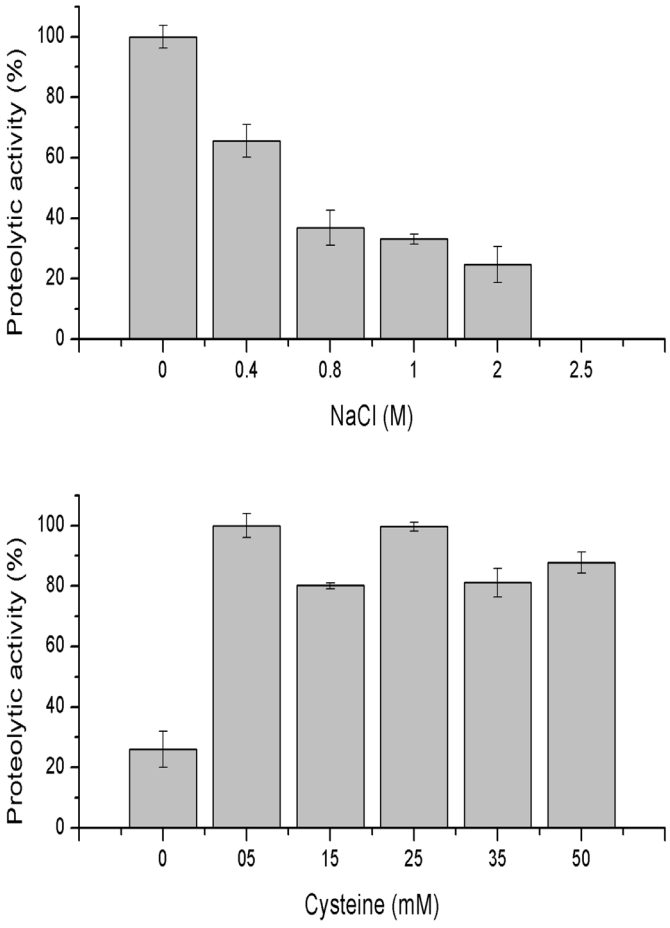

Figure 5. Effect of NaCl on proteolytic activity of enzyme extract (a). Effect of cysteine on the enzyme activity (b). Bars represent standard deviation (n=4).

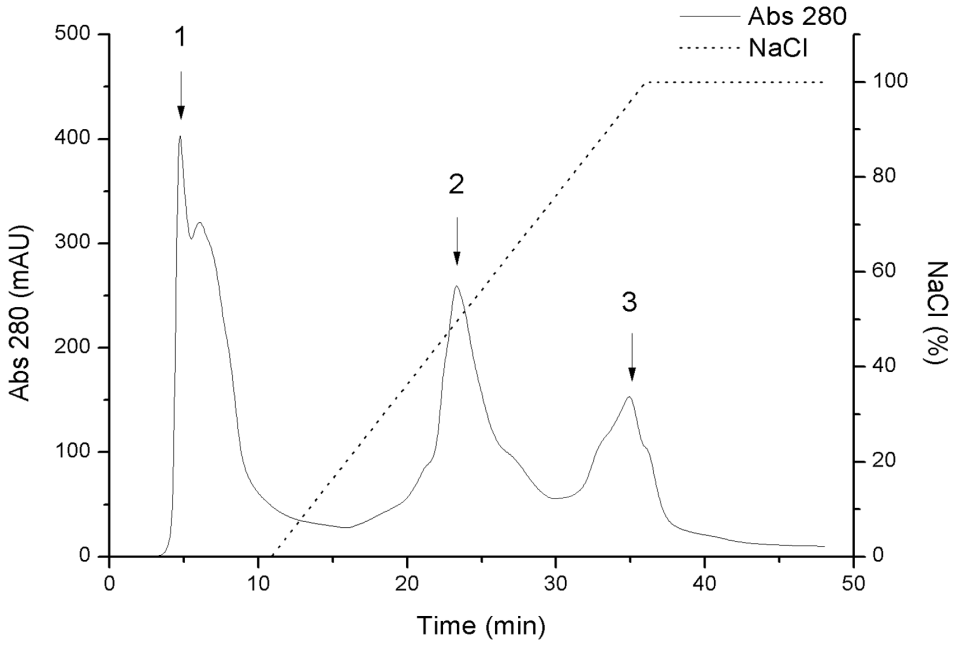

Figure 6. Anion exchange chromatography (Q-Sepharose High Performance) of EE. Column diameter: 1.6 cm; column height: 16.5 cm; starting buffer: Tris–HCl 50 mM, pH 8.5; elution saline gradient: 165 mL of 0.0–0.1 M of sodium chloride in the starting buffer. Flow rate, 1.0 mL min^{-1}. The arrows indicate proteolytic activity in the unretained fraction (peak 1) and retained fractions (peaks 2 and 3).

Size exclusion chromatography

As shown in Figure 7, the EE applied to Sephadex G-75 gel bed showed one major peak and several minor peaks. After testing all the fractions only four presented proteolytic activity against azocasein. The SDS-PAGE of Fraction I (FI), which was eluted at the void volume, showed two intense protein bands above 94 kDa and weak one at about 94 kDa. One of the proteins with high molecular weight of this fraction could be the enzyme with gelatinolytic activity observed in the zymogram. Fraction II (FII) presented one band at 54 kDa. The fraction III (FIII) revealed at least two intense protein bands at 29 and 32 kDa. Finally, the fraction IV (FIV) showed a protein band at 21 kDa. The fraction II and IV were also present in the SDS-PAGE of the EC and EE in reducing conditions. Proteins bands similar to FIV have been reported in several studies from Bromeliaceae proteases, suggesting the presence of cysteine proteases. On the other hand, only one previous report (Moreno-Hernández et al. 2017) found aspartic proteases in fruit extracts using inhibitors, but they could not assign this type of proteases with a MW. Prior studies, not related to enzymes from Bromeliaceae, detected the presence of aspartic proteases from *Withania coagulans*, studied for its milk clotting properties, that range from 36 to 66 kDa (Naz et al. 2009, Kazemipour et al. 2017). These results suggest that FII can be associated with aspartic proteases found in the inhibitors' essay.

CONCLUSIONS

Novel characterization of plant proteases was performed from leaves extract of *B. serra*, which showed similarities and differences with respect to a fruit extract previously reported. The enzymatic extract presented high proteolytic activity in acidic conditions and resistance to denaturalization when exposed to high temperatures. Also, the ionic strength of the enzymatic extract was tested and it showed to be inactivated with low concentrations of

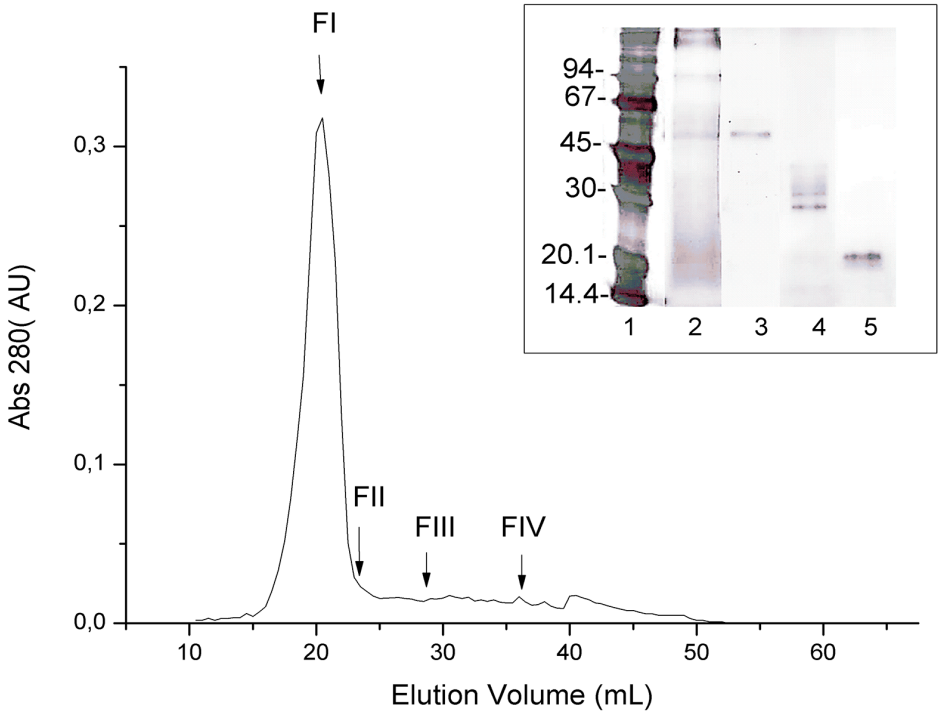

Figure 7. Size exclusion chromatography with Sephadex G-75. Fractions of 0,5 mL were collected at a flow rate of 0.250 mL /min and analyzed at 280 nm. The arrows indicate proteolytic activity in fraction I (FI), II (FII), III (FIII) and IV (FIV). Inset to Fig 7. SDS-PAGE of the fractions with proteolytic activity FI (lane 2), FII (lane 3), FIII (lane 4), FIV (lane 5) and the molecular weight standard (lane 1).

sodium chloride. Two proteolytic enzymes were purified with different molecular weights using different purification processes. Further studies of mass spectrometry are necessary to identify the purified enzymes. Due its characteristics and the abundant availability of *B. serra* leaves, this new enzymatic extract represents a promising source as an industrial catalyst.

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