RESEARCH ARTICLE

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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Antiproliferative effect in vitro; murine melanoma cells; cell culture; enzyme stability; bromelain; response surface methodology

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, anticoagulant, wound healing, debriding, digestive, expectorant, anti-obesity, antimicrobial, immunomodulatory, antibacterial and antifungal properties, as well as antineoplastic activity (Grabowska et al. 2015). This solution is prepared with bromelain from Ananas comosus var. comosus, which 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 Caseinase 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.

### Materials and methods

#### Chemicals and plant materials

The pineapple (*Ananas comosus* var. *comosus* AGB 772) plant was provided by Active Germplasm Bank.
**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 aforementioned, 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 NCBInr 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 spectroradiometer (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 x 10⁵ 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 24h. 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% was 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>
<thead>
<tr>
<th>Samples</th>
<th>Proteolytic activity (U/mL)</th>
<th>Specific activity (U/mg)</th>
<th>Protein content (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stem</td>
<td>27.99 (1.20)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>432.98 (18.62)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.0647 (0.0022)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Crown</td>
<td>40.15 (2.76)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1050.57 (72.31)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0382 (0.0009)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peel</td>
<td>57.11 (1.98)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1365.77 (47.23)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.0418 (0.0025)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pulp</td>
<td>62.50 (0.07)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1519.85 (1.60)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.0411 (0.0017)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Leaves</td>
<td>37.10 (1.66)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>859.44 (38.47)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.0432 (0.0020)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>F(397, 197.16, p < .0001).

<sup>b</sup>F(17, 297.70, p < .0001).

<sup>c</sup>F(403, 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.

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

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

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
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

![Figure 2](image)

**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.

<table>
<thead>
<tr>
<th>Name</th>
<th>Accession</th>
<th>Specie</th>
<th>Protein ID</th>
<th>Score</th>
<th>Sequence coverage</th>
<th>MM (kDa)</th>
<th>Peptide sequence</th>
</tr>
</thead>
</table>
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%).

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

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

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

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
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 Abilio 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 Lac and Phu Lao cultivars). The bromelain from the crown has shown higher activity than that of the other analyzed parts (Hebbar 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 Abilio 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 (Fraça-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. Fraça-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 prospect 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 Ta (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 neo-proliferative 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**


