Enzyme activity and partial characterization of proteases obtained from *Bromelia karatas* fruit and compared with *Bromelia pinguin* proteases

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Abstract The enzymatic activity and partial characterization of proteases from *Bromelia karatas* fruits were evaluated and compared with *Bromelia pinguin* proteases. The specific activity increased twofold after partial purification in both proteases. Partially purified proteases from *Bromelia karatas* showed good specific activity at pH 6.0–8.0 and residual activity of 70–100% for 60 min at 37–60 °C, similar to *Bromelia pinguin* proteases. The K_m value of proteases from *Bromelia karatas* was higher (253.32 μ M) than that of *Bromelia pinguin* proteases (234.94 μ M). The use of specific protease inhibitors indicated the presence of cysteine and serine proteases. Proteases with molecular weight of 66.2–97 and 21–31 kDa were detected. *Bromelia karatas* proteases registered 73% hydrolysis using a soy protein concentrate, similar to the enzyme activity of *Bromelia pinguin* proteases and commercial bromelain. These results demonstrate that *Bromelia karatas* proteases could be a potential alternative protease in the food industry.

Keywords *Bromelia karatas* fruit · Proteases · Enzyme activity · Enzyme characterization

Introduction

The Bromeliaceae family includes the *Bromelia* genus, which grows in neotropical zones in countries such as Mexico, Belize, Cuba, Puerto Rico, Guatemala, Panama, Brazil, the West Indies, and South America [1]. *Bromelia karatas* contains a good variety of minerals and vitamin C, and a high content of proteases [2, 3].

The number of proteases of plant origin that are used industrially is still small but is increasing because of their low production costs, relative abundance in some plants, and attractive biochemical characteristics. This has supported the screening of proteases from different wild plant species for isolation and characterization in order to evaluate their biotechnological potential for use in food [4, 5]. Active proteases from *B. pinguin* fruits have been detected in species cultivated in Puerto Rico [6], Cuba [7, 8], and Mexico [5]. However, only one study has been carried out on active proteases from *B. karatas* fruits [2]. These authors suggest that the yield, protein content, and enzymatic activity of the proteases vary according to the cultivar, the specific growing conditions, the extraction conditions, and the precipitation methods used for purification.

The development of techniques and methods for the purification of proteases is important for many biotechnological advances because the protease isolation treatment defines the kinetic behavior of these enzymes in different processes such as meat tenderization, brewing, protein hydrolysates [9], cheese production [10], and the pharmaceutical industry [11]. In the protocols for isolation of plant proteases, several extraction solutions have been used: water, phosphate buffer, Tris-HCl buffer, and sodium phosphate buffer. In addition, cysteine, as a protector of the active center, has been included in buffer solutions. On the

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other hand, acetone and ethanol are reported to be the solvents most commonly used for the precipitation process. The results of these studies demonstrate that the yield, enzymatic activity, kinetic parameters, and stability of isolated proteases depend on the extraction and precipitation conditions used to purify the enzymes [7, 12–15]. Therefore, more research is required to find the optimal conditions for the extraction and purification of proteases, especially from new plant sources. The aim of the present work was to evaluate the enzymatic activity and some kinetic characteristics of partially purified proteases from *B. karatas* fruits and compare their enzymatic characteristics with proteases from *B. pinguin* extracted under similar conditions.

Materials and methods

Plant material

Ripe fruits were collected from wild species of *B. karatas* (Cocuixitle) and *B. pinguin* (Guámara) in Santiago Ixcuintla, Nayarit, Mexico. The physicochemical characteristics of *B. pinguin* and *B. karatas* pulp were as follows: pH, 3.67 ± 0.05 and 3.14 ± 0.03 ; soluble solids, 12.4 ± 0.14 and 16.72 ± 0.10 °Brix; titratable acidity, 2.49 ± 0.05 and $3.35 \pm 0.27\%$; total protein, 8.04 ± 0.06 and $7.22 \pm 0.04\%$, respectively. The pulp was ultra-frozen and stored at $-80\text{ }^{\circ}\text{C}$ (926, Forma Scientific Inc, Marietta, OH, USA) until use.

Reagents

Sodium dodecyl sulfate (SDS) and polyacrylamide were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Bovine serum albumin, bromelain (EC 3.4.22.32), tyrosine standard, Coomassie brilliant blue R-250, and ethylenediaminetetraacetic acid (EDTA) were obtained from the Sigma-Aldrich (St. Louis, MO, USA). Ovalbumin protein was obtained from HYCEL de Mexico S.A de C.V. (Mexico City, Mexico). Milk and soy protein concentrates were purchased from Nestlé S.A. de C.V. (Mexico City, Mexico).

Preparation of enzymatic crude extract

Three solutions were used for protease extraction: distilled water (W); phosphate cysteine buffer (PCB) prepared with 0.1 M phosphate solution, 5 mM EDTA, and 5 mM cysteine adjusted to pH 6.1; Na_2S buffer (SSB) prepared with 2 mM Na_2S and 0.2 mM H_2SO_4 adjusted to pH 6.1. All solutions were kept cold ($4\text{ }^{\circ}\text{C}$). Fifty grams of frozen pulp ($-80\text{ }^{\circ}\text{C}$) was mixed and homogenized with 250 mL (1:5

w/v) of each extraction solution. The homogenate was filtered and then centrifuged (Z306, Hermle Labortechnik, Wehingen, Germany) at $12,000\times g$ for 15 min at $4\text{ }^{\circ}\text{C}$. Some of the supernatant, named crude extract (CEs) of proteases, was freeze-dried at $-50\text{ }^{\circ}\text{C}$ for 12 h under 12 Pa using a freeze dryer (77522020, Labconco Corporation, Kansas city, MI, USA). The lyophilized samples were stored at $-80\text{ }^{\circ}\text{C}$ for further analysis. The rest of the supernatant was used for precipitation.

Precipitation of proteases from enzymatic crude extract

CEs were precipitated with cold acetone ($-20\text{ }^{\circ}\text{C}$) in a ratio of 1:1 (v/v) or with cold ethanol ($-20\text{ }^{\circ}\text{C}$) in a ratio of 1:1 (v/v). The solvent was added slowly. The mixtures were kept for 1 h at $-20\text{ }^{\circ}\text{C}$. Next, the precipitates were separated by decantation and centrifugation at $12,000\times g$ and $4\text{ }^{\circ}\text{C}$ for 15 min. The precipitates were maintained at $5\text{ }^{\circ}\text{C}$ for 16 h to remove the residual solvent and then they were freeze-dried. The freeze-dried samples were stored at $-80\text{ }^{\circ}\text{C}$ for further analysis.

Purification of proteases

CEs were extracted from *B. karatas* and *B. pinguin* fruits using the best extracting solution according to their specific activity (EA). CEs (100 mL) were slowly added to 100 mL of the selected precipitation solvent ($-20\text{ }^{\circ}\text{C}$) with gentle agitation. The mixture was kept for 1 h at $-20\text{ }^{\circ}\text{C}$ to ensure protein precipitation and was then centrifuged at $6000\times g$ at $4\text{ }^{\circ}\text{C}$ for 10 min in order to obtain a protein pellet, which was called fraction one (FI). A further 100 mL of cold solvent was added to the supernatant and the process was repeated to obtain fraction two (FII). Another fraction (FIII) was obtained with 100 mL of CE and 100 mL of cold solvent slowly added and kept for 1 h at $-20\text{ }^{\circ}\text{C}$, then the mixture was centrifuged at $6000\times g$ for 10 min at $4\text{ }^{\circ}\text{C}$. The precipitate was saved and 200 mL of cold solvent was added to the supernatant, repeating the precipitation and centrifugation process. The precipitated proteins were combined. All fractions were frozen at $-80\text{ }^{\circ}\text{C}$ and freeze-dried. The samples were stored at $-80\text{ }^{\circ}\text{C}$ until further analysis.

Protein content

The protein content in the CEs, total precipitated proteins, partially purified protease, and proteins used as enzyme substrate in the reaction mixture, was determined according to the Bradford assay [16]. A sample of 2.6 mg was mixed with 1.5 mL of phosphate buffer (0.1 M, pH 7.0). Then 200 μL of the mixture was mixed with 600 μL of the

Bradford reagent and incubated at ambient temperature for 10 min. The absorbance was registered at 595 nm (Jenway 6705, Cole-Parmer Instrument Co., Felsted, UK) and the results were reported in milligrams per gram of dry matter (mg/g DM) or milligrams per milliliter (mg/mL).

Enzyme activity

The methodology proposed by Natalucci et al. [17] was followed with some modifications to measure the enzyme activity. Briefly, 1.1 mL of ovalbumin solution (10 g/L, dissolved in 0.1 M phosphate buffer at pH 7.0, heated to 90 °C in a water bath for 20 min, and centrifuged at 12,000×g, 10 min) was used as the substrate. Then, 200 µL (2.6 mg of lyophilized sample in 1.5 mL of phosphate buffer at pH 7.0 or 1.7 g/mL) of CE, precipitate, or partially purified protease was added to the substrate. The reaction mixture was incubated for 20 min at 37 °C, then 1.8 mL of trichloroacetic acid (TCA, 50 g/L) was added to stop the reaction. The samples were then placed on ice for 30 min and centrifuged at 14,000×g at 4 °C for 10 min, and the absorbance of supernatant was measured at 280 nm. A blank was prepared by adding TCA to the reaction mixture before incubation of the CE with the corresponding substrate. One activity unit (U) was defined as millimoles of tyrosine per minute per milliliter under the above assay conditions. The EA was calculated as units per milligram of protein (U/mg protein).

Kinetic enzyme characteristics

The enzymatic characteristics were determined only in partially purified proteases from both plant species. The effect of pH (5.0–10.0) on the enzyme activity of the samples was evaluated. The reaction mixture was prepared according to the method for enzyme activity mentioned above and varying the pH. Thermal stability was determined by measuring the enzyme activity of the samples at 37 °C and 40 °C to 75 °C at intervals of 5 °C for 2, 5, 10, 20, 40, 60, and 120 min at pH 7.0 [14]. The results are reported as residual enzymatic activity and calculated as follows:

$$RA = \frac{A_x}{A_0} \times 100 \quad (1)$$

where RA represents the residual activity, A_x is the enzyme activity after the heat treatment, and A_0 represents the enzyme activity without heat treatment.

The partially purified proteases were incubated in the presence of protease inhibitor compounds in order to elucidate the type of proteases associated with the enzyme activity. The proteases were incubated with different specific protease inhibitors such as phenylmethylsulfonyl

fluoride (PMSF, 1 mM), $HgCl_2$ (1 mM), and EDTA (10 mM) [14]. The enzyme activity was determined as mentioned above. The appropriate blanks and inhibitor solvents were used as controls. Inhibition of the activity was reported as percent (%) of residual activity [14].

The kinetic parameters of partially purified proteases were evaluated using ovalbumin as substrate. Different concentrations of ovalbumin (2–60 µM/mL) and protease solution (1.7 mg/mL) were used and controlled. The enzyme activity was determined as mentioned earlier. The Michaelis–Menten constant (K_m) and maximum reaction rate (V_{max}) were estimated from the Lineweaver–Burk double-reciprocal plot [18]. A bromelain protein standard was used as a positive control.

Polyacrylamide gel electrophoresis and zimogram

No-denaturing and denaturing polyacrylamide gel electrophoresis were performed in a Mini-PROTEAN Tetra Handcast Systems (Bio-Rad Laboratories, Hercules, CA, USA) according to Laemmli [19] with a constant current of 24 mA for 60 min. The gels (15% acrylamide/bisacrylamide) were stained with Coomassie brilliant blue R-250. The following molecular weight (MW) markers were used (SDS-standard Low Range, Bio-Rad Laboratories, Hercules, CA, USA): α -lactalbumin (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (30.0 kDa), ovalbumin (43.0 kDa), bovine serum albumin (67.0 kDa), and phosphorylase b (94.0 kDa).

A zimogram (no-denaturing electrophoresis) was performed in order to visualize the enzyme activity of partially purified proteases. A concentrator agarose gel with substrate (0.01 g ovalbumin) and separator agarose gel (0.005 g ovalbumin) were prepared each with 1 mg/mL of sample. After electrophoresis, the gels were collocated in Triton X-100 solution (2.5%) for 30 min; then in NaCl solution (0.2 M), $CaCl_2$ (5 mM) with Tris-HCl at pH 8.0 and 37 °C for 16–18 h. Finally, the gels were stained with Coomassie brilliant blue R-250 for 12 h and then faded with ethanol/acetic acid/water (40:10:50 v/v) for 16 h.

Degree of hydrolysis

The hydrolysis reaction was carried out according to Kim et al. [20] with modification in the protein determination. Briefly, 10 mL of the solutions of ovalbumin, milk, and soy proteins was incubated with 1 mL of proteases (1.7 g/mL) at 60 °C for 35 min. The hydrolysis was stopped with 1.8 mL of TCA (50 g/L). The samples were then centrifuged at 16,000×g at 4 °C for 10 min. The protein content in the supernatants and precipitates after hydrolysis was measured by the Bradford method [16]. The

percentage degree of hydrolysis (DH) was calculated using the following equation:

$$\text{DH (\%)} = \frac{(\text{PCS}_{\text{to}} - \text{PCP}_{\text{tx}})}{\text{PCS}_{\text{to}}} \times 100 \quad (2)$$

where Pas_{tor} is the initial protein content in the supernatant without hydrolysis at time zero and PCP_{tx} is the protein content without hydrolysis in precipitates in hydrolysis time.

Statistical analysis

All experiments were carried out in triplicate with three replicates. The data were expressed as mean $\pm$ standard deviation. The data were analyzed by analysis of variance (ANOVA) using the STATISTICA software (v.10 StatSoft, Tulsa, OK, USA). Multiple means comparisons were performed with Tukey's test. Statistically significant differences were identified when $p < 0.05$.

Results and discussions

Protein content and enzyme activity of crude extracts

Table 1 shows significant differences ($p < 0.05$) in the protein content, activity units, and EA of the CEs. The CE from *B. karatas* obtained with PCB had a higher total protein content (8.79 mg/g DM) than the CE obtained with SSB (7.78 mg/g DM) and water (7.56 mg/g DM). On the other hand, the CE from *B. pinguin* obtained with SSB had higher total protein content (9.35 mg/g DM) compared with the CE obtained with PCB (8.74 mg/g DM) and water (8.46 mg/g DM). Therefore, the CE from *B. karatas* obtained with PCB and the CE from *B. pinguin* obtained with SSB also had the highest activity units (2.05 and 3.64 U) and EA (4.45 and 7.91 U/mg protein, respectively). The

most studies on extraction of *Bromelia* proteases have used cysteine as an active center protector. Also, the addition of Na_2S was used in the extracting buffer of bromelain and the enzyme activity increased up to five times [12, 21]. The enzyme activity of *B. karatas* proteases has not been well investigated, unlike *B. pinguin* proteases. Therefore, studies using different extracting solutions are important in order to find out the optimal extraction conditions for each protease. Also, the differences in catalytic activities of endopeptidases depend on their structure and genetic differences in the species [22]. The *B. karatas* CE presented a lower enzyme activity than *B. pinguin* CE; but the EA was higher than *B. pinguin* CE (1.3 U/mg protein) from harvested fruits from Culiacan, Sinaloa, Mexico [5]. Andrade et al. [23] reported that protein synthesis, which is caused by the ripening process of the fruits, depends on specific growing conditions. Therefore, the enzyme activity of proteases depends on the location.

Protein content and enzyme activity of precipitate proteases

The total protein content increased from 115 to 200% in the precipitates in comparison with the CEs, independently of the extraction solutions and plant species (Table 2). These results were expected because it is known that acetone and ethanol decrease the solubility of proteins, causing precipitation. Analyzing the effect of each sample, it was observed that the concentration of proteins was dependent on the plant species because the species are genetically different. In the *B. karatas* precipitates, the total protein content increased when the CEs were obtained with PCB and precipitated with acetone (21.63 mg/g DM) or with PCB and SSB (20.83 and 21.82 mg/g DM, respectively) when they were precipitated with ethanol. Meanwhile, in the *B. pinguin* precipitates, the total protein content was higher when the CEs were extracted with SSB and precipitated with acetone or ethanol (25.41 and 25.50 mg/g

Table 1 Protein content, activity units, and specific activity of crude extracts obtained with water, phosphate cysteine buffer (PCB) and sodium sulfide buffer (SSB) from *Bromelia karatas* and *Bromelia pinguin* fruits

Parameter	<i>Bromelia karatas</i>			<i>Bromelia pinguin</i>		
	Water	PCB	SSB	Water	PCB	SSB
Total protein (mg/g DM)	7.56 $\pm$ 0.14c	8.79 $\pm$ 0.24b	7.78 $\pm$ 0.12c	8.46 $\pm$ 0.20b	8.74 $\pm$ 0.12b	9.35 $\pm$ 0.39a
Protein (mg/mL) ¹	0.38 $\pm$ 0.01a	0.46 $\pm$ 0.01a	0.46 $\pm$ 0.01a	0.46 $\pm$ 0.01a	0.46 $\pm$ 0.01a	0.46 $\pm$ 0.01a
Activity units (U)	0.72 $\pm$ 0.08b	2.05 $\pm$ 0.11b	1.47 $\pm$ 0.13c	1.60 $\pm$ 0.09c	2.47 $\pm$ 0.22b	3.64 $\pm$ 0.02a
Specific activity (U/mg protein)	1.89 $\pm$ 0.36e	4.45 $\pm$ 0.25c	3.19 $\pm$ 0.30d	3.48 $\pm$ 0.20d	5.37 $\pm$ 0.48b	7.91 $\pm$ 0.05a

Means $\pm$ standard deviations ($n \geq 3$) with different letters within rows are statistically different ($\alpha = 0.05$)

DM: dry matter, U: activity unit (mM/mL/min)

¹Protein (mg/mL) is the protein content in 200 μL of lyophilized crude extract resuspended in phosphate buffer (pH 7.0)

Table 2 Protein content, activity units, and specific activity of precipitated proteases extracted with water, phosphate cysteine buffer (PCB), and sodium sulfide buffer (SSB) from *Bromelia karatas* and *Bromelia pinguin* fruits

Plant species	Parameter	Extraction solutions					
		Water Precipitated with acetone	PCB	SSB	Water Precipitated with ethanol	PCB	SSB
<i>Bromelia karatas</i>	Total protein (mg/g DM)	18.41 ± 0.21bc	21.63 ± 0.28a	19.45 ± 0.24b	16.80 ± 0.3c	20.83 ± 0.25a	21.82 ± 2.35a
	Protein (mg/mL) ¹	0.10 ± 0.02d	0.21 ± 0.01c	0.23 ± 0.03c	0.43 ± 0.05a	0.32 ± 0.03b	0.11 ± 0.03d
	Activity units (U)	0.14 ± 0.03e	1.46 ± 0.09a	1.19 ± 0.06c	0.08 ± 0.01e	1.33 ± 0.08b	0.43 ± 0.09e
	Specific activity (U/mg protein)	1.41 ± 0.92d	6.33 ± 0.41a	5.17 ± 0.26b	0.19 ± 0.03e	4.56 ± 0.03b	3.91 ± 0.86c
<i>Bromelia pinguin</i>	Total protein (mg/g DM)	24.22 ± 0.21ab	23.70 ± 0.13b	25.41 ± 0.31a	18.37 ± 0.30c	23.58 ± 0.82b	25.50 ± 0.55a
	Protein (mg/mL) ¹	0.12 ± 0.01c	0.10 ± 0.01d	0.16 ± 0.02c	0.35 ± 0.03a	0.26 ± 0.01b	0.27 ± 0.02b
	Activity units (U)	0.30 ± 0.02e	1.47 ± 0.05c	3.00 ± 0.11a	0.78 ± 0.10d	1.73 ± 0.14b	1.64 ± 0.19bc
	Specific activity (U/mg protein)	2.56 ± 0.37d	14.72 ± 0.28b	18.75 ± 1.60a	2.23 ± 0.29d	6.65 ± 0.54c	6.07 ± 0.71c

Means ± standard deviations (n ≥ 3) with different letters within rows are statistically different ($\alpha = 0.05$)

DM: dry matter, U: activity unit (mM/mL/min)

¹Protein (mg/mL) is the protein content in 200 μ L of lyophilized precipitates resuspended in phosphate buffer (pH 7.0)

DM, respectively). Although the same quantity of precipitate was used to evaluate the total protein content in the reaction mixture, the content was statistically different ($p < 0.05$). This indicated that the purity of the precipitates was poor, because the proteins could be linked to polyphenols or carbohydrates principally when the CEs were precipitated with ethanol [24].

The enzyme activity coincided with the protein content. In the proteases precipitated from *B. karatas*, the PCB solution and acetone as the precipitation agent were more effective to maintain the highest activity units (1.46 U) and EA (6.33 U/mg protein). In this same way, the proteases precipitated from *B. pinguin* presented the highest activity units and EA (3.0 U and 18.75 U/mg protein, respectively) when they were extracted with SSB and precipitated with acetone. In comparison with the enzyme activity of CEs, the EA increased 42% for the proteases precipitated from *B. karatas* and 137% for the proteases precipitated from *B. pinguin*. Similar behavior in the EA of *Bromelia hieronymi* proteases was reported by Bruno et al. [24] when they used acetone and ethanol for the precipitation of proteases. Thus, water is not effective to maintain the enzyme activity of proteases, whereas ethanol can cause higher denaturalization or can carry other compounds that affect the enzyme activity [24]. Therefore, the CE from *B. karatas* was obtained using PCB and the CE from *B. pinguin* was obtained with SSB and acetone as a precipitation solvent for both.

Protein content and enzyme activity of partially purified proteases

The highest total protein content was registered in FIII from both plant species (Table 3), this content was higher than in the precipitates (Table 2). This was attributed to the higher volume of acetone used to precipitate the proteases in this stage. The purification of proteases provided two retained active fractions (FII and FIII) from *B. karatas* and *B. pinguin* with different EA. Fractions FII and FIII from *B. karatas* had an EA of 9.60 and 10.0 U/mg protein, respectively, whereas FII and FIII from *B. pinguin* showed an EA of 21.16 and 22.83 U/mg protein. Therefore, there was 35–37% more EA in the fractions from *B. karatas* and 11–18% more EA in the fractions from *B. pinguin*. The highest purification factors were 2.24- and 2.88-fold. The significant differences ($p < 0.05$) in the EA were probably due to the fact that each Bromeliaceae species has different proteases. Purification schemes for other *Bromelia* have been reported and the purity varied from 1.5- to 2.9-fold when acetone was used for precipitation [14, 24, 25]. Also, a lower EA (7.11–11 U/mg protein) has been found in purified proteases of *B. pinguin* [7, 8] than in this experiment.

Enzymatic characteristics

The pH effect on enzyme activity is shown in Fig. 1A. The *B. karatas* proteases had the highest EA (6.94–7.62 U/mg protein) at pH 6.0 and 8.0. Meanwhile, the *B. pinguin*

Table 3 Protein content, activity units, and specific activity of purified proteases from *Bromelia karatas* and *Bromelia pinguin* fruits

Vegetal species	Step	Total protein (mg/g DM)	Protein (mg/mL) ¹	Activity units (U)	Specific activity (U/mg protein)	Purification (n-fold)
<i>Bromelia karatas</i>	CE	8.79 ± 0.24d	0.46	2.05 ± 0.11a	4.45 ± 0.25b	1.0
	Fraction I	14.16 ± 0.57c	0.06	0.10 ± 0.05d	1.66 ± 0.79c	0.37
	Fraction II	15.93 ± 2.03b	0.02	0.20 ± 0.04c	9.60 ± 1.12a	2.15
	Fraction III	27.7 ± 0.64a	0.05	0.48 ± 0.06b	10.00 ± 1.71a	2.24
<i>Bromelia pinguin</i>	CE	9.35 ± 0.39d	0.46	3.64 ± 0.02c	7.91 ± 0.05c	1.0
	Fraction I	22.33 ± 2.66c	0.06	0.13 ± 0.01e	2.16 ± 0.27d	0.27
	Fraction II	34.76 ± 0.10b	0.06	1.27 ± 0.02b	21.16 ± 0.24b	2.67
	Fraction III	37.72 ± 0.31a	0.06	1.37 ± 0.04a	22.83 ± 0.62a	2.88

Means ± standard deviations (n ≥ 3) with different letters within columns are statistically different ($\alpha = 0.05$)

DM: dry matter, U: activity unit (mM/mL/min)

¹Protein (mg/mL) is the protein content in 200 μ L of lyophilized crude extract or fractions resuspended in phosphate buffer (pH 7.0)

Fig. 1 Effect of pH (A), temperature (B, C), and inhibitors (D) on the specific activity or residual activity of partially purified proteases obtained from *B. pinguin* and *B. karatas* fruits

proteases exhibited the maximum EA (16.57 U/mg protein) at pH 7.0. The enzyme activity of *B. karatas* proteases at different pH values has not been reported. *B. karatas* proteases have a wide range of optimum pH, unlike *B. pinguin* proteases. Payrol et al. [7] reported that 90% (6.8 U) of the enzyme activity of *B. pinguin* extracts was reached around pH 7.0–8.5. Moreno-Hernández et al. [5] reported that the highest enzyme activity for *B. pinguin* CE was detected at pH 2.0 (3.2 U/mg). However, at alkaline

pH, the highest enzyme activity was recorded at pH 7.5 (1.3 U/mg), concluding that the CE could contain acid, neutral, and alkaline proteases. The highest enzyme activity of *B. pinguin* proteases found in this experiment was probably a result of the purification method or differences in crops. Others authors have reported that the pH range of the maximum enzyme activity of *Bromelia balansae* [10], *Bromelia hieronymi* [24] and *Bromelia anthi-acanta* [14] proteases is between 5.0 and 10.0 U/mg

protein. In this work, the high enzyme activity was found at neutral-alkaline pH values, which could be related to the fact that plants from the Bromeliaceae family generally produce a large amount of cysteine proteases in their fruits [5].

The effect of the temperature on the stability of partially purified proteases is presented in Fig. 1B, C. The *B. karatas* proteases retained 68–95% of their activity after incubation at a temperature range of 37–60 °C for 60 min, whereas a gradual decrease in enzyme activity was observed at higher temperatures after prolonged incubation times (120 min). On the other hand, the *B. pinguin* proteases showed from 70 to 93% RA at a temperature range from 37 to 60 °C during 60 min of incubation, but at a temperature and incubation time higher than 70 °C and 60 min, respectively, the enzyme activity decreased perhaps due to heat denaturation. Payrol et al. [7] reported that *B. pinguin* proteases retained over 80% of their enzyme activity at a temperature range of 21–66 °C. On the other hand, Moreno-Hernández et al. [5] found *B. pinguin* proteases present in the CE were quickly inactivated at 60 °C. Other purified proteases from *Bromelia* fruits were stable at temperatures of 60 °C [14, 24]. The high enzyme activity exhibited by *B. karatas* proteases after 60 min of incubation at 37–60 °C and pH of 6.0–8.0 is an important feature for application in food processes.

The inhibitory effect of PMSF is characteristic of serine proteases. *B. karatas* and *B. pinguin* proteases were inhibited 64 and 22%, respectively, by PMSF (Fig. 1D); the higher inhibition of *B. karatas* proteases is possibly because there is a higher concentration of serine proteases in the fruits. The metalloprotease inhibitor, EDTA, did not inhibit *B. karatas* and *B. pinguin* proteases, in agreement with previous studies on the characterization of *Bromelia* proteases [5, 7, 21, 24]. The inhibition of *B. karatas* proteases by mercury chloride (HgCl_2) was 62%, whereas *B. pinguin* proteases were inhibited 81%. Thus, there are probably cysteine proteases in both plant species, although may be at a lower concentration in *B. karatas* fruits than in *B. pinguin* fruits.

The results for the kinetic parameters are shown in Table 4. The K_m value varied considerably among proteases. Payrol et al. [8] reported a K_m of 182.1–233.1 μM for partially purified proteases from *B. pinguin* fruits using casein (Hammarsten type) as substrate. Similar results were registered in this experiment for *B. karatas* (253.32 μM) and *B. pinguin* (234.94 μM) partially purified proteases when ovalbumin was used as substrate. In the same way, the K_m value in bromelain was 163.43 μM . The $V_{\max}$ value was similar for the enzyme activity of *B. karatas* and bromelain proteases. $V_{\max}$ is dependent on the affinity of the enzyme for the substrate, enzyme quantity, and catalytic power; therefore normally the values vary [8]. These

results coincided with data reported for *B. pinguin* proteases [8], although similar data do not exist for other endopeptidases of the Bromeliaceae family. The $V_{\max}/K_m$ value is called the enzyme catalytic power parameter [26] and *B. karatas* proteases registered the lowest value coinciding with their EA.

Molecular weight and zimogram

The MW of *B. karatas* and *B. pinguin* proteases obtained by gel electrophoresis is shown in Fig. 2A. Different protein bands with approximate MWs of 66.2–97 and 21–31 kDa were observed. The bands with MW between 21 and 31 kDa could be associated with the presence of cysteine proteases reported in plants from the Bromeliaceae family [10, 27, 28]; the bands with the highest MW could be serine proteases. Although serine proteases have not been reported in *Bromelia* species, similar data were demonstrated in serine proteases extracted from *Morus indica*, which had a MW of 66–120 kDa in non-denaturing conditions [29]. Zimograms demonstrated that the enzyme activity of partially purified proteases of both plant species was conserved after the purification process (Fig. 2B). The clear or white zones in the zimogram (Fig. 2A) indicate that there was enzyme activity because during electrophoresis proteases are added in the substrate and they are capable to present activity in these non-denaturing conditions [30].

Degree of hydrolysis

The percentage DH is shown in Table 4. *B. karatas* proteases had the highest %DH in soy protein concentrate (73.38%), followed by the milk protein concentrate (61.64%), and ovalbumin (49.85%). The DH was similar when bromelain was used. However, the %DH of *B. pinguin* proteases was 65.71–68.98% in ovalbumin and milk protein concentrate, and 81% in soy protein concentrate. The DH depends on the process conditions such as substrate concentration, ratio of enzyme to substrate, incubation time, pH, temperature, and nature of the enzyme activity and specificity [31, 32]. In this experiment, it was clear that *B. pinguin* proteases had higher DH than *B. karatas* proteases. However, the *B. karatas* proteases can be considered to have good hydrolysis capacity [31]. All proteases had a high DH in the soy protein concentrate, probably because they are plant proteins and their chemical structure is simpler than that of ovalbumin glycoproteins and milk globulins.

The DH obtained in this experiment is higher than the DH of bean protein (28–30%) with bromelain reported by Oseguera-Toledo et al. [33]. Thus, the nature of the

Table 4 Enzymatic parameters and degree of hydrolysis (%) of ovalbumin, milk, and soy proteins caused by purified proteases from *Bromelia karatas* and *Bromelia pinguin* fruits and bromelain

Proteases	K_m (μM)	$V_{\max}$ ($\mu\text{M}/\text{min}$)	$V_{\max}/K_m$ (min^{-1})
<i>Bromelia karatas</i>	$253.32 \pm 2.23\text{a}$	$5.81 \pm 1.13\text{b}$	0.023b
<i>Bromelia pinguin</i>	$234.94 \pm 2.35\text{b}$	$8.03 \pm 1.51\text{a}$	0.034a
Bromelain	$163.43 \pm 2.24\text{c}$	$5.50 \pm 1.02\text{b}$	0.034a
Degree of hydrolysis (%)			
	Ovalbumin	Milk	Soy
<i>Bromelia karatas</i>	$49.85 \pm 1.63\text{cY}$	$61.64 \pm 1.44\text{bY}$	$73.38 \pm 2.95\text{aY}$
<i>Bromelia pinguin</i>	$65.71 \pm 2.49\text{bX}$	$68.98 \pm 1.85\text{bX}$	$81.74 \pm 1.74\text{aX}$
Bromelain	$55.53 \pm 0.59\text{cZ}$	$61.64 \pm 4.78\text{bY}$	$76.71 \pm 0.62\text{aY}$

K_m , Michaelis-Menten constant; $V_{\max}$, maximum velocity. Means $\pm$ standard deviations ($n \geq 3$) with different letters within rows (a, b, c) and columns (X, Y, Z) are statistically different ($\alpha = 0.05$)

Fig. 2 Denaturing and non-denaturing electrophoresis (A) and zimogram (B) of partially purified proteases obtained from *B. pinguin*, *B. karatas*, and bromelain protease standard. M-WM, molecular weight marker

enzymes influences the DH and the structures of the peptides produced [33].

In conclusion, the partially purified proteases from *B. karatas* fruits had good specific activity over of wide range of pH and temperature. They had similar enzymatic characteristics as *B. pinguin* proteases and bromelain. The presence of inhibitors revealed that the proteolytic properties of proteases are related to the presence of cysteine and serine proteases. In addition, they presented a high degree of hydrolysis with soy protein concentrate. *B. karatas* proteases could be used alone or in combination with other plant proteases in the food industry.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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