Separation and Purification Technology 98 (2012) 389–395

Contents lists available at SciVerse ScienceDirect

Separation and Purification Technology

journal homepage: www.elsevier.com/locate/seppur

Purification of bromelain from pineapple wastes by ethanol precipitation

Paulo A.G. Soares [a,b], Antônio F.M. Vaz [a], Maria T.S. Correia [a], Adalberto Pessoa Jr. [b], Maria G. Carneiro-da-Cunha [a,*]

[a] Departamento de Bioquímica, Universidade Federal de Pernambuco (UFPE), Av. Prof. Moraes Rego, s/n, Cidade Universitária, CEP 50670-420, Recife, PE, Brazil
[b] Faculdade de Ciências Farmacêuticas, Universidade de São Paulo (USP), Av. Lineu Prestes, 580, Butantã, CEP 05508-000, São Paulo, SP, Brazil

ARTICLE INFO

Article history:
Received 3 April 2012
Received in revised form 26 June 2012
Accepted 29 June 2012
Available online 7 July 2012

Keywords:
Bromelain
Ethanol precipitation
Enzyme purification
Downstream processing

ABSTRACT

Bromelain is an aqueous extract of pineapple that contains a complex mixture of proteases and non-protease components. These enzymes perform an important role in proteolytic modulation of the cellular matrix in numerous physiologic processes, including anti-inflammatory, anti-thrombotic and fibrinolytic functions. Due to the scale of global production of pineapple (*Ananas comosus* L.), and the high percentage of waste generated in their cultivation and processing, several studies have been conducted on the recovery of bromelain. The aim of this study was to purify bromelain from pineapple wastes using an easy-to-scale-up process of precipitation by ethanol. The results showed that bromelain was recovered by using ethanol at concentrations of 30% and 70%, in which a purification factor of 2.28 fold was achieved, and yielded more than 98% of the total enzymatic activity. This enzyme proved to be susceptible to denaturation after the lyophilization process. However, by using 10% (w/v) glucose as a cryoprotector, it was possible to preserve 90% of the original enzymatic activity. The efficiency of the purification process was confirmed by SDS–PAGE, and native-PAGE electrophoresis, fluorimetry, circular dichroism and FTIR analyzes, showing that this method could be used to obtain highly purified and structurally stable bromelain.
© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Many techniques have been used for the isolation and purification of enzymes, including obsolete ones such as precipitation, solvent extraction and filtration, which usually have high concentration power and low purification. On the other hand, modern techniques like affinity chromatography, ionic exchange, gel-filtration, aqueous two phase extraction and extraction by using reversed micelle, recover and purify biomolecules, often to the level of homogeneity. In many biotechnological industries (food, medical and pharmaceutical) the selective separation of an enzyme from fermentation broths, animal or vegetable sources has been a primary research interest for downstream processing operations [1]. It is difficult and expensive to selectively recover a targeted enzyme from a crude extract due to the low protein concentration among various contaminants and the similarity of the physical properties between proteins present in the same solution. Furthermore, of the steps involving isolation and purification of an enzyme, taking into account both economical and technical aspects, the purification steps correspond to 70–90% of the total production costs [2,3].

The effectiveness of each step in a purification process does not necessarily include the application of a unique operation. For example, after protein precipitation by ammonium sulfate it is necessary to perform a dialysis to adjust the ionic strength to levels that allow for ionic exchange chromatography. On the other hand, there are products such as organic acids and some industrial enzymes whose application does not require a high degree of purity, in which case chromatographic operations are not necessary. Nevertheless, reducing the number of steps is of fundamental importance to the technological and economical viability of a purification process [4,5].

Separation of biomolecules by precipitation from aqueous broth is the most traditional method for the recovery and partial purification of enzymes. It is an easy technique with simple equipment requirements, low energy needs, easy scale-up and the possibility of using a large number of precipitants, including some inexpensive ones, such as ethanol, which is widely produced in Brazil and worldwide [4]. Organic solvents that do not denature biological products, like enzymes, can also be used, and the precipitate formed is often more stable than the soluble material. Interactions between the solvent and internal hydrophobic areas may cause an irreversible denaturation of the enzyme that disrupts the normal secondary forms (α-helix and β-sheets), and uncoils it into a random shape [6]. This can be minimized by reducing the temperature to values around zero or below, because at low temperature the flexibility of the biomolecule is less, reducing the penetration capability of the solvent and any irreversible denaturation of enzymes, minimizing the loss of activity [4]. Ethanol precipitation is a promising technique that can be applied to the purification of different enzymes [6–9].

* Corresponding author. Tel.: +55 81 21268547; fax: +55 81 21268576.
E-mail address: mgcc@ufpe.br (M.G. Carneiro-da-Cunha).

1383-5866/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.seppur.2012.06.042

Moreover, ethanol can be recycled in the final process by a simple distillation, reducing environmental impacts through the release of effluents, such as precipitation with ammonium sulfate or acids.

Cysteine proteases comprise a family of enzymes widely distributed in nature and several have been isolated from a number of plant sources. One of them is bromelain, a mixture of cysteine proteases extracted from the pineapple plant (*Ananas comosus*), in particular, from the stem [10]. This proteolytic enzyme is used as a phytomedical compound and demonstrates, *in vitro* and *in vivo*, anti-inflammatory, antiedemic [11,12], anti-thrombotic [13], anti-cancer and anti-metastatic properties [14,15], in addition to strong immunogenicity [16] and wound healing in burns [17]. Bromelain proteases are usually unstable and sensitive under stress conditions in the presence of elevated temperature, organic solvents and chemicals, which may result in a decrease in enzymatic activity of this health-promoting enzyme, limiting its pharmacological, industrial and biotechnological applications [18,19].

The primary structure of stem bromelain is constituted by a single polypeptide chain with 212 amino acids folded into two structure domains stabilized by disulphide bridges and numerous hydrogen bonds. The active site is located on the surface molecules between domains, with two catalytic residues, Cys25 and His159, for hydrolysis of cleaved bonds and substrate specificity [20,21]. The first N-terminal domain of stem bromelain contains mainly β-sheets while the C-terminal domain is composed of α-helices, which allows classifying it within the α + β protein class such as other cystein proteases like papain, actinidin and chymopapain [21–24].

Ethanol can alter the secondary, tertiary or quaternary structure of the enzyme, distorting the binding sites of amino acid residues arranged along the catalytic site, yet there are few studies that have been made to confirm if the purification process using this solvent was efficient without modifying the molecular structure of the enzymes. The aim of this study was to purify bromelain from pineapple wastes by ethanol precipitation, and evaluate the efficiency of the process by SDS–PAGE, native-PAGE electrophoresis, fluorimetry, circular dichroism and FTIR analyzes.

2. Materials and methods

2.1. Chemicals and plant materials

Mature pineapples (cv. Perola) were purchased at a local retail outlet (Recife, Brazil). Casein, L-tyrosine and purified commercial bromelain were purchased from Sigma (St. Louis, USA). The concentrated reagent of Bradford for protein assay was purchased from BioAgency (São Paulo, Brazil). All the buffers and reagents were of analytical grade.

2.2. Preparation of crude extract from pineapple stem and bark

The stem and bark of ripe pineapple fruit were separated from the fleshy fruit. The stem and bark portions were then cut into small pieces and crushed in an industrial blender (Siemsen TA-04, Brazil) with deionised water (1:1 w/w) to get approximately 500 ml of juice, which was filtered through a Vual tissue to remove the fibrous material. The filtered juice was centrifuged (Jouan BR4i, France) at 15,000*g* for 15 min at 4 °C to remove insoluble materials. The clear supernatant obtained, called crude extract, was stored in aliquots (2 mL) at −18 °C, and used whenever required.

2.3. Evaluation of the temperature variation over time when ethanol is added to the aqueous medium

Two tests were conducted in test tubes with deionized water to observe the change in temperature of the aqueous medium as a function of contact time with absolute ethanol. The tests were conducted in a cooled water bath (PolyScience, USA) at 0 °C containing a 40% (w/v) ethylene glycol solution. The temperature was measured by a precision digital thermometer (Omega 5831, USA). The first test was conducted by a single application of 9.0 mL of absolute ethanol in 1.0 mL of deionized water to a final ethanol concentration of 90%. In the second test, the addition of absolute ethanol in 1.0 mL of deionized water was performed by three consecutive applications to obtain ethanol concentrations of 20% ($t = 0$ min), 50% ($t = 1$ min) and finally 90% ($t = 2$ min). The temperature variation with time was monitored every 15 s, and the total elapsed time for both tests was 15 min.

2.4. Bromelain purification from crude extract by ethanol precipitation

The precipitation of bromelain from the crude extract was performed in a single step using different concentrations of ethanol (20–90%). Test tubes containing 1.0 mL of crude extract were cooled to 0 °C using a refrigerated water bath (PolyScience, USA). The volumes of absolute ethanol at 0 °C were added to the tubes in accordance with the temperature control tests as a function of contact time with the solvent (15 min). After precipitation, the sample was centrifuged at 2000*g* for 20 min at 4 °C, and the precipitate was resolubilized in 1.0 mL of phosphate buffer (0.03 M, pH 7.0) and thus called purified bromelain. All precipitations were performed in triplicate.

2.5. Effect of the cryoprotectors on enzymatic activity of bromelain

The purified bromelain was solubilized in different cryoprotectants (pH 7.0 phosphate buffer as control; 10% w/v of glucose in phosphate buffer of pH 7.0; 10% w/v of glycerol in phosphate buffer of pH 7.0; 10% w/v of PEG 4000 in phosphate buffer of pH 7.0; 10% w/v of sucrose in phosphate buffer of pH 7.0), in order to evaluate the product stability during the lyophilization process. After this process, the purified bromelain was resolubilized in phosphate buffer of pH 7.0, and the enzymatic activity was determined before and after the process.

2.6. Measurements of protein content and enzymatic activity

Protein content was measured spectrophotometrically according to Bradford [25] and bovine serum albumin was used as a standard. All measurements were performed in triplicate. The enzyme activity was estimated according to modifications of the method described by Kunitz [26] and Walter [27], as follows: 2% casein (w/v) in 0.1 M phosphate buffer (pH 7.5) was used as a substrate. Aliquots of 50 μL of the samples were added to a centrifuge tube containing 0.625 mL of buffered solution of casein. The mixture was maintained for 10 min in a water bath at 37 °C. Subsequently, 1.25 mL of a solution of trichloroacetic acid (TCA) was added and after 10 min at room temperature (25 °C), the mixture was centrifuged at 4000*g* for 20 min. The absorbance of the supernatant was determined at 280 nm using a UV/visible spectrophotometer (Beckman DU 640, USA).

One unit (U) of enzyme was defined as the amount of bromelain necessary to produce 1 μmol of tyrosine in 1 min at 37 °C and expressed as U/ml. The specific activity (S_A) was determined by the ratio of enzyme activity (U/ml) and protein concentration (mg/ml) and expressed as U/mg.

2.7. Determination of the carbohydrate content

The determination of total sugars was carried out using the phenol–sulfuric acid method described by Dubois et al. [28]. The absorbance at 490 nm of a colored aromatic complex formed be-

tween phenol and the carbohydrate content of the samples was determined using a UV/visible spectrophotometer (Beckman DU 640, USA) by comparison with a glucose calibration curve.

2.8. SDS–PAGE electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was conducted by using 14% (w/v) polyacrylamide according to Laemmli [29]. Polypeptide bands of crude extract (10 μg), commercial bromelain (10 μg), purified bromelain (5 μg) and standards (5 μg) from BenchMark™ Protein Ladders from Life Technologies (California, USA) were stained with silver using a silver staining kit purchased from Bio-Rad laboratories Inc. (Sao Paulo, Brazil).

2.9. Native-PAGE electrophoresis

A native-PAGE with activity staining was conducted, using the modification of the method described by Garcia-Carreno et al. [30] in order to show the presence of active protease in purified, cryoprotected bromelain. During activity staining, the gel was removed to soak in 2% casein (w/v) in 0.1 M phosphate buffer (pH 7.5) for 30 min at 4 °C after electrophoresis. Then the temperature was raised to 37 °C and gel was incubated for 90 min to digest protein by the active fraction. After incubation, the clear zones on the dark blue background by Coomassie blue staining indicate active protease.

2.10. Fluorescence measurement

Intrinsic fluorescence assay were performed using a spectrofluorometer (Jasco FP-6300, Japan). All the samples (commercial bromelain and purified, cryoprotected bromelain) were in the range of 0.5 mg/mL with a 1 cm path length. Excitation and emission slit widths were 5 nm. An excitation wavelength of 280 nm was used and emission spectra were recorded in the range from 300 to 400 nm.

2.11. Circular dichroism (CD) measurements

CD measurements were carried out on a spectropolarimeter (JASCO J-815, Japan). The protein concentrations were: commercial bromelain (2.5 μM) and purified, cryoprotected bromelain (4.2 μM) in phosphate buffer of pH 7.0 at 25 °C. CD spectra were measured in the far-UV range (190–250 nm) in a 10 mm pathlength quartz cuvette. The data were averaged for 5 scans performed at a speed of 50 nm/min, and collected in 0.5 nm steps. The baselines (buffer alone) were subtracted from the protein spectra. Results were expressed as mean residue ellipticity, $[\theta]$, defined as $[\theta] = \theta_{obs}/(10 \cdot C \cdot l \cdot n)$, where θ_{obs} is the CD in millidegrees, C is the protein concentration (M), l is the path-length of the cuvette (cm) and n is the number of amino acid residues assuming a mean number of 212 residues.

2.12. Fourier transform infrared (FTIR) spectroscopy

Fourier transform infrared (FTIR) spectra were measured using a spectrometer (Bruker FT-IR Vertex 70, USA) and analyzed using PE-GRAMS/32 1600 software, as described by Liao et al. [31]. Briefly, 0.5 mg of the dry commercial bromelain and purified, cryoprotected bromelain were mixed with 300 mg ground potassium bromide and compressed to get a pellet. The spectra were smoothed with a 15-point Savitsky–Golay function to remove any possible white-noise. The baseline of the spectrum in the amide I region was leveled and zeroed, then the spectrum of the sample was normalized for area in the region and the intensity of α-helical and β-sheet bands was recorded.

3. Results and discussion

3.1. Purification of bromelain from crude extract by ethanol precipitation

As it is widely known, the temperature has an important effect on the extraction process of biomolecules, mainly when ethanol is used as solvent. To avoid the denaturation of bromelain by the ethanol, a preliminary study was conducted to evaluate the temperature variation over time when ethanol was being added to the system. The temperature variation in the aqueous medium as a function of contact time with ethanol is shown in Fig. 1. It can be seen in Fig. 1A that when performing a single application of 90% (v/v) of ethanol, the highest temperature was 5.2 °C. Within about 2 min after the addition of ethanol the temperature was 1.0 °C, and suitable for precipitation without denaturation of the bromelain. According Kilikian and Pessoa [4], the addition of ethanol in a single application can lead to denaturation of enzymes by forming "pockets" of ethanol in the solution, even if the temperature is below the critical temperature (>10.0 °C). For this reason, the addition of ethanol was performed with three consecutive applications over 2 min.

The results presented in Fig. 1B show that with the addition of 20% (v/v) ethanol (0 min), the highest temperature recorded was 4.8 °C. After 1.0 min, the temperature decreased to 2.2 °C and when the ethanol concentration was completed to 50% (v/v), the highest temperature reached 3.7 °C, a reduction of 21.0% of the temperature after the addition of the first aliquot of ethanol. At the end of 2.0 min, the temperature decreased to 1.7 °C and when the ethanol concentration was completed to 90% (v/v), the temperature continued decreasing until 0.1 °C in about 7.0 min, when it stabilized.

Considering the results here obtained, for the precipitation of bromelain, the first application (0 min) was set at 0.25 mL (20% v/v ethanol), at concentrations of ethanol between 20% and 50% (v/v), the volume was completed to the desired concentration in the 1st min, and for concentrations above 50% (v/v), the volume of ethanol was completed in the 2nd min.

The results of enzyme activity present in the supernatant and fractions precipitated by ethanol are shown in Fig. 1C. The 100% recovery of proteins with a yield of ⩾99.1% in enzymatic activity was obtained from the tests of precipitation that showed volumes ⩾60% (v/v) of ethanol. It was observed that at 30% (v/v) ethanol, the bromelain did not yet precipitate, however, under these conditions, approximately 26.0% of contaminating proteins were precipitated, and with 70% (v/v) ethanol, all bromelain present in the crude extract was precipitated with an activity yield of 99.7% (Table 1).

In an attempt to improve the extraction process of bromelain, a precipitation test was then performed in two steps, the fraction of F30–70% (v/v). The results showed that it was possible to extract all the bromelain (100.0%) allowing an increase in purification factor of 1.18–2.28 as well as an increase of 2–3 times in the initial specific activity (see Table 1), which is quite considerable for a technique with a low purification power, as in this precipitation. It was also observed that there was a large reduction of about 99.4% in the level of carbohydrate content (14.43–0.08 mg/mL), one of the main contaminants in the crude extract of pineapple, corresponding to about 35–40% of total impurities [32]. Increasing by a hundred times the scale purification of the 30–70% (v/v) fraction of bromelain precipitated with ethanol, it was possible to recover 18.33 mg of bromelain with an activity yield of 99.2% of its initial activity.

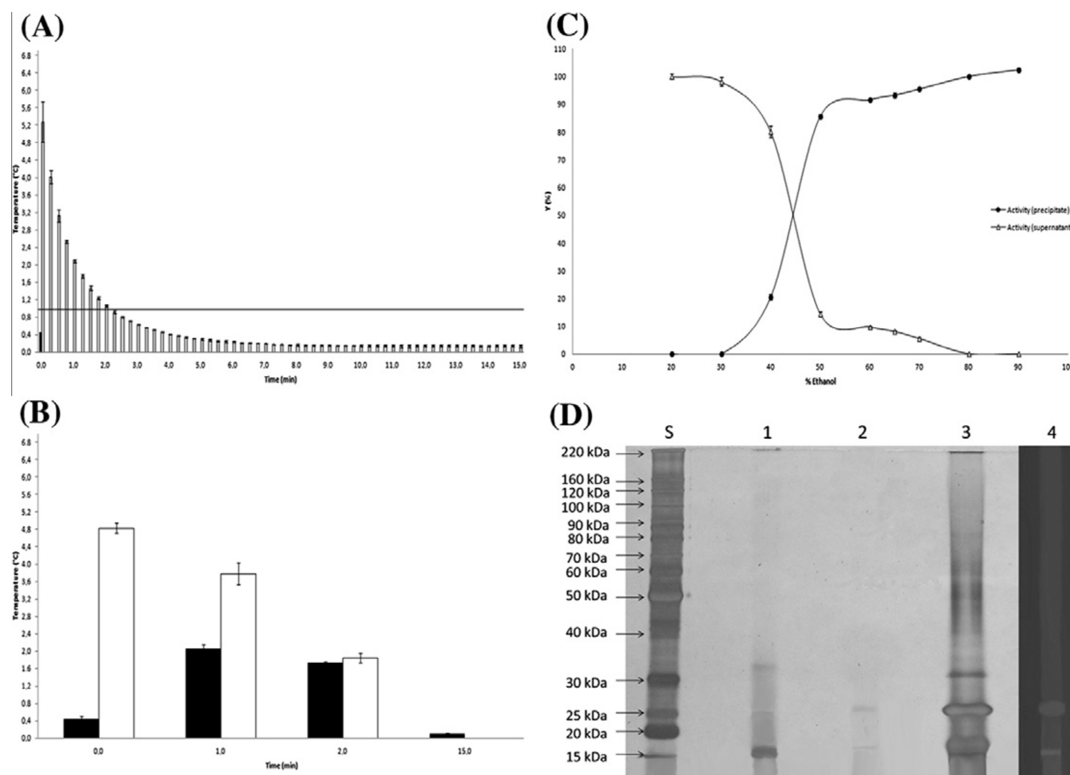

Fig. 1. Effect of 90% (v/v) ethanol on the temperature of aqueous medium (initial temperature = 0.4 °C) in only one stage (A) and in three stages (B): Temperature before (■) and after (□) adding ethanol. Effect of ethanol concentration on bromelain activity (C). SDS/PAGE analysis and native-PAGE of bromelain using a 14% (w/v) polyacrylamide gel: lane S, molecular mass standards (in kDa); lane 1, commercial bromelain (10 μg); lane 2, purified bromelain (5 μg); lane 3, crude extract (10 μg); lane 4, native-PAGE of the purified, cryoprotected bromelain (5 μg) (D).

Table 1
Effect of ethanol concentration on bromelain precipitation.

Samples	PC (mg/mL)	EA (U/mL)	SA (U/mg)	CC (mg/mL)	*Y* (%)	PF
CE	0.23 ± 0.01	2.86 ± 0.15	12.43 ± 0.21	14.23 ± 0.61	–	–
F30%	0.03 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.21 ± 0.02	0.00 ± 0.00	0.00 ± 0.00
F70%	0.20 ± 0.01	2.77 ± 0.07	13.85 ± 0.09	0.31 ± 0.10	96.85 ± 2.56	1.11 ± 0.02
F30–70%	0.10 ± 0.02	2.82 ± 0.04	28.20 ± 0.15	0.08 ± 0.01	98.60 ± 1.49	2.27 ± 0.07

CE – crude extract; PC – protein content (mg/mL); EA – enzymatic activity (U/mL); SA – specific activity (U/mg); CC – carbohydrate content (mg/mL); *Y* (%) – activity yield (%); PF – purification factor.

Devakate et al. [5] achieved a bromelain recovery of about 80.0% in the 34.0% of total protein content extracted with fractions collected at 40–60% and 60–80% (w/v) ammonium sulfate saturation levels. Other alternative methods with low power purification have also been used in the extraction of bromelain and showed satisfactory results, as in the work done by Rabelo et al. [33], who recovered 79.5% of the bromelain present in the flesh and stem of the pineapple using an aqueous two-phase system containing block copolymers of polyoxide ethylene and propylene, which obtained a purification factor of 1.25, and Hebbar et al. [34] who achieved a recovery of 106.0% in the extraction of bromelain from pineapple wastes, with a purification factor of 5.2 using a liquid–liquid extraction system by reverse micelles.

The use of the ethanol precipitation method for enzyme extraction has also been applied successfully as in the work reported by Golunski et al. [6], who achieved an increase in purification factor (2.0 fold) similar to that obtained in this work, for the precipitation of inulinases derived from Kluyveromyces marxianus with 55% (v/v) ethanol with an activity yield of 86.1%, and the work by Cortez and Pessoa Jr. [9], who obtained a recovery of approximately 100.0% of the xylanase originating from Penicillium janthinellum by precipitation with 80% (v/v) ethanol.

The SDS–PAGE analysis (Fig. 4D) revealed the efficiency of the protocol used to purify bromelain from pineapple wastes by ethanol precipitation. Two polypeptide bands of 27.1 and 14.9 kDa were shown, although some authors have reported that up to six enzyme forms can be obtained from the fractionation of stem bromelain [35]. Similar results were reported by Hebbar et al. [34], Takahashi et al. [36] and Wharton [37], which revealed a molecular weight of 26 kDa, 28 kDa and 28.4 kDa for polypeptide bands of bromelain, respectively.

3.2. The effect of cryoprotectors on the enzymatic activity of bromelain

The bromelain fraction precipitated by 30–70% (v/v) ethanol was lyophilized in order to maintain the enzyme stability in the long-term. It was observed that after the lyophilization process, a loss of 48.6% of the initial enzyme activity occurred (Fig. 2). During lyophilization, the water around a protein is extracted into a matrix of ice crystals and the presumed hydration layer would be

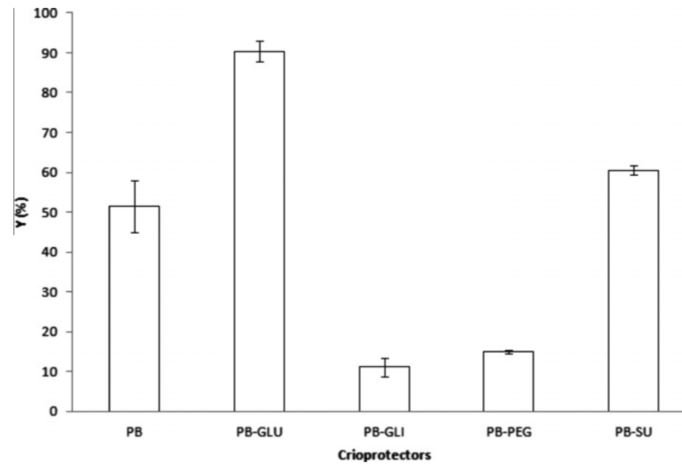

Fig. 2. The effect of the use of cryoprotectors on the enzymatic activity of bromelain purified by ethanol precipitation after lyophilization. PB – phosphate buffer pH of 7.0, PB-GLU – 10% (w/v) glucose in phosphate buffer of pH 7.0; PB-GLY – 10% (w/v) glycerol in phosphate buffer of pH 7.0, PB-PEG – 10% (w/v) PEG 4000 in phosphate buffer of pH 7.0; PB-SU – 10% (w/v) sucrose in phosphate buffer of pH 7.0.

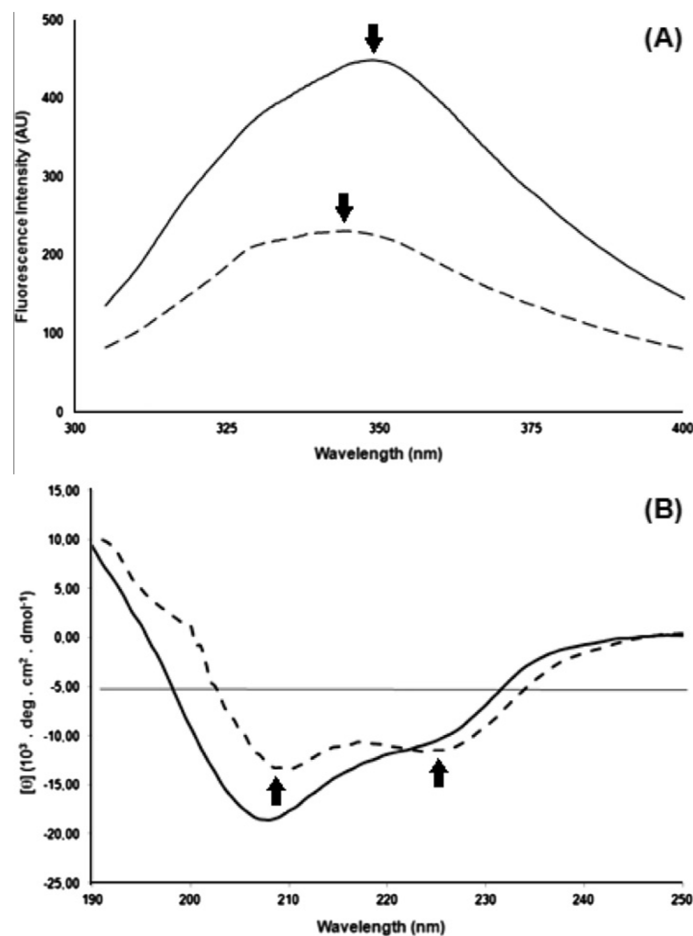

Fig. 3. Fluorescence emission spectra (A) and CD spectra (B) of bromelain recorded in phosphate buffer (pH 7.0) at 25 °C: (—) commercial bromelain; (- - -) purified, cryoprotected bromelain. The arrows represent the signals that were more intense in both fluorescence and CD spectra.

interrupted. So the hydration, under the same environment, would cease to exist in solution and during lyophilization, the passage of the water from solid to gas states could denature some proteins sensitive to the process, especially enzymes [38].

To compensate for the loss of enzymatic activity of bromelain after the lyophilization process, some cryoprotectors were used in order to decrease the tension force generated by the ice crystals on the bromelain. The best cryoprotector for bromelain was observed for 10% (w/v) glucose in a phosphate buffer of pH 7.0, which allowed for a 90.3% recovery of initial activity. The efficiency of the process was confirmed in the native-PAGE shown in Fig. 1D, in which the presence of white bands is related to the maintenance of activity after lyophilization. It was also possible to recover about 61.0% of the enzymatic activity of bromelain in the presence of 10%

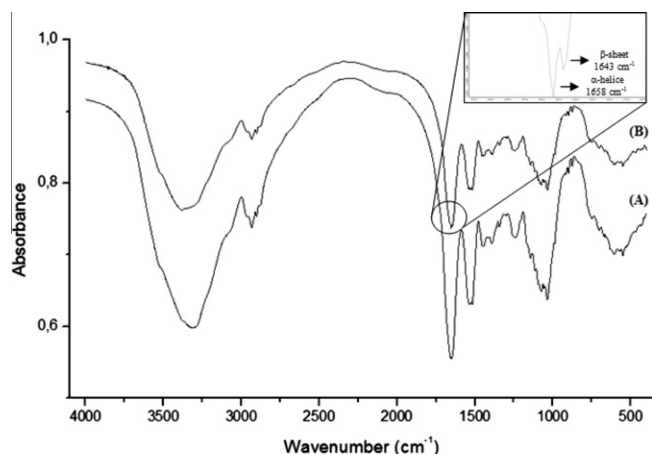

Fig. 4. FTIR spectra of commercial bromelain (A) and purified, cryoprotected bromelain (B); insert shows the representative bands of the secondary structures of α-helical and β-sheet for purified, cryoprotected bromelain.

sucrose (w/v) in a phosphate buffer of pH 7.0. Sugars, mainly monosaccharides and some disaccharides, are considered good cryoprotectors because they act by reducing the water that can be frozen on the surface of protein molecules, helping to reduce injury caused by water crystallization [39].

In the presence of glycerol and PEG 4000, the loss of enzyme activity was greater than 80.0% for both cases, indicating that they were not effective at cryopreserving bromelain. Several investigators have proposed that PEGs function as chaperones, which are molecules that favor the folding of polypeptide chains into their native structures. It has been assumed that this was only possible to perform when protein molecules were in direct contact with the PEG molecules [38,40]. In this specific case, bromelain could not be interacting with PEG-4000 during the lyophilization process, which explains the loss in enzymatic activity. In a solution containing glycerol, when temperatures reach below 0 °C, an increase in viscosity occurs that allows more water to remain unfrozen than in a solution without glycerol [41]. As the enzyme will not freeze, it will remain active to exert its catalytic properties, which explains, once again, the loss of enzyme activity during the lyophilization process.

3.3. Fluorescence and CD measurements

Ethanol can alter the secondary, tertiary or quaternary structure of enzymes, distorting the binding sites of amino acid residues arranged along their catalytic site. The fluorescence of the purified, cryoprotected bromelain remained unaltered after freeze-drying (Fig. 3A). Bromelain contains five tryptophan residues [42] and extensive sequence homology with papain, which suggests that three of the residues are buried in the hydrophobic core whereas two may be located near the surface of the molecule [43,44]. The maximum peak of purified, cryoprotected bromelain at pH 7.0 was recorded at 345 nm, similar to commercial bromelain (349 nm), showing the integrity of the hydrophobic centers of the bromelain after ethanol precipitation.

The same results have been reported by Gupta et al. [45] in their studies for the interference caused to the tertiary structure of bromelain by methanol. Thus, the tertiary structure of the purified, cryoprotected bromelain was not affected by the precipitation process, as the maintenance of enzymatic activity was confirmed by native-PAGE electrophoresis (Fig. 1D).

The CD spectra of commercial bromelain and purified, cryoprotected bromelain are shown in Fig. 3B. In the far-UV region, both curves are practically coincident, suggesting that there is no interference in secondary structures during the precipitation process. The CD spectra of bromelain obtained in this work are comparable with those reported elsewhere [43,46]. In the aromatic absorption region (250–320 nm), data not shown, CD curves of the bromelains are very similar to the spectrum reported earlier by Arroyo-Reyna et al. [47]. On the other hand, in the far-UV region those authors observed considerably weaker bands than those shown in this work.

In general, the spectral characteristics displayed by commercial bromelain and purified, cryoprotected bromelain are typical of α + β proteins, the CD signal of which is more intense at 210 nm than at 223 nm, as was observed in the case of different fruit proteinases such as papain and proteinase Q [48,49]. Thus it is probable that bromelain forms may have the same folding pattern shown in other members of the papain family, namely a bilobal structure with all α-helical and β-sheet domains [50]. In spite of the aforementioned similarities, fluorescence and CD curves for purified, cryoprotected bromelain show one discreet dislocation to the left at 200 nm when compared to commercial bromelain. This discrepancy could be caused by the two N-acetylglucosamine residues present in the carbohydrate moiety linked to the polypeptide chain of bromelain that clearly appears in other fruit proteinases such as papain and proteinase Q [49,51].

3.4. FTIR analyses

Infrared spectroscopy is one of the oldest and well established experimental techniques for the analysis of secondary structure of polypeptides and proteins. FTIR spectra for commercial bromelain and purified, cryoprotected bromelain are shown in Fig. 4. There are no differences in the spectra. The spectrum at 3338–3380 cm^{-1} shows the presence of NH— stretching vibrations. The characteristic C—N stretching vibration frequencies are assigned to observed IR bands at 1517–1587, 1255–1290 and 1179–1149 cm^{-1}. The band at 1640–1700 cm^{-1} shows the presence of C═O stretching groups (amide I region at 1600–1690 cm^{-1}). We also observed the maintenance of secondary structures of α-helical and β-sheet for purified bromelain by ethanol precipitation by the presence of bands at 1658 and 1643 cm^{-1}, respectively.

These results are in agreement with Byler and Susi [52], Dong et al. [53] and Kong and Yu [54], who reported bands of secondary structure of some proteins by deconvolved FTIR spectra. Devakate et al. [5] extracted bromelain using ammonium sulfate and purified it using ion exchange chromatography. The freeze-dried bromelain obtained by these authors shows similar FTIR spectra as those shown in this work.

4. Conclusion

Bromelain was purified by ethanol precipitation with success and cryopreserved using glucose after the lyophilization process. Moreover, ethanol precipitation produces a high purification factor using only a two-step precipitation process (F30–70%). The maintenance of tertiary and secondary structure and enzymatic activity was confirmed by SDS–PAGE and native-PAGE electrophoresis, spectrofluorimetry, circular dichroism and FTIR analysis. Based on process time, low ionic strength (which is desirable for further purification steps) and local ethanol prices, as Brazil is one of the largest ethanol producers, this technique could be useful to easily obtain bromelain.

Acknowledgments

Author Paulo A.G. Soares was a recipient of a scholarship from Conselho Nacional de Desenvolvimento Científico e Tecnológico

(CNPq). We are grateful to the Centro de Tecnologias Estratégicas do Nordeste (CETENE) of the Ministério da Ciência e Tecnologia for technical assistance and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/PROCAD/NF/1415/2007) for financial support.

References

[1] M.A. Desai, Downstream Processing of Proteins: Methods and Protocols, first ed., Humana Press, New Jersey, 2000.
[2] B. Hans-Jörg, P. Stephan, Industrial Scale Natural Products Extraction, first ed., Wiley-VCH, Weinheim, 2011.
[3] R. Hutti-Kaul, B. Mattiasson, Isolation and Purification of Proteins, first ed., Marcel Dekker, New York, 2003.
[4] A. Pessoa Jr., B.V. Kilikian, Purificação de Produtos Biotecnológicos, first ed., Manole, São Paulo, 2005.
[5] R.V. Devakate, V.V. Patil, S.S. Waje, B.N. Thorat, Purification and drying of bromelain, Sep. Purif. Technol. 64 (2009) 259–264.
[6] S. Golunski, V. Astolfi, N. Carniel, D. Oliveira, M. Di Luccio, M.A. Mazutti, H. Treichel, Ethanol precipitation and ultrafiltration of inulinases from Kluyveromyces marxianus, Sep. Purif. Technol. 78 (2011) 261–265.
[7] L. Cui, G. Du, D. Zhang, H. Liu, J. Chen, Purification and characterization of transglutaminase from a newly isolated *Streptomyces hygroscopicus*, Food Chem. 105 (2007) 612–618.
[8] P.K. Gill, R.K. Manhas, P. Singh, Purification and properties of a heat-stable exoinulinase isoform from *Aspergillus fumigates*, Bioresour. Technol. 97 (2006) 894–902.
[9] E.V. Cortez, A. Pessoa Jr., Xylanase and β-xylosidase separation by fractional precipitation, Process Biochem. 35 (1999) 277–283.
[10] R.M. Heinicke, W.A. Gortner, Stem bromelain – a new protease preparation from pineapple plants, Econ. Bot. 11 (1957) 225–234.
[11] L.P. Hale, P.K. Greer, C.T. Trinh, M.R. Gottfried, Treatment with oral bromelain decreases colonic inflammation in the IL-10-deficient murine model of inflammatory bowel disease, Clin. Immunol. 116 (2005) 135–142.
[12] J.M. Braun, B. Schneider, H.J. Beuth, Therapeutic use, efficiency and safety of the proteolytic pineapple enzyme Bromelain-POS in children with acute sinusitis in Germany, In Vivo 19 (2005) 417–422.
[13] C.M. Ley, A. Tsiami, Q. Ni, N. Robinson, A review of the use of bromelain in cardiovascular diseases, J. Chin. Integr. Med. 9 (2011) 702–710.
[14] K. Chobotova, A.B. Vernallis, F.A.A. Majid, Bromelain's activity and potential as an anti-cancer agent: current evidence and perspectives, Cancer Lett. 290 (2010) 148–156.
[15] K. Bhui, S. Prasad, J. George, Y. Shukla, Bromelain inhibits COX-2 expression by blocking the activation of MAPK regulated NF-kappa B against skin tumor-initiation triggering mitochondria, Cancer Lett. 282 (2009) 167–176.
[16] L.P. Hale, P.K. Greer, G.D. Sempowski, Bromelain treatment alters leukocyte expression of cell surface molecules involved in cellular adhesion and activation, Clin. Immunol. 104 (2002) 183–190.
[17] L. Rosenberg, A. Barezovski, R. Gurfinkel, E. Silverstein, Y. Krieger, Safety & efficacy of bromelain extract in debriding deep burns: recent summary of all available data, Burns 35 (2009) S17.
[18] S.S. Poh, F.A.A. Majid, Thermal Stability of free bromalain and bromelain-polyphenol complex in pinneaple juice, Int. Food Res. J. 18 (2011) 1051–1060.
[19] Y. Xue, C.Y. Wu, C.J. Branford-White, X. Ning, H.L. Nie, L.M. Zhu, Chemical modification of stem bromelain with anhydride groups to enhance its stability and catalytic activity, J. Mol. Catal. B: Enzym. 63 (2010) 188–193.
[20] H. Ishihara, N. Takahashi, S. Oguri, S. Tejima, Complete structure of the carbohydrate moiety of stem bromelain, J. Biol. Chem. 254 (1979) 10715–10719.
[21] J. Yon-Kahn, G. Herve, Examples of structure-function relationships in enzymatic systems, in: J. Yon-Kahn, G. Herve (Eds.), Molecular and Cellular Enzymology, Springer, New York, 2010, pp. 451–542.
[22] L.W. Cohen, V.M. Coghlan, L.C. Dihel, Cloning and sequencing of papain-encoding cDNA, Gene 48 (1986) 219–227.
[23] A. Carne, C.H. Moore, The amino acid sequence of the tryptic peptides from actinidin, a proteolytic enzyme from the fruit of *Actinidia chinensis*, Biochem. J. 173 (1978) 73–83.
[24] D.C. Watson, M. Yaguchi, K.R. Lynn, The amino acid sequence of chymopapain from *Carica papaya*, Biochem. J. 226 (1990) 75–81.
[25] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[26] M. Kunitz, Crystalline soybean trypsin inhibitor II. General properties, J. Genet. Physiol. 30 (1946) 291–310.
[27] H.E. Walter, Proteinases: methods with hemoglobin, casein, and azocoll as substrates, in: H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, Verlag Chemie, Weinheim, 1984, pp. 270–277.
[28] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356.
[29] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[30] F.L. Garcia-Carreno, L.E. Dimes, N.F. Haard, Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors, Anal. Biochem. 214 (1993) 65–69.
[31] Y. Liao, M.B. Brown, G.P. Martin, Investigation of the stabilisation of freeze-dried lysozyme and the physical properties of the formulations, Eur. J. Pharm. Biopharm. 58 (2004) 15–24.
[32] R.M. Heinicke, W.A. Gortner, Stem bromelain-a new protease preparation from pineapple plants, Econ. Bot. 11 (1957) 225–234.
[33] A.P.B. Rabelo, E.B. Tambourgi, A. Pessoa Jr., Bromelain partitioning in two-phase aqueous systems containing PEO–PPO–PEO block copolymers, J. Chromatogr. B 807 (2004) 61–68.
[34] H.U. Hebbar, B. Sumana, K.S.M.S. Raghavarao, Use of reverse micellar systems for the extraction and purification of bromelain from pineapple wastes, Bioresour. Technol. 99 (2008) 4896–4902.
[35] S. Ota, E. Muta, V. Katahira, Y. Okamoto, Reinvestigation of fractionation and some properties of the proteolytically active components of stem and fruit bromelains, J. Biochem. 98 (1985) 219–228.
[36] N. Takahashi, Y. Yasuda, K. Goto, J. Miyak, T. Murachi, Multiple molecular forms of stem bromelain. Isolation and characterization of two closely related components, SB1 and SB2, J. Biochem 74 (1973) 355–373.
[37] C.W. Wharton, A. Cornish-Bowden, K. Brocklehurst, E.M. Crook, Kinetics of the hydrolysis of *N*-benzoyl-L-serine methyl ester catalysed by bromelain and by papain. Analysis of modifier mechanisms by lattice nomography, computational methods of parameter evaluation for substrate-activated catalyses and consequences of postulated non-productive binding in bromelain- and papain-catalysed hydrolyses, Biochem. J. 141 (1974) 365–381.
[38] Y. Mi, G. Wood, L. Thoma, Cryoprotection mechanisms of polyethylene glycols on lactate dehydrogenase during freeze–thawing, AAPS J. 6 (2004) 1–10.
[39] E.G. Aisen, V.H. Medina, A. Venturino, Cryopreservation and post-thawed fertility of ram semen frozen in different trehalose concentrations, Theriogenology 57 (2002) 1801–1808.
[40] B.I. Kurganov, I.N. Topchieva, Artificial chaperone-assisted refolding of proteins, Biochemistry 63 (1998) 413–419.
[41] J.K. Graham, Cryopreservation of stallion spermatozoa, Vet. Clin. North Am. Equine Pract. 12 (1996) 131–147.
[42] A. Ritonja, A.D. Rowan, D.J. Buttle, N.D. Rawlings, V. Turk, A.J. Barett, Stem bromelain: amino acid sequence and implications for weak binding of cystatin, FEBS Lett. 247 (1989) 419–424.
[43] S.K. Haq, S. Rasheedi, R.H. Khan, Characterization of a partially folded intermediate of stem bromelain at low pH, Eur. J. Biochem. 269 (2002) 47–52.
[44] P. Gupta, R.H. Khan, M. Saleemuddin, Trifluoroethanol-induced "molten globule" state in stem bromelain, Arch. Biochem. Biophys. 413 (2003) 199–206.
[45] P. Gupta, M. Saleemuddin, R.H. Khan, Hydrophobic interactions are prevalent forces between bromelain: Fab', Complex. Biochem. 71 (2006) S31–S37.
[46] K. Welfl, R. Misselwitz, G. Hausdorf, W. Höhne, H. Welfl, Conformation, pH-induced conformational changes, and thermal unfolding of anti-p24 (HIV-1) monoclonal antibody CB4-1 and its Fab and Fc fragments, Biochim. Biophys. Acta 1431 (1999) 120–131.
[47] A. Arroyo-Reyna, A. Hernandez-Arana, R. Arreguin-Espinosa, Circular dichroism of stem bromelain: a third spectral class within the family of cysteine proteinases, Biochem. J. 300 (1994) 107–110.
[48] P. Manavalan, W.C. Johnson, Sensitivity of circular dichroism to protein tertiary structure class, Nature 305 (1983) 831–832.
[49] S. Solis-Mendiola, A. Arroyo-Reyna, A. Hernandez-Arana, Circular dichroism of cysteine proteinases from papaya latex. Evidence of differences in the folding of their polypeptide chains, Biochim. Biophys. Acta 1118 (1992) 288–292.
[50] I.G. Kamphuis, K.H. Kalk, M.B.A. Swarte, J. Drenth, Structure of papain refined at 1.65 A resolution, J. Mol. Biol. 179 (1984) 233–257.
[51] J.B. Bowstra, E.C. Spoelstra, P. De Waard, B.R. Leeflang, R. Bastian, J.P. Kamerling, J.F.G. Vliegenthart, Conformational studies on the N-linked carbohydrate chain of bromelain, Eur. J. Biochem. 190 (1990) 113–122.
[52] D.M. Byler, H. Susi, Examination of the secondary structure of proteins by deconvolved FTIR spectra, Biopolymer 25 (1986) 469–487.
[53] A. Dong, B. Caughey, W.S. Caughey, K.S. Bhat, J.E. Coe, Secondary structure of the pentraxin female protein in water determined by infrared spectroscopy: effects of calcium and phosphorylcholine, Biochemistry 31 (1992) 9364–9370.
[54] J. Kong, S. Yu, Fourier transform infrared spectroscopic analysis of protein secondary structures, Acta Biochim. Biophys. Sin. 39 (2007) 549–559.