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Purification of bromelain from pineapple wastes by ethanol precipitation

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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.

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].

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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-