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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).