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A new procedure based on column chromatography to purify bromelain by ion exchange plus gel filtration chromatographies

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ABSTRACT

Bioproducts separation and purification processes are an important segment of the biotechnical industry. Bromelain is an enzyme which has great commercial value and is of wide interest in the pharmaceutical, alimentary, and textile industries, among others. The goal of this study was to develop a new method for bromelain purification from the stem residues resulting from agricultural processing of the pineapple plant. Bromelain was purified using two liquid chromatography steps, ion exchange plus gel filtration chromatography. Use of the methodology which was developed produced an enzyme with a molecular weight of 30 kDa (confirmed by SDS-PAGE), high recovery of enzymatic activity (89%), and with a purification factor of 16.93, a result superior to the methodologies described in the literature. HPLC showed the presence of two peaks in the ion exchange chromatogram and only one protein in the gel filtration chromatogram. Results indicate that, depending on the destination of the bromelain, the process can be stopped after the first purification step. The MALDI-TOF MS provided the peptide mass fingerprint of bromelain and MALDI-MS/MS the fragmentation profile and sequencing of the ions of *m/z* 951 and 1584. Thus, the connectivity and chemical structure of bromelain was confirmed. Moreover, besides its superiority to other methodologies, it can be applied to take advantage of the agricultural and industrial pineapple plant residues.

1. Introduction

The isolation and purification of bioproducts are very important processes in the biotechnology industry, representing 80–90% of total production costs. Furthermore, the development of simple, viable methods for protein purification has been an essential prerequisite for many advances in biotechnology (Harikrishna et al., 2002; Costa et al., 2009).

Liquid chromatography (LC) is a technique commonly used for protein purification. However, other analytical techniques also could be used such as liquid–liquid extraction by aqueous two-phase systems (Babu et al., 2008), reverse micellar systems (Hebbar et al., 2011), and separation by membrane and precipitation (Doko et al., 2005; Silveste et al., 2012). In liquid chromatography, proteins in solution (mobile phase) are isolated and purified through their interaction with a stationary phase (Cao, 2005). Generally, the products obtained by LC purification are very expensive and have high added value due to the costs of the materials used in the production process. The development of low-cost LC purification methods has been challenging (Costa et al., 2009).

Proteolytic enzymes, or proteases, are a class of hydrolytic enzymes capable of cleaving the peptide bonds of protein chains and are essential in physiological processes. In addition, proteases are among the most relevant enzymes from an industrial standpoint because of their involvement in several technological applications (Bon et al., 2008).

Bromelain is a proteolytic enzyme belonging to the cysteine peptidase family. This enzyme can be found in the tissues of plants of the Bromeliaceae family, and pineapple (*Ananas comosus* var. *comosus*) is its main source (Rowan et al., 1990; Hebbar et al., 2008). Bromelain has a number of biotechnological applications, especially in the food, cosmetics and pharmaceutical industries, as

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well as several clinical applications, such as an antitumor agent, immune response modulator and an enhancer of antibiotic effects and mucolytic and gastrointestinal action (Maurer, 2001; Bala et al., 2012). Furthermore, the enzyme has an effect on cardiovascular and circulatory diseases and may have potential uses in surgical procedures and wound care (Costa et al., 2009).

The worldwide application of bromelain increases the importance of determining a viable extraction and purification method for this enzyme. Such a method may help to minimize losses in the pineapple agribusiness because the agricultural and industrial wastes of pineapple (stems, leaves and crowns) are rich in this enzyme. Usually, this residue is not used and has no appropriate destination (Bardiya et al., 1996; Costa et al., 2009). Therefore, the present study presents a new, viable method for the purification of bromelain from pineapple stems using LC as its main technique.

2. Methods

2.1. Materials

2.1.1. Pineapple wastes

Pineapple wastes from the cv. Vitória were obtained from the Incaper Sooretama Experimental Farm, at Sooretama-ES, Brazil.

2.1.2. Chemicals

All chemicals products used in this study were purchased from Sigma, Merck or GE (USA). HPLC grade chemical products were purchased from Tedia Brazil.

2.2. Crude extract

A known quantity (450 g) of waste (stem of pineapple cv. Vitória) was crushed for five minutes along with 600 mL of extractor solution (0.4 M H_2SO_4/2 mM Na_2SO_4 pH 4.5 at 4 °C). The extract was then filtered and centrifuged (Eppendorf centrifuge 5804 R, Germany) at 14,750 × *g* for 20 min at 4 °C, and the supernatant (crude enzyme extract) obtained was used for the following experiments. The crude extract was stored frozen at −20 °C.

2.3. Protein determination

The protein concentrations from enzymatic solutions were determined according to the method described by Bradford (1976) using bovine serum albumin as the standard.

2.4. Bromelain activity

The bromelain activity was determined according to the casein digestion unit (CDU) method, which uses casein as a substrate in the presence of cysteine and EDTA (Murachi, 1976 with modifications). The assays were based on the proteolytic hydrolysis of the casein substrate.

The absorbance of the clear filtrate (solubilized casein) was measured at 280 nm using a spectrophotometer (Thermo Spectronic® BIOMATE 3, USA). One unit of bromelain activity was defined as 1 μg of tyrosine released in 1 min per mL of sample when casein was hydrolysed under the standard conditions of 37 °C and pH 7.0 for 10 min.

The sample analyses were performed against their respective blank solutions. The protein concentration readings were taken in triplicate, and the average value was used for the calculation of the extraction efficiencies. The specific activity, activity recovery (%) and purification fold were estimated by the following equations:

$$\text{Specific activity} = \frac{\text{proteolytic activity (U/mL)}}{\text{protein content (mg/mL)}} \quad (1)$$

$$\text{Bromelain recovery (\%)} = \frac{\text{bromelain activity after purification}}{\text{bromelain activity of the crude extract}} \times 100 \quad (2)$$

$$\text{Purification fold} = \frac{\text{specific activity after purification}}{\text{specific activity of the crude extract}} \quad (3)$$

2.5. Ion exchange chromatography

A 25 mm ID glass column, 110 mm long was packed with caboxymethy-celullose and equilibrated with four column volumes of 5×10^{-3} M acetate buffer. Then 10 mL of the crude extract was submitted and remained in contact with the resin for one hour. After, sample was eluted using a 1 M acetate buffer pH 4.5 at a flow rate of 0.5 mL/min. The fractions were then collected in 4 mL aliquots. The presence of protein was monitored using recording spectrophotometer at 280 nm (Biomate®), using acetate buffer pH 4.5 as blank. The procedures for the estimation of protein content and enzyme activity are described below.

Finally, the column was then washed with 2 M NaCl solution until no further protein eluted. All procedure was conducted in a refrigerated environment at 4 °C. Purified enzyme was stored in frozen at −20 °C (Cabral et al., 2000; Hernández et al., 2005 with modifications).

2.6. Gel filtration chromatography

Aliquots from ion exchange chromatography were collected and submitted to gel filtration chromatography. A glass column with a 17.5 mm ID and 200 mm long was packed with Sephadex G-50® and equilibrated with one and half column volumes of acetate buffer. The sample was applied and left in contact with the resin for one hour. The sample was then eluted with 1 M acetate buffer pH 4.5 at flow rate of 0.7 mL/min, and the eluent was collected as previously described. The column was then washed with 5×10^{-3} M acetate buffer pH 4.5 solution until no further protein eluted. All procedure was conducted in a refrigerated environment at 4 °C Purified enzyme was stored in frozen at −20 °C. (Cabral et al., 2000; Hernández et al., 2005 with modifications).

2.7. SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970) using 15% (w/v) polyacrylamide gels. The staining of the gels was performed using Coomassie Brilliant Blue G-250. Chromatography measurements and SDS-PAGE were performed in triplicate.

2.8. HPLC

A 20 μL aliquot of the sample obtained from the ion exchange and gel filtration chromatography was subjected to analytical high performance liquid chromatography (HPLC) performed on a Shimadzu Prominence (Kyoto, Japan) apparatus with a UV/vis LC-20A detector. The fractions were eluted with a Shimadzu Shim Pack CLC (M) C18 (4.6 mm ID × 250 mm long) column using a linear gradient between TFA/H_2O (1:1000, v/v) and TFA/acetonitrile (1:1000, v/v) over 80 min at a flow rate of 0.5 mL/min, and the absorbance were read at 280 nm (Hernández et al., 2005 with modifications).

2.9. Mass spectrometry

The protein spot of interest was excised out of the gel (Shevchenko et al., 1996). The spot was washed three times with a washing solution (1:1, v/v 25 mM ammonium carbonate–acetonitrile) at 25 °C for 2 h. The washing solution was then removed and the spot was now covered with acetonitrile for 20 min at 25 °C. Finally, the acetonitrile was removed and the excess evaporated, thus providing, the drying of the spot. The dehydrated gel particles were then rehydrated for 10 min with 10 μL of digest buffer (25 mM ammonium carbonate) containing 20 ng of trypsin (sequencing grade modified porcin trypsin, Promega, Madison, WI, USA). After, 20 μL of digest buffer were added, and the sample was incubated for 20 h at 37 °C. The resulting tryptic peptides were twice extracted with 50 μL of 0.1% trifluoroacetic acid (TFA) in 50% (v/v) acetonitrile with 20 min sonication. The products from the two extracts were combined, vacuum-dried and then dissolved in 0.1% TFA in 50% (v/v) acetonitrile.

The matrix (10 mg/mL CHCA (α-cyano-4-hydroxycinnamic acid) in 0.1% TFA and 50%, v/v acetonitrile) and the sample (1:1, v/v) were spotted and cocrystallised on a target plate. Matrix-assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS, model 4700 Explorer Proteomics Analyser, Applied Biosystem®, USA) has been applied to Peptide Mass Fingerprint (PMF) and peptide sequencing. For MALDI-MS/MS measurements, N2 was used as collision gas in the collision cell at 2.8×10^{-6} Torr. Trypsin autolysis peptides of masses 842.5 and 2211.1 and calibration mixtures 1 and 2 (Sequazyme Peptide Mass Standard kit, PerSeptive Biosystems®, Foster City, CA, USA) were used, respectively, as internal and external standards in both the MALDI MS and MALDI MS/MS procedures.

For database searching, ppw files were submitted to the Mascot search engine using Daemon 2.1.0 (Matrix Science: http://www.matrixscience.com) on a Mascot server version 2.2.1. The data were searched against the latest version of the public non-redundant protein database of the National Center for Biotechnology Information (NCBI), downloaded on August 2008, with a mass accuracy of 15 ppm for the parent ion (MS) and 0.2 Da for the fragment ions (MALDI MS/MS).

The search was constrained to tryptic peptides with a maximum of one missed cleavage. The carbamidomethylation of cysteine was considered a fixed modification, whereas the oxidation of methionine residues was considered as a variable modification. An initial list of proteins was generated and formed the basis for further analysis. A "positive list" was generated by considering only those proteins that contained at least one unique peptide (minimum 10 aa) with a Mascot score above 67 (p-value < 0.05). HPLC and MS experiments were performed in duplicate (Rodrigues et al., 2011).

3. Results and discussion

The crude extract was obtained with 1.77 mg/mL of total protein, 40.84 U mL^{-1} of proteolytic activity and 23.07 U mg^{-1} of specific activity (Table 1). After the purification steps, the crude extract was applied to the ion exchange chromatography.

3.1. Ion exchange chromatography

Bromelain purification with carboxymethylcellulose (CMC) exhibited a purification factor of 3.01, with 93% recovery of the proteolytic activity. No activity was detected after the washing flow. The ion exchange chromatography of bromelain resulted in a prominent peak in the sixth fraction (Fig. 1). The specific activities of the applied crude extract and the sixth eluted fraction were 23.07 and 69.50 U/mg protein, respectively.

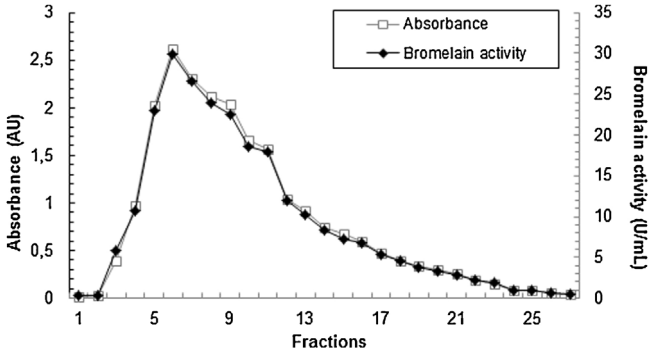

Fig. 1. Ion exchange chromatographic purification profile for bromelain from pineapple stems.

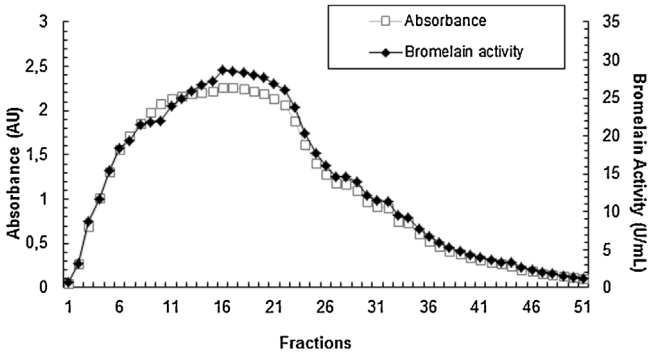

Fig. 2. Gel filtration chromatographic purification profile for bromelain from pineapple stems after ion exchange chromatography.

We observed a higher bromelain activity than those reported for existing methodologies of ion exchange chromatography. This activity is due to the use of 1 M acetate buffer pH 4.5 in this step. Costa (2010), compared different buffers for the maintenance of bromelain, and noted that this buffer at pH 4.5 contributed to bromelain activity. Moreover, previous works have indicated that differences in the ion chromatography resin are important to bromelain activity (Devakate et al., 2009; Costa, 2010).

3.2. Gel filtration chromatography

The fractions obtained by ion exchange chromatography were pooled (fractions 3–22) and subjected to molecular exclusion chromatography using Sephadex G-50 resin, and a total of 51 fractions were collected (Fig. 2). The specific activity of bromelain was 390.75 U/mg, whereas that the proteolytic activity was 89% of recovery with a purification factor of 16.93.

The coupling of CMC resin with the Sephadex® G-50 resin is a powerful chromatography method to obtain Bromelain in its pure form. Suh et al. (1992) purified bromelain from pineapple fruit and stem until homogeneity using gel filtration chromatography, obtaining a yield of only 23% of activity.

Hernández et al. (2005) reported a purification method for bromelain similar to our study. Authors conducted the Bromelain purification however using opposite treatment: they performed the gel filtration followed by ion exchange chromatography. The gel-filtration resin used was Sephadex® G-100, yielding 87.81% activity. The second purification step was used the CMC, which the yield of bromelain activity decreased to 41.15%.

The Sephadex® G-50 is cheaper than Sephadex® G-100 (reduction of 50% of cost), becoming the purification process of bromelain economically viable. Additionally, the purification methodology developed presents a higher yield of proteolytic activity.

Table 1
Yield in total protein, proteolytic activity, specific activity and purification factor for each bromelain purification step.

Steps	Fraction volume (mL)	Total protein (mg/mL)	Proteolytic activity (U/mL)	Specific activity (U/mg)	PF[a]
Crude extract	8	1.77	40.84	23.07	1
CMC	50	0.20	13.90	69.50	3.01
Sephadex	5	0.093	36.34	390.75	16.93

[a] PF: purification factor.

Fig. 2 shows fractions which have proteolytic activity (fraction 2–45). After 51th fraction non proteolytic activity was observed corresponding to non-targets proteins.

3.3. SDS-PAGE

In the SDS-PAGE analysis, the molecular weight of bromelain was estimated as 30 kDa, and predicted by Gautam et al. (2010) and other previous studies. Bromelain may not be completely pure when the extract is eluted only with ion exchange, as suggested by the double gel band in the 30 kDa region (Lane 1 – Fig. 3). The result of the gel filtration purification of bromelain was confirmed by the SDS-PAGE result, in which only one band can be observed in the 30 kDa region (Lane 2 – Fig. 3).

3.4. HPLC

Aliquots from the same fractions used for the preparation of the SDS-PAGE gels were then prepared for HPLC. The HPLC analysis corroborated with the SDS-PAGE results. The reversed phase chromatography confirmed the presence of two peaks in the ion exchange chromatogram (Fig. 4a) and only one protein (Peak 1) in the gel filtration chromatogram, which was eluted in the first 10 min (Fig. 4b).

Bromelain is a protein with polar characteristics, as indicated by the peak appearing during the first few minutes when water is the main solvent in the non-polar column (C-18), causing it to be rapidly eluted. Napper et al. (1994), in a comparison of pineapple proteases, ascribed the polarity of bromelain mainly to its contents of the amino acids lysine and arginine, which are present in larger amounts in comparison with other proteases.

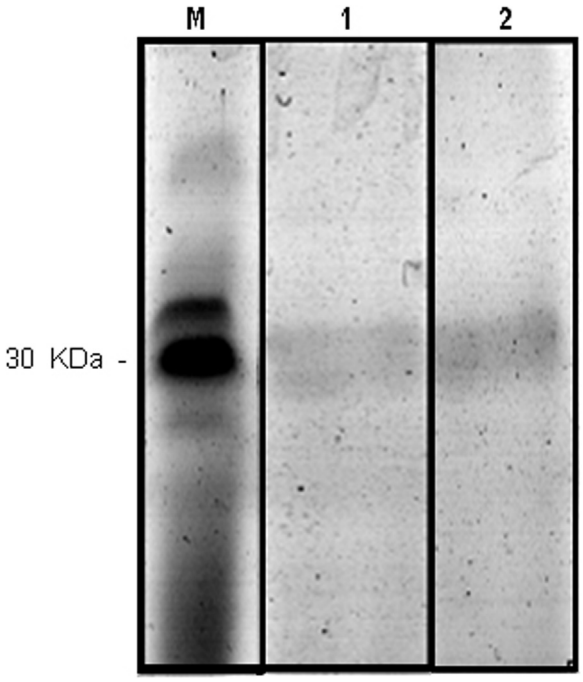

Fig. 3. SDS-PAGE analysis of ion exchange (Lane 1) and gel filtration (Lane 2) chromatography. (M – Molecular Weight Marker).

3.5. Mass spectrometry

The SDS-PAGE previously shown (Fig. 3, lane 2) was excised and trypsinised to obtain a PMF. The MALDI-TOF MS confirmed the purity of the bromelain (Fig. 5a) from identification of 10 more abundant peptides with *m/z* values of 951.44, 963.44, 1000.49, 1016.48, 1032.48, 1068.52, 1103.52, 1526.70, 1566.73 and 1584.75. Among signals identified, the ions of *m/z* 951 and 1584 were submitted to MALDI-MS/MS experiments for determination of fragmentation profile and sequencing (Fig. 5b and c). The Mascot program has been used for the MALDI-MS/MS data, thus providing, the most likely sequence for the peptides of *m/z* 951 (Probable sequence: WGEAGYIR) and 1584 (Probable sequence: TNGVPNSAYITGYAR).

The NCBI (http://blast.ncbi.nlm.nih.gov) database analysis of the two peptides identified a FBSB precursor [*Ananas comosus*] with the access code gi2463584, which has a 95–100% identity with bromelain. Additionally, the SwissProt (http://blast.ncbi.nlm.nih.gov) database was also used, confirming again the purity of the

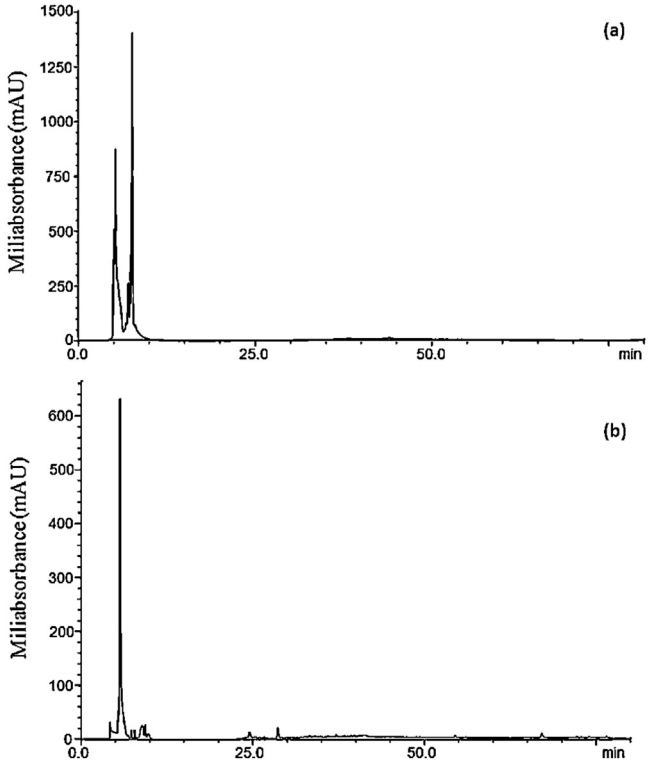

Fig. 4. HPLC chromatograms obtained from the fraction with the highest proteolytic activity in the C18 column using a gradient of 0–80% water–acetonitrile at a flow of 0.5 mL/min for 80 min (a) ion exchange chromatogram and (b) gel filtration chromatogram.

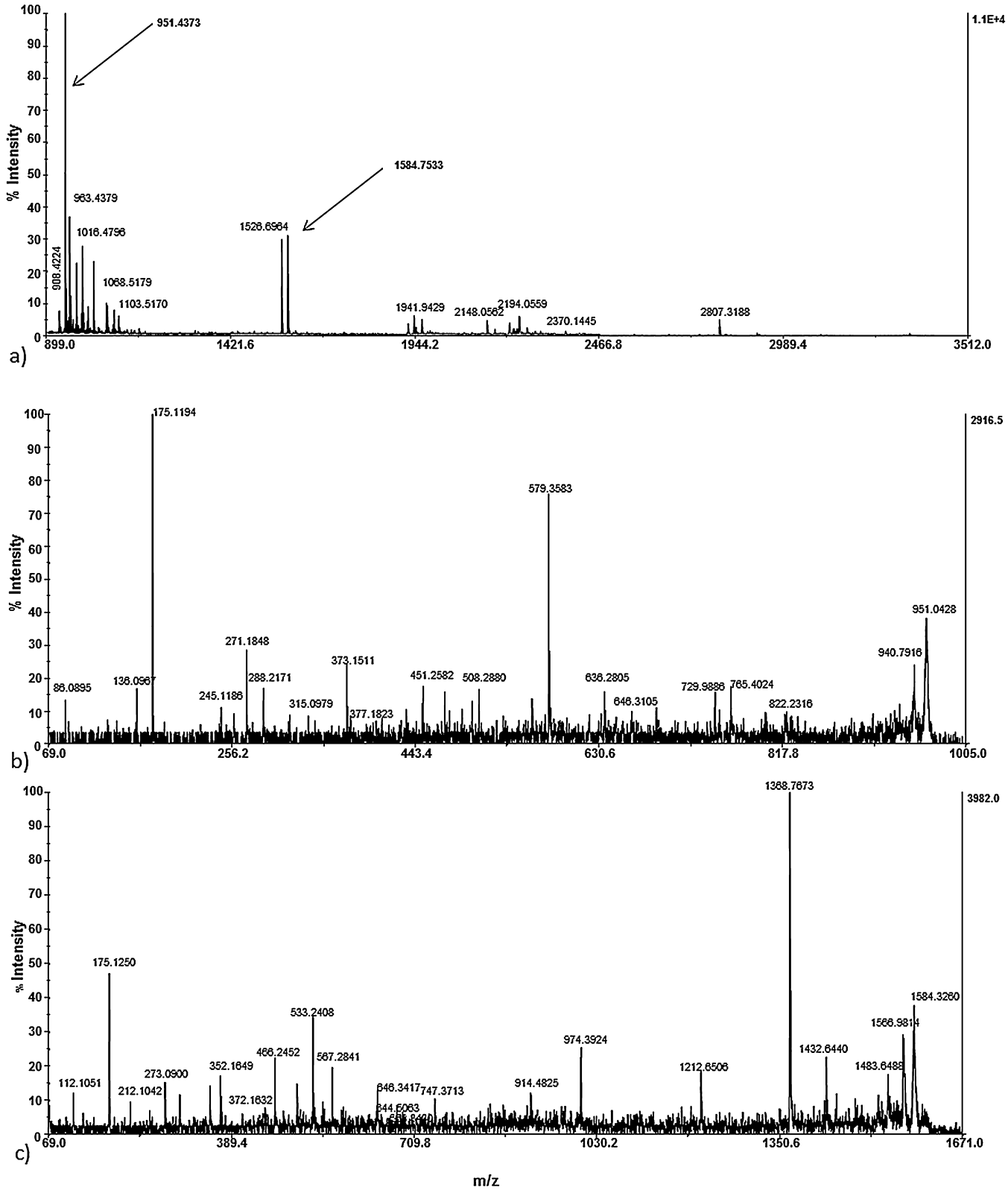

Fig. 5. (a) MALDI-TOF MS spectrum from bromelain purified, (b) MALDI TOF MS/MS from peptide m/z 951 and (c) MALDI TOF MS/MS from peptide m/z 1584.

bromelain as an identity of 95–100% between bromelain and a peptide with the access code BROM2_ANACO.

The MSDB database was also used to analyze the two peptides. The peptide with the m/z 951 showed high homology with ananain, identified as an AN8 precursor.

Rowan et al. (1990) described the presence of four main proteases in pineapple (*Ananas comosus*): fruit bromelain, stem bromelain, ananain and comosain.

Ananain shows a 77% amino acid sequence identity with bromelain (Lee et al., 1997). This peptide (*m/z* 951) is present at the

N-terminus region of bromelain, which is a portion identical to that of other cysteine proteases, as it comprises mainly the region of the catalytic site. If we align only one fragment of the two proteins (bromelain and ananain – see below), the peptides differ by only one amino acid: bromelain has an alanine (A), whereas the ananain has a glycine (G).

Stem bromelain	WGEAGYIR
Ananain	WGEGGYIR
Peptide *m/z* 951	WGEAGYIR

As proteases of the same family, the proteins were expected to share several identical regions in their primary sequences, and some peptides would have a high identity with ananain or other proteases. The most likely sequence identified for the peptide mass of *m/z* 951 is the bromelain. Moreover, Larocca et al. (2010) also confirmed the chemical identity of peptide of *m/z* 951 by MALDI-TOF MS.

3.6. Purification process

The bromelain purification method using carboxymethylcellulose was able to purify the compound approximately 3.01 times the level in the crude extract, and the Sephadex® column purified the compound approximately 16.93 times (Table 1).

The results indicate that, depending on the destination of the bromelain, the process can be stopped after the first purification step using ion exchange chromatography. This method is possible because the CMC purified the compound 3.01 times the level in the crude extract (Table 1), with this result confirmed by the SDS-PAGE and HPLC results.

In many cases, the commercial use of bromelain in the food, cosmetics, and nutritional, medicinal and pharmacological supplement industries (Ketnawa et al., 2012) does not require a high purity bromelain enzyme preparation. The process described in the present study enables producers to choose the desired level of bromelain purity, thereby further reducing the costs of the production process.

Proteolytic activity is the main parameter for quality control, and the bromelain purified by the process demonstrated in the present study had a proteolytic activity yield of 89%, which is higher than that obtained from current purification processes.

Finally, it is estimated that from 1 kg of stem pineapple, it is possible to obtain approximately 267 mg of pre-purified bromelain and 124 mg of purified bromelain.

4. Conclusions

The bromelain purification method of the present study employs two LC steps: ion exchange chromatography (carboxymethylcellulose), followed by gel filtration chromatography (Sephadex® G-50). The purified bromelain has a molecular weight of approximately 30 kDa and is of high purity, and the purification method yields a higher proteolytic activity value (89%) at a lower cost than the existing methodologies. Therefore, the method demonstrated in the present study can be used to transform the agricultural and industrial wastes of pineapple crop into a product with high added value and significant biotechnological interest.

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