RESEARCH ARTICLE

Q1 The biochemical characterization, stabilization studies and the antiproliferative effect of bromelain against B16F10 murine melanoma cells

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ABSTRACT

The current study aims to extract bromelain from different parts (stem, crown, peels, pulp and leaves) of *Ananas comosus* var. *comosus* AGB 772; to determine of optimum pH and temperature; to test bromelain stability in disodium EDTA and sodium benzoate, and to investigate its pharmacological activity on B16F10 murine melanoma cells *in vitro*. The highest enzymatic activity was found in bromelain extracted from the pulp and peel. The optimum bromelain pH among all studied pineapple parts was 6.0. The optimum temperature was above 50°C in all bromelain extracts. The fluorescence analysis confirmed the stability of bromelain in the presence of EDTA and sodium benzoate. Bromelain was pharmacologically active against B16F10 melanoma cells and it was possible verifying approximately 100% inhibition of tumor cell proliferation *in vitro*. Since bromelain activity was found in different parts of pineapple plants, pineapple residues from the food industry may be used for bromelain extraction.

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Introduction

Bromelain is a mix of thiol endopeptidases and other components such as phosphatases, glycosidases,

peroxidases, cellulases, escarases, glycoproteins, carbohydrates and protease inhibitors found in different tissues of plants belonging to family Bromeliaceae and

pineapple (*Ananas comosus*) is the best known bromelain source in this family (Maurer 2001; Ketnawa et al. 2011; Pavan et al. 2012). Pineapples and bromelain have an importance of folk and modern medicinal use (Amini et al. 2016).

Bromelain has been exploited commercially in many applications in the food, beverage, tenderization, cosmetic, pharmaceutical and textile industries (Arshad et al. 2014). Among its therapeutic effects, the following stand out: anti-inflammatory, anti-oedematous, anti-coagulant, wound healing, debriding, digestive, expectorant, anti-obesity, antimicrobial, immunomodulatory, antibiotic absorption potentiation, antineoplastic activity among others, have been attributed to bromelain (Taussig & Batkin 1988; Maurer 2001; Hale et al. 2005; Aiyegbusi et al. 2011a, 2011b; Borelli et al. 2011; Hu et al. 2011; Dave et al. 2012; Aichele et al. 2013; Dutta & Bhattacharyya 2013; Errasti et al. 2013). A recent study shows a new application to bromelain, on vaccine synthesis (Wang et al. 2012). Bromelain has been accepted as herbal medicine for oral administration, due to its efficacy, safety and lack of adverse effects (Maurer 2001). It has been commercialized in the oral solution form, in Brazil. This solution is prepared with bromelain extracted from *Ananas comosus* pulp and used as mucolytic and expectorant in respiratory conditions.

The commercially available bromelain is extracted from the stem and the pulp. However, it is known that this enzyme is also found in other pineapple plant tissues. Therefore, bromelain extraction from different parts of the plant and its kinetic features are of interest due to its potential for biotechnological use and reduce costs of enzyme production. Disodium EDTA and sodium benzoate are commonly used as oral preservative formulations, and EDTA is also used as antioxidant agent.

With respect to previous studies about the action of proteases on melanoma cells (Grabowska et al. 1997; Guimarães-Ferreira et al. 2007; Bhui et al. 2012; Dittz et al. 2015), the efficacy of bromelain from AGB 772 plants (an unexplored French Guiana variety) in inhibiting the proliferation of B16F10 murine melanoma cells was investigated here. Also, this manuscript aims to study the optimum temperature and pH conditions; and to test the stability of bromelain extracted from the pulp and the peel in commonly used additives.

Materials and methods

Chemicals and plant materials

The pineapple (*Ananas comosus* var. *comosus* AGB 772) plant was provided by Active Germplasm Bank

(AGB) of Brazilian Agricultural Research Corporation (EMBRAPA – *Empresa Brasileira de Pesquisa Agropecuária*), Cruz das Almas Country, Brazil. Originally, it was collected in French Guiana (Lat: 0537N; Lon: 5350W) in 1993. Its collection record is BRA-009610 and the access to the bank is AGB 772. The plant was washed, air-dried and its different parts (stem, crown, peels, pulp and leaves) were manually separated. The commercial bromelain and the Casein were supplied by Sigma (St. Louis, MO). Coomassie Brilliant Blue G-250 was supplied by Merck (Darmstadt, Germany). All buffers and reagents were of analytical grade.

Preparation of the crude enzymatic extracts from the stem, crown, peel, pulp and leaf

The plant parts were chopped into small pieces before being blended in domestic blender using cold distilled water at 4:1 (w:v) ratio. The resulting blend was filtered in cheese cloth and then centrifuged at 8000×g (Centrifuge 5804R – Eppendorf, São Paulo, Brazil), at 4°C, for 20 min. The supernatant, herein called bromelain, was kept frozen until the time to be analyzed (Soares et al. 2012).

Protein content and enzymatic activity measurements

The protein content was measured in spectrophotometer (Cary Varian, São Paulo, Brazil), according to the method by Bradford (1976), bovine serum albumin was used as standard. The enzyme activity was estimated according to modifications in the method described by Murachi (1976), 0.5% casein (w/v) in 0.2 M phosphate buffer (pH 6.0) was used as substrate. Sample aliquots of 100 µL were added to tubes containing 1 mL of casein buffered solution. The mixture was kept in water bath at 37°C for 20 min. Subsequently, a 40 µL aliquot of the mixture was removed from the reaction and added to 2 mL of Bradford reagent. The absorbance was determined at 595 nm, using UV/visible spectrophotometer (Cary Varian, São Paulo, Brazil). One enzyme unit (U) was the bromelain amount defined as necessary to consume 1 µg of casein per minute (expressed as U/mL). The specific activity was determined by the ratio between enzyme activity (U/mL) and protein concentration (mg/mL) – expressed as U/mg. All measurements were performed in triplicate.

Temperature and pH profile assay

The proteolytic activity of the crude extract was conducted at different temperatures (30, 40, 50, 60, 70, 80 and 90 °C). The assay was conducted as afore mentioned, using casein as substrate. The pH profile was determined through proteolytic activity assays using different pH values (pH 5–9). Citrate-phosphate (pH 5–7) and tris-HCl buffers (pH 8–9) were used. The temperatures were controlled by means of a circulating water bath. Blanks without enzyme sample were made for each determination.

Circular dichroism (CD) analysis

The CD measurements were carried out in spectropolarimeter (JASCO J-815, Tokyo, Japan), under the camera cuvette controlled temperature by Peltier (PTC-423S/15). The CD spectra were measured in far-UV range (190–250 nm), using 1 mm path length quartz cuvette. The samples were read at 50 nm/min and collected in 0.5 nm steps. The analyses were monitored in the Spectra Measurement (Jasco, Tokyo, Japan) software. The bromelain samples from the pulp and the peel were previously purified through the ethanol precipitation method described by Soares et al. (2012). The final contents of bromelain from the peel were 0.2 mg.mL⁻¹ and 0.4 mg.mL⁻¹ for the control, respectively, the bromelain from the pulp recorded in phosphate buffer, at pH 6.0, was 0.2 M.

Mass spectrometry analysis (MS/MS)

To confirm the identity of the protein, the band observed on the SDS-PAGE gel after protein purification was cut and treated for subsequent mass spectrometry analysis in order to confirm protein identity, as similarly described by Camillo et al. (2012). Shortly after, the tryptic digestion of the Coomassie-stained gel was conducted according to Shevchenko et al. (2006). The peptide mixtures were analyzed through online nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS) in a nanoAcquity chromatograph (Waters, Milford, MA) coupled to the Q-ToF micromass spectrometer (Waters, Milford, MA) (de Oliveira et al. 2011). The raw data of MS and MS/MS were processed in the Protein Lynx v2.3 software (Waters, Milford, MA) and compared to the NCBI nr databank in the MASCOT server v2.3. (<http://www.matrixscience.com/>). The following parameters were used for the search: trypsin digestion with one missed cleavage, at maximum; carbamidomethyl (Cys) as fixed modification; oxidation (Met), as variable

modification; peptide mass tolerance of 0.3 Da for the parent ion, and 0.10 Da, for the fragment ions.

The thermal stability of bromelain extracted from the peel – unfolding and refolding analysis

The bromelain from the peel was subjected to thermal stability analysis. It was monitored through far-UV CD spectroscopy, using the “Temperature Measurement” software. Readings were made every 5 °C increase, at 208 nm. The “unfolding” readings were performed at 23–95 °C and the “refolding” ones at 95–23 °C.

Stabilization studies

Stabilization studies were done using bromelain from the pulp and the peel. The enzymatic reaction was conducted under the same conditions described above, except for the addition of different disodium EDTA and sodium benzoate concentrations. A Doehlert matrix was applied to find the best conditions for the enzymatic activity. The EDTA disodium concentrations (m/w) were conducted at five levels: 0.005%, 0.055%, 0.03%, 0.08% and 0.105% (0.1–2.8 mM). The sodium benzoate was assessed at three levels: 0.05%, 0.075% and 0.1% (3.5–6.9 mM). Three central point (c) repetitions, in a total of nine experiments, were performed in order to estimate the possible pure error. The proteolytic activity was measured at each point. The ANOVA (analysis of variance) and response surface graphs were plotted in the Statistica software, version 7.0 (Statsoft, Tulsa, OK).

Fluorescence measurements

Intrinsic fluorescence assays were performed using a spectrofluorometer (Quimis, Tarrytown, NY)-prototype 1 (UV-Vis – Q798FIL) – in the Optical Properties Laboratory (LaPO) of the Physics Institute at Federal University of Bahia. The value of the fluorescence radiation area was measured through the curve integration provided by the fluorometer, using the Origin[®] software. The samples were previously purified through the ethanol precipitation method described by Soares et al. (2012) and showed bromelain content 0.4 mg/mL. The Disodium EDTA and sodium benzoate concentrations used were 0.055% and 0.075%, respectively.

Proliferation assay

The B16F10 murine melanoma cells were grown in RPMI 1640 medium (Roswell Park Memorial Institute

Medium) supplemented with 10% fetal bovine serum, incubated at 37 °C, in 5% CO₂ atmosphere. The cells were distributed in 24 plates and the cell concentration was adjusted to 2×10^5 cells/well. The extracts from the pulp and the peel were added to the culture medium at 25 µg/mL and 50 µg/mL, respectively (these concentrations were determined by previous studies; data not shown). Next, they were incubated for 24 h. The antiproliferative effects were analyzed through colorimetric test (Lshai-Michaeli et al. 1990); the treated cells were fixed using methanol, for 10 min. Then, 500 µL of Methylene Blue at 0.1% were added to the plates, for 10 min. Borate buffer (pH 8.7) was used to wash the plates. Subsequently, 500 µL of HCl 0.1 M were added and the mixture was incubated for 10 min. The optical density was assessed in Microplate Reader at wavelength 655 nm.

Statistical analysis

The ANOVA (analysis of variance) was used to analyze data from the triplicate measurements. Differences between means were tested through the Tukey test, at 0.1% probability, in the BIOSTAT 5 software. The statistical significance test applied to test the antiproliferative effect of bromelain was conducted in the GraphPad Prism 5.0 software, using the non-parametric ANOVA and the Dunnett post-test. All measurements were compared to the results of the bromelain-free control. Whenever the *p* value was less than .05, the result was considered significant. The following standards were set to the graphics in comparison to the control: **p* < .05; ***p* < .001; ****p* < .0001.

Results

Enzymatic activity of bromelain extracted from the stem, crown, peel, pulp and leaf

Table 1 shows the enzymatic activity and the protein content of bromelain extracted from different parts of the *Ananas comosus* var. *comosus* AGB 772 plant.

The bromelain extracted from the pulp has shown the highest specific activity, whereas the bromelain extracted from the residues such as peel and crown, has shown high activity, but lower than that of the pulp. The bromelain from the stem has shown the lowest activity and the highest protein content.

The effect of temperature and pH on the proteolytic activity of bromelain

Temperature and pH are very important factors to enzyme featuring studies. They have direct influence

on bromelain activity and on its stability. The optimum temperature was tested from 30 to 90 °C in test tubes (selected to be equal in weight and size) and were incubated in a water bath. The results were obtained in U/mL and plotted in relative activity.

The bromelain from the stem showed higher activity at 60 °C, whereas the enzyme from the peel showed higher activity at temperatures ranging from 60 to 90 °C (Figure 1(A)). The pulp did not face activity decrease above 40 °C. The bromelain from the crown showed higher activity between 50 and 70 °C, whereas the leaves showed higher activity between 60 and 80 °C. The pH is also an essential parameter in enzyme featuring studies, since small pH changes may lead to different ionization degrees in the biomolecule and cause conformational changes able to increase the affinity between the active site and the substrate or even to cause macromolecule denaturation. The pH in the current study ranged from 5 to 9, at 60 °C. The results are shown in Figure 1(B). The optimum pH to achieve the highest activity of bromelain extracted from all studied pineapple parts was 6.0, whereas the leaves presented their two peaks at pH 6.0 and 8.0.

Circular dichroism (CD) analysis

The CD spectra of the samples are shown in Figure 2. Both curves are practically coincident in the far-UV region and it suggests that there is no difference in the secondary structures of control, peel and pulp bromelains. The spectra are typical of alpha-helix unfolding proteins, and it was indicated by the negative peak at 208 nm.

The identity of the bromelain extracted from the peel was confirmed through mass spectrometry (Table 2). The profile of tryptic peptides obtained through mass spectrometry (LC/MS/MS) showed 11% peptide coverage between the bromelain extracted from the peel and the *Ananas comosus* bromelain

Table 1. Activities and protein content of bromelain extracted from different parts of *Ananas comosus* var. *comosus* BGA 772.

Samples	Proteolytic activity (U/mL) ¹	Specific activity (U/mg) ²	Protein content (mg/mL) ³
Stem	27.99 (1.20) ^c	432.98 (18.62) ^e	0.0647 (0.0022) ^a
Crown	40.15 (2.76) ^b	1050.57 (72.31) ^b	0.0382 (0.0009) ^b
Peel	57.11 (1.98) ^a	1365.77 (47.23) ^b	0.0418 (0.0025) ^b
Pulp	62.50 (0.07) ^a	1519.85 (1.60) ^a	0.0411 (0.0017) ^b
Leaves	37.10 (1.66) ^b	859.44 (38.47) ^d	0.0432 (0.0020) ^b

^a(*F* = 197.16, *p* < .0001).

^b(*F* = 297.70, *p* < .0001).

^c(*F* = 40.39, *p* < .0001).

Averages are shown with their respective deviations in parenthesis. Values followed by different letters in the same column represent significant difference (*p* < .01) by Tukey test.

deposited in the gene bank (Access number: gi|2463588|BAA22546.1).

Stabilization studies

The present report has studied the stability of bromelain from the pulp and the peel in sodium benzoate and disodium EDTA, using the Doehlert experimental matrix with two variables (Table 3). The response surface plots and the contour lines are shown in Figure 3(A,B).

Equations (1) and (2) illustrate the relation between these two variables and the response R (enzymatic activity %) of the bromelain from the pulp and the peel, respectively; E and B are the disodium EDTA and sodium benzoate concentrations (%), respectively.

$$R = 83.34(\pm 5.4467) + 93.93 * E(\pm 56.0547) - 808.48 * E^2(\pm 279.9237) + 381.61 * B(\pm 131.3018) - 2652.32 * B^2(\pm 839.7711) + 172.22 * E * B(\pm 613.2821) \quad (1)$$

$$R = 82.06(\pm 5.0218) - 109.34 * E(\pm 51.6814) - 385.87 * E^2(\pm 258.0845) + 745.21 * B(\pm 121.0579) - 6298.99 * B^2(\pm 774.2536) + 2449.36 * E * B(\pm 565.4349) \quad (2)$$

The stabilization results showed that bromelain remained stable, and presented high proteolytic capacity increase at critical points. The bromelain from the pulp presented maximum activity in the presence of 0.0658% (0.2 mM) disodium EDTA and of 0.0704% (4.9 mM) sodium benzoate, whereas the bromelain from the peel shows its maximum catalytic capacity in the presence of 1.1203% (30 mM) disodium EDTA and of 0.0825% (5.7 mM) sodium benzoate.

The Dohler design described the well-adjusted behavior of the variables concerning bromelain stabilization.

The variance analysis presented in Table 4 shows that the results of the model adjustment was verified

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Figure 1. Relative proteolytic activity of bromelain extracted from different parts of *Ananas comosus* var. *comosus* AGB 772 under temperature (A) and pH (B) variations.

Figure 2. (A) CD spectra of bromelain extracted from the control, the pulp and the peel recorded in phosphate buffer (pH 6.0) at 20 °C: (—) standard; (---) pulp; (···) peel; (B) thermal stability of bromelain from the peel through CD. The spectra show enzyme unfolding (from 23 to 95 °C) and refolding (from 95 to 23 °C) recorded in phosphate buffer (pH 6.0).

Table 2. Data of the identification of SDS-PAGE band by mass spectrometry.

Name	Accession	Specie	Protein ID	Score	Sequence coverage	MM (kDa)	Peptide sequence
Peel bromelain	gi 2463588 BA-A22546.1	<i>Ananas comosus</i>	FB1035 precursor	165	11	36,414	R.NSWGSSWGEGG-YVR.M K.TGYLVSLSEQEVL-DCAVSYGCK.G K.IKTGYLVSLSEQEVL-DCAVSYGCK.G

by the determination coefficient (0.89 and 0.80), which indicated that 89.0% and 80.0% of the total variation in the response in bromelain extracted from the pulp and from the peel was explained by the adjusted

model. Moreover, the quadratic equation significance was evaluated by *F* test (Table 4). The Fisher-based test indicated that the adjusted equation was statistically significant ($F_{cal} > F_{tab}$) and the lack of

Table 3. Doehlert matrix used in the stabilization of bromelain from the pulp and the peel of *Ananas comosus* var. *comosus* BGA 772.

Assay	Disodium EDTA (%)	Sodium benzoate (%)	Pulp bromelain activity (%)	Peel bromelain activity (%)
1	0.03 (−0.5)	0.1 (+0.866)	97.78	95.79
2	0.08 (+0.5)	0.1 (+0.866)	98.47	103.48
3	0.005 (−1)	0.075 (0)	97.35	104.39
4c	0.055 (0)	0.075 (0)	99.60	105.29
5c	0.055 (0)	0.075 (0)	101.08	106.21
6c	0.055 (0)	0.075 (0)	100.70	104.82
7	0.105 (+1)	0.075 (0)	99.52	104.56
8	0.03 (−0.5)	0.05 (−0.866)	98.34	102.10
9	0.08 (+0.5)	0.05 (−0.866)	98.59	103.66

(c): central point; coded values are presented in parenthesis.

Figure 3. Disodium EDTA surface response versus the sodium benzoate influence on the activity of bromelain extracted from the pulp (A) and the peel (B) of *Ananas comosus* var. *comosus* AGB 772.

adjustment showed good agreement ($F_{cal} < F_{tab}$) between the predicted response model and the experimental values in each analyzed variable.

Fluorescence measurements

The fluorescence analysis was conducted to test the effect of these additives on the conformation of bromelain extracted from the pulp and the peel of *Ananas comosus* var. *comosus* AGB 772.

The bromelain from the pulp and from the peel was purified to minimize the interference of other crude extract constituents in the fluorescence analysis. Figure 4 shows bromelain fluorescence in presence and in absence of additives, and it evidenced that fluorescence remained unchanged.

Bromelain from the pulp and from the peel peaked at approximately 500 nm and it is similar to the control peak. It showed the conformational integrity of bromelain in stabilizers. Therefore, the bromelain tertiary structure was not affected by disodium EDTA (0.055%) and sodium benzoate (0.075%).

Bromelain antiproliferative effect on B16F10 murine melanoma cells

The present study assessed the effect of bromelain on the growth of B16F10 murine melanoma cell cultures, for 24 h (Figure 5). The results showed more than 90% growth-inhibitory effects by all parts tested at both studied concentrations. Bromelain from the leaves was more effective at higher concentration (50 µg/mL), it showed 99.4% inhibition versus 51.7% inhibition at 25 µg/mL.

Discussion

Enzymatic activity, temperature and pH optimum profile of bromelain

Brazil is endowed with the world's greatest genetic diversity of the genus and the country is considered one of the centers of origin and dispersion of this important fruit plant. The country has the largest *ex situ* collection under field conditions, held by the Embrapa Cassava & Fruits (AGB-Pineapple), with approximately 600 accessions. This gene bank houses varieties that have never been studied before. On the other hand, preliminary assays showed the potential that this variety, *Ananas comosus* var. *comosus* – AGB 772 has for prospecting bromelain, justifying the extension of these studies. Diversity in plant genetic

Table 4. Variance analysis of the stabilization bromelain extracted from the pulp and the peel of *A. comosus* var. *comosus* BGA 772.

Variation source	ANOVA									
	Pulp bromelain					Peel bromelain				
	SS	df	MS	Fcal	Ftab	SS	df	MS	Fcal	Ftab
Regression	11.4659	5	2.2932	4.8238	9.01	60.6263	5	12.1253	2.4690	28.24
Residual	1.4262	3	0.4754			14.7332	3	4.9111		
Lack-of-fit	0.2508	1	0.2508	0.4268	18.51	13.7341	1	13.7341	27.4925	98.50
Pure error	1.1754	2	0.5877			0.9991	2	0.4996		
Total SS	12.8920	8				75.3595	8			

SS: sum of squares; df: degree of freedom; MS: mean square; Fcal: calculated *F* value; Ftab: tabulated *F* value.

Figure 4. Fluorescence emission spectra of bromelain from the control (A), the pulp (B) and the peel (C) of *Ananas comosus* var. *comosus* AGB 772; (—) with and (—) without additives.

Figure 5. The antiproliferative effect of bromelain extracted from *Ananas comosus* var. *comosus* AGB 772 on B16F10 melanoma cells after 24 h with 25 µg/mL (A) and 50 µg/mL (B) bromelain extract.

resources (PGR) provides opportunity for plant breeders to develop new and improved cultivars with desirable characteristics, which include farmer-preferred traits (yield potential, etc.) and breeders preferred traits (pest and disease resistance and photosensitivity, etc.) (Govindaraj et al. 2014). The bromelain extracted from both the pulp and the peel has shown activity higher than that of the other studied parts. Given that the pulp is widely used in food industry process, it is possible extracting bromelain from pineapple wastes. Similar result was found by França-Santos et al. (2009), who found high activity

in bromelain from the pulp. The study of Abílio et al. (2009) showed high activity in the peel. However, the studies of Ketnawa et al. (2012) found higher activity in bromelain from the crown in comparison to the activity in bromelain from the peel, core fruit, stem and crown of *Ananas comosus* (Nang Lae and Phu Lae cultv.). The bromelain from the crown has shown higher activity than that of the other analyzed parts (Hebbbar et al. 2008). Cysteine proteases are involved in many aspects of plant's physiology and development. Bromelain's capability to inhibit fungal growth was related to its proteolytic activity (López-García et al. 2012). Studies of Abílio et al. (2009) showed that the specific activity of bromelain contributes to the occurrence of known resistance to *Fusarium* and the different specific activity of each plant part seemed to be related to the role played by bromelain in plant metabolism and its defense against pathogens.

The optimum temperature was above 50 °C in all bromelain extracts. Other authors have reported higher activity in the pulp at 59 °C (Corzo et al. 2012); in the stem, between 50 and 60 °C; in the pulp, between 37 and 70 °C (Bala et al. 2012), at 40 °C (França-Santos et al. 2009) and at 30 °C; and fast activity loss above 60 °C (Khan et al. 2003). The optimum pH was approximately 6, and this is in agreement with results by other authors; for example, Bala et al. (2012) found optimum pH between 6 and 7 in bromelain from the stem, and between 3 and 8, in bromelain from the fruit. Corzo et al. (2012) found optimum pH 7.7 in bromelain from the fruit. França-Santos et al. (2009) found optimum pH 5.0 in bromelain from the fruit, and Khan et al. (2003) found optimum pH 6.0 in bromelain from the fruit. Thus, the results of optimum pH showed that bromelain has maximum activity at pH close to that of human skin pH and this indicates that bromelain is active at physiological temperature of skin. These biochemical properties (pH and temperature optimum) are important to the prospection for the bromelain use on skin, for example, at optimized formulations to melanoma treatment.

CD analysis

The CD spectra of the here in assessed bromelain were similar to those reported by other authors (Arroyo-Reyna et al. 1994; Haq et al. 2002; Soares et al. 2012). They were comparable to the CD pattern from other papain such as cysteine protease (Hernández-Arana & Soriano-García 1988; Sathish et al. 2002), chymopapain (Solis-Mendiola et al. 1989), ficin (Deveraj et al. 2011) and baupain from *Bauhinia*

forficata leaves (Silva-Lucca et al. 2014). These results have suggested that bromelain may have the same folding pattern of other papain family members. Figure 2(B) shows the thermal stability of bromelain extracted from the peel through far-UV CD spectroscopy. The results have shown that bromelain is thermo-instable and that it does not refold after unfolding. The T_a (mean fusion temperature when 50% of the structure is lost) was 77.5 °C. Similar results were reported by Jutamongkon and Charoenrein (2010), who found almost complete bromelain activity loss after incubation at 80 °C for 8 min. A thermo-stable recombinant bromelain was produced; however, it was completely denatured after 60 min at 90 °C (Amid et al. 2011).

Stabilization studies

The enzymatic formulation stabilization adds additives to formulations containing enzymes able to enhance the formulations' biocatalyst activity or stabilization. Enzymes presenting oxidizable amino acid residues such as cysteine proteases (bromelain, papain), in the active site, require special protection in order to prevent inactivation. Different additives and strategies are used to improve the storage stability of formulations containing enzymes. Microbial contamination is a problem for liquid enzymatic compositions. Therefore, the filtering and addition of preservatives are alternatives to prevent microbial contamination. Such a procedure is essential to keep storage stability and to preserve the native structure (Illanes 2008). Sodium benzoate and disodium EDTA are widely used as preservatives in food additives, especially in orally administered medicines. The importance of benzoate and EDTA lies on their antimicrobial and antioxidant actions, which are necessary for the conservation and shelf life of food and medicines. The crude aqueous extract of *A. comosus* has preservatives and other additives in its formulation, and it has been used as oral solution for mucolytic activity.

The present results are in agreement with those found by other authors (Heinicke 1966; Moretto 1992) who have proved the effect of bromelain stabilized through ion benzoate; benzoate keeps bromelain stable for longer periods. Therefore, in the long term, it has great potential to stabilize powders, solutions and pastes containing bromelain (Heinicke 1966). Benzoate also works as antioxidant when it is added during extract preparation; thus, it decreases the normal oxidation of the extracts (Heinicke 1966). On the other hand, EDTA is often used as a bromelain activator, that is why it is used by many researchers in

bromelain-catalyzed enzymatic reactions or even in the bromelain extraction process (Babu et al. 2008; Ketnawa et al. 2009; Xue et al. 2010; Ketnawa et al. 2011; Kumar et al. 2011; Silvestre et al. 2012). Bromelain activity increase was also observed in the presence of higher EDTA (20%) and benzoate (30%) amounts (Moretto 1992). However, the present study was based on usual doses of these additives in formulations developed for oral administration (0.005–0.1% EDTA and 0.05–0.1% sodium benzoate). It is known that the activator mechanism of EDTA is associated with its metal chelating effect, which leads to bromelain activity increase (Zhao et al. 2011).

Fluorescence measurements

Fluorescence analyses are able to show different aspects of the bromelain structure, including structural changes (Johnson 2005). The presence of additives such as disodium EDTA and sodium benzoate can alter the bromelain native structure by changing its catalytic site and by compromising its activity. The present results show that the three-dimensional (3D) structure of bromelain was not affected by the disodium EDTA (0.055%) and sodium benzoate (0.075%) stabilizers. Bromelain fluorescence in the presence of these additives was not reported in the literature. However, the fluorescence intensity of bromelain from the stem decreased, when urea (2 M) or guanidine hydrochloride (6 M) was used (Haq et al. 2002). The fluorescence of bromelain from the stem was monitored under pH variations (Khan et al. 2003).

Proliferation assay

The antineoplastic activity of bromelain has long been known. The pioneers in this field were Taussig and Batkin who made, in 1988, a review addressing this application, among other therapeutic actions for the enzyme. Our results (Figure 6(A)) show bromelain's effectiveness against the proliferation of murine melanoma cells and are in accordance with the literature reports. There are numerous studies about the antiproliferative activity of bromelain in different cancer types *in vitro* and about its antitumor activity *in vivo* (Grabowska et al. 1997; Báez et al. 2007; Bhui et al. 2010, 2012; Amini et al. 2013, 2015, 2016).

Figure 6(A) also shows that the proliferation of murine melanoma cells was inhibited at both concentrations and in all studied bromelain types and the values were statistically equal in all parts. It was possible to see that the highest specificity of bromelain was found in the pulp but the anti-proliferative effect

was not so different between the parts of *Ananas comosus*. Since bromelain is a crude, aqueous extract from the pineapple plant containing sulfhydryl proteolytic enzymes and non-proteolytic constituents (Amini et al. 2016), it is known that the beneficial effects of bromelain involve its multiple constituents, besides its proteolytic fraction, due to multiple factors (Alternative Medicine Review 2010; Maurer & Eschmann 2015). Although proteases are the major constituents, the non-protease components have important role on bromelain activities (Amini et al. 2016). Thus, the non-proteolytic fraction may have a synergic effect, since the specific activity had no significant influence on the proliferative assays. Similar result was published by Grabowska et al. (1997) who indicated that crude bromelain (which had the lowest proteolytic activity), was the most active component, when compared with the other proteases applied. These result suggested an involvement of other non-protease components on bromelain activity. A previous study by Bhui et al. (2012) has shown that bromelain is effective against the growth and proliferation of A375 melanoma cells, depending on the dose and the time (IC₅₀ of 400 mg/mL for 48 h exposure). However, our results show that bromelain from AGB 772 was much more effective, since at a concentration as low as 25 µg/mL it almost completely inhibited the proliferation of B16F10 neoplastic cells. These results are similar to findings previously reported about the *in vitro* inhibition of B16F10 melanoma cells by bromelain and papain (Grabowska et al. 1997).

The present study about the antiproliferative effect of bromelain from *Ananas comosus* var. *comosus* AGB 772 are in line with other reports. Studies by Amini et al. (2013) conducted *in vitro* involved other neoplastic cell lines, such as gastrointestinal tumor cells, and found dose-dependent inhibitory effects of bromelain on cell proliferation; *in vivo*, Báez et al. (2007) showed antitumor effects of bromelain in different animal models following intraperitoneal administration. The effect *in vivo* of a mixture of proteases (trypsin, chymotrypsin and papain) was assessed in rats with B16 melanoma and it showed tumor growth inhibition and relapse (Wald et al. 2001).

The potential of bromelain, on its own or in combination with N-acetylcysteine, to treat the peritoneal dissemination of gastrointestinal mucin-producing malignancies was shown (Amini et al. 2016). Studies *in vitro* and *in vivo* suggest that the bromelain and N-acetylcysteine combination has dual effect on growth and mucin products of mucin-expressing tumor cells (Amini et al. 2015). Bromelain and N-acetylcysteine have inhibitory effects on growth and

proliferation of cancer cells, both *in vitro* and *in vivo*, holding preclinical promise for locoregional treatment of human gastrointestinal carcinoma as an adjunct in chemotherapy (Amini et al. 2015). The mechanisms underlying antiproliferative activity of bromelain include the interference with cell growth and the induction of apoptosis (Bhui et al. 2012). Its effect on malignant growth were described and summarized by Amini et al. (2016). It involves a lot of synergic mechanisms and different aspects of tumor biology: cell survival, growth, proliferation, differentiation, migration, adhesion and invasion are affected by bromelain treatment. They reported bromelain actions as: a chemopreventive effect; able to regulate key cellular pathways responsible for invasive behavior of cancer cells; to induce cell growth arrest or apoptosis; to alter cell surface molecules of adhesion and invasion; it is able to prevent the malignant transformation, angiogenesis and metastasis due to its anti-inflammatory and immunomodulatory activity. Therefore, proteases such as bromelain have proven anticancer activity in different models and types of neoplasia, with a synergic mechanisms. Pineapple is a candidate for being used in the development of new anticancer therapy drugs, either alone or in combination with other cytotoxic drugs.

Conclusions

All *Ananas comosus* var. *comosus* AGB 772 (an unexplored French Guiana variety) parts show bromelain activity. The highest enzymatic activity was found in the pulp and in the peel. All extracts inhibited B16F10 murine melanoma cells proliferation *in vitro*. Further studies could provide scientific information about the development and application of bromelain extracted from residues parts of *Ananas comosus* var. *comosus* in the biotechnological and pharmaceutical fields.

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Disclosure statement

The authors report no conflicts of interest.

References

- Abílio GMF, Holschuh HJ, Bora PS, Oliveira EF. 2009. Extração, atividade da bromelina e análise de alguns

- parâmetros químicos em cultivares de abacaxi. *Rev Brasil Fruticult.* 31:1117–1121.
- Aichele K, Bubel G, Deubel G, Pohlemann M, Oberringer M. 2013. Bromelain down-regulates myofibroblast differentiation in an *in vitro* wound healing assay. *Naunyn-Schmiedeberg's Arch Pharmacol.* 386:853–863.
- Aiyegbusi AI, Duru FI, Anunobi CC, Noronha CC, Okanlawon AO. 2011a. Bromelain in the early phase of healing in acute crush Achilles tendon injury. *Phytother Res.* 25:49–52.
- Aiyegbusi AI, Olabiyi OO, Duru FI, Noronha CC, Okanlawon AO. 2011b. A comparative study of the effects of bromelain and fresh pineapple juice on the early phase of healing in acute crush Achilles tendon injury. *J Med Food.* 14:348–352.
- Alternative Medicine Review. 2010. Bromelain Monograph. *Altern Med Rev.* 15:361–368.
- Amid A, Ismail NA, Yusof F, Salleh HM. 2011. Expression, purification, and characterization of a recombinant stem bromelain from *Ananas comosus*. *Process Biochem.* 46:2232–2239. doi:10.1016/j.procbio.2011.08.018.
- Amini A, Ehteda A, Moghaddam SM, Akhter J, Pillai K, Morris DL. 2013. Cytotoxic effects of bromelain in human gastrointestinal carcinoma cell lines (MKN45, KATO-III, HT29-5F12, and HT29-5M21). *Oncotargets Ther.* 6:403–409.
- Amini A, Masoumi-Moghaddam S, Ehteda A, Liauw W, Morris DL. 2015. Depletion of mucin in mucin-producing human gastrointestinal carcinoma: results from *in vitro* and *in vivo* studies with bromelain and N-acetylcysteine. *Oncotarget.* 6:33329–33344.
- Amini A, Masoumi-Moghaddam S, Morris DL. 2016. Utility of bromelain and N-acetylcysteine in treatment of peritoneal dissemination of gastrointestinal mucin-producing malignancies. Cham, Switzerland: Springer International Publishing.
- Arroyo-Reyna A, Hernández-Arana A, Arreguin-Espinosa R. 1994. Circular dichroism of stem bromelain: a third spectral class within the family of cysteine proteinases. *Biochem J.* 300:107–110.
- Arshad ZI, Amid A, Yusof F, Jaswir I, Ahmad K, Loke SP. 2014. Bromelain: an overview of industrial application and purification strategies. *Appl Microbiol Biotechnol.* 98:7283–7297.
- Babu BR, Rastogi NK, Raghavarao KSMS. 2008. Liquid-liquid extraction of bromelain and polyphenol oxidase using aqueous two-phase system. *Chem Eng Process.* 47:83–89.
- Báez R, Lopes MT, Salas CE, Hernández M. 2007. *In vivo* antitumoral activity of stem pineapple (*Ananas comosus*) bromelain. *Planta Med.* 73:1377–1383.
- Bala M, Ismail NA, Mel M, Jami MS, Salleh HM, Amid A. 2012. Bromelain production: current trends and perspective. *Arch Sci.* 65:369–399.
- Bhui K, Tyagi S, Prakash B, Shukla Y. 2010. Pineapple bromelain induces autophagy, facilitating apoptotic response in mammary carcinoma cells. *Biofactors.* 36:474–482.
- Bhui K, Tyagi S, Srivastava AK, Singh M, Roy O, Singh R, Shukla Y. 2012. Bromelain inhibits nuclear factor kappa-B translocation, driving human epidermoid carcinoma A431 and melanoma A375 cells through G(2)/M arrest to apoptosis. *Mol Carcinog.* 51:231–243.

- Borelli F, Capasso R, Severino B, Fiorino F, Aviello G, Rosa GD, Mazzella M, et al. 2011. Inhibitory effects of bromelain, a cysteine protease derived from pineapple stem (*Ananas comosus*), on intestinal motility in mice. *Neurogastroenterol Motil.* 23:745–e331.
- Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem.* 72:248–254.
- Corzo CA, Waliszewski KN, Chanes JW. 2012. Pineapple fruit bromelain affinity to different protein substrates. *Food Chem.* 133:631–635.
- Dave S, Kaur NJ, Nanduri R, Dkhar HK, Kumar A, Gupta P. 2012. Inhibition of adipogenesis and induction of apoptosis and lipolysis by stem bromelain in 3T3-L1 adipocytes. *PLoS One.* 7:1–12.
- De Oliveira GA, Pereira EG, Dias CV, Souza TL, Ferretti GD, Cordeiro Y, Camillo LR, Cascardo J, Almeida FC, Valente AP, et al. 2012. *Moniliophthora perniciosa* necrosis- and ethylene-inducing protein 2 (MpNep2) as a metastable dimer in solution: structural and functional implications. *PLoS One.* 7:e45620.
- Deveraj KB, Kumar PR, Prakash V. 2011. Comparison of activity and conformational changes of ficin during denaturation by urea and guanidine hydrochloride. *Process Biochem.* 46:458–464.
- Dittz D, Figueiredo C, Lemos FO, Viana CT, Andrade SP, Souza-Fagundes EM, Fujiwara RT, et al. 2015. Antiangiogenesis, loss of cell adhesion and apoptosis are involved in the antitumoral activity of proteases from *V. cundinamarzensis* (*C. candamarcensis*) in murine melanoma B16F1. *Int J Mol Sci.* 16:7027–7044.
- Dutta S, Bhattacharyya D. 2013. Enzymatic, antimicrobial and toxicity studies of the aqueous extract of *Ananas comosus* (pineapple) crown leaf. *J Ethnopharmacol.* 150:451–457.
- Errasti ME, Caffini NO, Pelzer LE, Rotelli AE. 2013. Anti-inflammatory activity of *Bromelia hieronymi*: comparison with bromelain. *Planta Med.* 79:207–213.
- França-Santos A, Alves RS, Leite NS, Fernandes RPM. 2009. Estudos bioquímicos da enzima bromelina do *Ananas comosus* (abacaxi). *Sci Plena.* 5:1–6.
- Govindaraj M, Vetriventhan M, Srinivasan M. 2015. Importance of genetic diversity assessment in crop plants and its recent advances: an overview of its analytical perspectives. *Genet Res Int.* Article ID 431487, doi:10.1155/2015/431487.
- Grabowska E, Eckert K, Fichtner I, Schulze-forster K, Maurer H. 1997. Bromelain proteases suppress growth, invasion and lung metastasis of B16F10 mouse melanoma cells. *Int J Oncol.* 11:243–248.
- Guimarães-Ferreira CA, Rodrigues EG, Mortara RA, Cabral H, Serrano FA, Ribeiro-dos-Santos R, Travassos LR. 2007. Antitumor effects *in vitro* and *in vivo* and mechanisms of protection against melanoma B16F10-Nex2 cells by fastuosain, a cysteine proteinase from *Bromelia fastuosa*. *Neoplasia.* 9:723–733.
- Hale LP, Greer PK, Trinh CT, James CL. 2005. Proteinase activity and stability of natural bromelain preparations. *Int Immunopharmacol.* 5:783–793.
- Haq SK, Rasheedi S, Khan RH. 2002. Characterization of a partially folded intermediate of stem bromelain at low pH. *Eur J Biochem.* 269:47–52.
- Hebbar HU, Sumana B, Raghavarao KSMS. 2008. Use of reverse micellar systems for the extraction and purification of bromelain from pineapple wastes. *Bioresour Technol.* 99:4896–4902.
- Heinicke RM. 1966. Stabilization of bromelain preparations. United States Patent US 3,293,143A.
- Hernández-Arana A, Soriano-García M. 1988. Detection and characterization by circular dichroism of a stable intermediate state formed in the thermal unfolding of papain. *Biochim Biophys Acta.* 954:170–175.
- Hu W, Wang AM, Wu SY, Zhang B, Liu S, Gou YB, Wang JM. 2011. Debriding effect of bromelain on firearm wounds in pigs. *J Trauma.* 71:966–972.
- Illanes A. 2008. Enzyme production. In: *Enzyme biocatalysis*. Brazil: Springer Science and Business Media VB, 69. p. 57–106.
- Johnson AE. 2005. Fluorescence approaches for determining protein conformations, interactions and mechanisms at membranes. *Traffic.* 6:1078–1092.
- Jutamongkon R, Charoenrein S. 2010. Effect of temperature on the stability of fruit bromelain from Smooth Cayenne pineapple. *Kasetsart J (Nat Sci).* 44:943–948.
- Ketnawa S, Chaiwut P, Rawdkuen S. 2011. Extraction of bromelain from pineapple peels. *Food Sci Technol Int.* 17:395–402.
- Ketnawa S, Chaiwut P, Rawdkuen S. 2012. Pineapple wastes: a potential source for bromelain extraction. *Food Bioprod Process.* 90:385–391.
- Ketnawa S, Sai-Ut S, Theppakorn T, Chaiwut P, Rawdkuen S. 2009. Partitioning of bromelain from pineapple peel (*Nang Lae* cultv.) by aqueous two phase system. *Asian J Food Agro-Ind.* 2:457–468.
- Khan RH, Rasheedi S, Haq SK. 2003. Effect of pH, temperature and alcohols on the stability of glycosylated and deglycosylated stem bromelain. *J Biosci.* 28:709–714.
- Kumar S, Hemavathi AB, Hebbar HU. 2011. Affinity based reverse micellar extraction and purification of bromelain from pineapple (*Ananas comosus* L. Merryl) waste. *Process Biochem.* 46:1216–1220.
- López-García B, Hernández M, Segundo BS. 2012. Bromelain, a cysteine protease from pineapple (*Ananas comosus*) stem, is an inhibitor of fungal plant pathogens. *Lett Appl Microbiol.* 55:62–67.
- Lshai-Michaeli R, Eldor A, Vlodavsky L. 1990. Heparanase activity expressed by platelets, neutrophils, and lymphoma cells releases active fibroblast growth factor from extracellular matrix. *Cell Regul.* 1:833–842.
- Maurer HR. 2001. Bromelain: biochemistry, pharmacology and medical use. *Cell Mol Life Sci.* 58:1234–1245.
- Maurer R, Eschmann K. 2015. Use of bromelain for adjuvant therapy during wound healing process. United States Patent US 7,731,958B2.
- Moretto LD. 1992. Effect of temperature and of activators on the extraction process of bromelain [Doctoral thesis]. São Paulo, Brazil: University of São Paulo.
- Murachi T. 1976. Bromelain enzymes. In: Lorand L, editor. *Methods in enzymology*. New York: Academic Press, XLV. p. 475–485.
- Pavan R, Jain S, Shraddha KA. 2012. Properties and therapeutic application of bromelain: a review. *Biotechnol Res Int.* 1–6.

- Sathish HA, Kumar PR, Prakash V. 2002. Effect of urea at lower concentration on the structure of papain-formation of a stable molten globule and its characterization. *Indian J Biochem Biophys.* 39:155–162.
- Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. 2006. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc.* 1:2856–2860.
- Silva-Lucca RA, Andrade SS, Ferreira RS, Sampaio MU, Oliva ML. 2014. Unfolding studies of the cysteine protease baupain, a papain-like enzyme from leaves of *Bauhinia forficata*: effect of pH, guanidine hydrochloride and temperature. *Molecules.* 19:233–246.
- Silvestre MPC, Carreira RL, Silva MR, Corgosinho FC, Monteiro MRP, Morais HA. 2012. Effect of pH and temperature on the activity of enzymatic extracts from pineapple peel. *Food Bioprocess Technol.* 5:1824–1831.
- Soares PAG, Vaz AFM, Correia MTS, Pessoa Jr, Carneiro-da-Cunha MG. 2012. Purification of bromelain from pineapple wastes by ethanol precipitation. *Sep Purif Technol.* 98:389–395.
- Solis-Mendiola S, Zubillaga-Luna R, Rojo-Dominguez A, Hernandez-Arana A. 1989. Structural similarity of chymopapain forms as indicated by circular dichroism. *Biochem J.* 257:183–186.
- Taussig S, Batkin S. 1988. Bromelain, the enzyme complex of pineapple (*Ananas comosus*) and its clinical application. An update. *J Ethnopharmacol.* 22:191–203.
- Wald M, Olejár T, Sebková V, Zadinová M, Boubelík M, Poucková P. 2001. Mixture of trypsin, chymotrypsin and papain reduces formation of metastases and extends survival time of C₅₇Bl₆ mice with syngeneic melanoma B16. *Cancer Chemother Pharmacol.* 47:S16–S22.
- Wang W, Suguitan AL, Jr, Zengel J, Chen Z, Jin H. 2012. Generation of recombinant pandemic H1N1 influenza virus with the HA cleavable by bromelain and identification of the residues influencing HA bromelain cleavage. *Vaccine.* 30:872–878.
- Xue Y, Wu CY, Branford-White CJ, Ning X, Nie HL, Zhu LM. 2010. Chemical modification of stem bromelain with anhydride groups to enhance its stability and catalytic activity. *J Mol Catal B: Enzym.* 63:188–193.
- Zhao LC, Wang Y, Chen JL, Liu X. 2011. Effects of additives on bromelain activity. *Food Sci.* 32:225–229.

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