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Characterization of Bromelain from *Ananas Comosus* Agroindustrial Residues Purified by Ethanol Factional Precipitation

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Brazil produces over than 70,000 ha of pineapple, being the world leader in pineapple production, and Minas Gerais State alone produces over than 600 kt/y. The pineapple is the marketable plant part, which represents only 23 % of the plant itself, and the remaining plant, stem, leaves, bark and crown, being considered an agroindustrial residue. This residue contains great amount of bromelain, a vegetable protease group with several applications in food and pharmaceutical industries, which has high market value. These enzymes performs important role in proteolytic modulation at cellular matrix, and in numerous physiologic processes, including tissue morphogenesis, tissue repair, angiogenesis and tissue modulation, decreasing bruises, swelling, pain and healing time. This work reports the ethanol fractional precipitation of bromelain from pineapple stem, bark and leaves, and its characterization after the recovery process. Aqueous extract of bromelain was prepared by processing pineapple's stem, bark and leaves in a common juice extractor. Ethanol fractional precipitation studies were performed under refrigeration (4 ºC), and it was carried out stepwise, where several concentrations of ethanol were added to perform a fractional precipitation. Bromelain was characterized before and after precipitation to determine its optimal pH, temperature and stability. Results showed that bromelain was precipitated successfully in the 30-70 % ethanol fraction, in which were achieved a purification factor of 2.07 fold and yielded over than 98 % of enzyme recovery. After the purification, the bromelain optimal pH changed from 7.0 to 8.0, and it kept activity even after 60 ºC, however its optimum were 50 ºC. These results showed that bromelain recovery with ethanol fractional precipitation is a viable process, in which results in a good quality enzyme for industrial applications.

1. Introduction

The pineapple is native of South America, probably in the South and South-eastern Brazil, Argentina and Uruguay (Crestani et al., 2010). Its fruit is consumed natural, or in form of ice cream, candy, lollies, soft drinks and homemade juices. The Brazil cultivates over 70,000 hectares of pineapple (IBGE, 2011) and thus considered one of the largest producers in the world. In 2008, the production area was estimated in 62,862 ha spread over several states, and Minas Gerais being one of the major producers, with an area of 7,600 ha and production estimative of 597,895 t (Crestani et al., 2010).
The pineapple fruit is the marketable part of the plant, representing only 23 % of total plant, the remainder, composed of stem, leaf, bark and crown, is considered agricultural waste and is usually disposed or used in composting, losing completely its economic potential to extract high valued products (Crestani et al., 2010).

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The quality of pineapple fruits is referred to their physical features external, such as peel colour, size and shape of the fruit, and internal conferred by broad range of constituents, especially by the high levels of sugars and proteolytic enzymes, particularly bromelain (Abílio et al., 2009).
The large percentage of waste generated during industrial processing, about 60 of total weight of the fruit, contains appreciable amounts of bromelain, a protease that has numerous applications in the food and pharmaceutical industries, which have high aggregate value and does not disappear when the fruit ripens (Coelho et al., 2013). Bromelain is chemically known since 1875 and used as a phytotherapeutical agent (Pavan et al., 2012). It is included in the classification of hydrolase, more specifically from the group of cysteine proteinase, being able to break peptide bonds, separating proteins and amino acids.
The raw material most often used to obtain bromelain are the mature stalks of pineapple utilizing them after harvesting the fruit, however, also leaves, juice, peel and waste can be utilized (Crestani et al., 2010). Bromelain has a wide range of therapeutic benefits, but the mode of its action is not well understood. It is proved that Bromelain is well absorbed in body after oral administration and has not important side effects even after prolonged use (Pavan et al., 2012).
This enzyme is used in different sectors, all based on your activity proteolytic, among which stand out the property to facilitate the digestion of proteins and are therefore added in digestive drugs, and the ability to softening meat (Abílio et al., 2009). Its economic importance is related to the production of pharmaceuticals, their effect on the digestive system, replacing pepsin and trypsin in treatment of pancreatic insufficiency. It is also used in the treatment of heart disease, rheumatoid arthritis, surgical trauma, sinusitis, due mainly to its ability to facilitate blood clotting, reducing edema and also provide an anti-inflammatory effect (Ketnawa et al., 2012).
And furthermore has an extensive use in the food industry, such as in clarification of beers, in cheese making, the softening of meat, in preparation of infant and dietetic foods, among others, in the treatment of digestive disorders, wounds and inflammations, preparation of hydrolysed collagens, in textile industries, for softening the fibres and also in the production of detergents (Ferreira et al., 2011b).
The commercial production process obtains bromelain from cooled pineapple juice using ultrafiltration, centrifugation and lyophilisation yielding a yellowish powder with 30 to 40 % protein content, and uncertain biological activity (Coelho et al., 2013, Larocca et al., 2010), which price exceeds 1800 US$/kg (Sigma, 2014). The continuing interest in bromelain, due to its numerous applications, both in food industry, and in the pharmaceutical industry, makes this enzyme one of best protease of vegetal origin. But the Brazilian companies interested in its use must resort to importing bromelain, once it is not produced locally. Thus, several studies have been performed either to optimize bromelain biological activity (Godoi, 2007) and purify it using cross-flow filtration (Lopes et al., 2006), reversed micelles (Fileti et al., 2009) and Aqueous Two-Phase Systems (ATPS) (Ferreira et al., 2011c, Ferreira et al., 2011a). This, it is essential to develop a feasible process to purify bromelain based on national technology where the big challenge is obtaining bromelain enzyme and maintain its stability during all steps involved and fractional precipitation has been used successfully to obtain biomolecules (Longo et al., 2010).

2. Materials and Methods

2.1 Enzyme Extract

The enzyme extract was obtained from the bark, stem and leaves of pineapple (*Ananas comosus*) from local industries. The plant tissue, bark, stem and leaves were processed in a kitchen extractor and then centrifuged at 10,000 g for 20 min at 4 °C to remove insoluble material.

2.2 Determination of Enzyme Activity

The enzyme activity assayed by the azocasein method as described by Oliveira et al. (2006), where azocasein 1.0% (w/v) (Sigma) was solubilized in 4% ethanol (v/v) and 0.1 M phosphate buffer, pH 7.0, and used as substrate. The assay mixture, containing 125 µL of substrate and 125 µL of extract enzymatic was incubated for 10 min at 37 °C and the reaction stopped by addition of 750 µL of 5 % trichloroacetic acid (w / v). The samples were centrifuged at 4000g for 10 minutes and at a temperature 5 °C. One unit of activity was defined as the amount of enzyme required to produce the increase in optical density by one unit within 1 h.

2.3 Precipitation by Ethanol

Bromelain precipitation was performed according to methodology described Englard and Seifter (1990). Ethanol 98 % (w/w) cooled to 0 °C was added dropwise until concentrations of 30 and 70 % (w/w) was

reached. The solution was then centrifuged at 10,000 for 20 min at 4 °C and the resulting pellet was suspended in 20 mM buffer phosphate pH 7.0.

2.4 Enzyme Partial Characterization

Optimum pH and temperature was evaluated before and after ethanol precipitation, as well as pH and temperature stability. Optimum pH assays were performed as described in item 2.2 with exchanged buffers so the desired pH could be reached. Optimum temperature was accessed by changing the incubation temperature. Bromelain stability was evaluated before and after purification for 180 min in all pH tested. For all tests bromelain standard solutions (as described in Section 2.1) were prepared for comparison.

3. Results and Discussion

3.1 Ethanol Fractional Precipitation of Bromelain

Table 1 shows the effects of ethanol concentration on bromelain precipitation. It was observed when ethanol concentration reached 30 % there was a reduction of protein concentration, however there was no bromelain precipitation. Ethanol concentrations over 65 % showed an enzymatic activity recovery near its initial value. Based on these early results, bromelain was precipitated in two steps, in which was possible to recover over than 98 % of its initial activity and to obtain a purification factor of 2.34.

Table 1: Ethanol fractional precipitation

Fraction	Protein concentration ($mg \cdot mL^{-1}$)	Bromelain activity ($U \cdot mL^{-1}$)	Specific activity ($U \cdot mg^{-1}$)	Recovery (%)	Purification Factor
Crude Extract	0.201	16.25	80.85	N.A.	N.A.
0-30 %	0.052	0.00	0.00	0.00	0.00
30-70 %	0.084	15.96	189.5	98.21	2.34
70-100 %	0.065	0.290	4.46	1.79	0.05

Devakate et al. (2009) achieved, 2.97-fold purification factor using ammonium sulphate precipitation, but could only recover 69,7 % of its initial activity. Rabelo et al. (2004) described bromelain purification using aqueous two-phase systems, in which was obtained a purification of 1.25-fold. Umesh Hebbar et al. (2008) achieved 106 % of enzyme activity recovery using reversed micelles systems.

Although the ethanol precipitation is a technique, considered by many to be obsolete, when compared to more modern techniques, such as affinity chromatography and ionic exchange, it is still a common method for recovery and partial purification of enzymes. The main purpose of a precipitation method is to concentrate the target molecule. Concentration is a necessary step to reduce volume, and thus, making it easier for transportation and handling. Considering the economics, it is always advisable to include concentration steps, even though if there is a compromise in the yield. Ethanol precipitation uses simple equipment, easy technique, and ethanol is an inexpensive organic solvent, largely produced in Brazil that can still be recovered by simple distillation. Moreover, the results showed here, showed that the recovery yield loss was insignificant when compared with other processes.

3.2 pH and Temperature Effect on Bromelain

The influence of pH on crude and purified bromelain is shown in Figure 1. The pH change showed an increase of crude bromelain activity until it reached pH 7.0, where it showed a little decline in activity, and then exhibiting a second peak of activity at pH 10.0. Although, after purification, pH change showed a shifted pattern, when compared to the crude extract, whereas activity reached its maximum at pH 8.0.

The two-peak pH profile is probably due to different types of proteolytic enzymes presented at the initial residue. Since, at the pineapple processing, it is not possible to remove all the fruit from the bark, it is possible to identify a two-peak profile at the crude extract. Similar results were found by Vallés et al. (2007). Ketnawa et al. (2012) analyzing two varieties of *Ananas comosus*, described that high enzyme activity was observed in pH ranging from 6.5 to 8.0, and the maximum activity was near pH 7.0. Depending on the variety studied, it was possible to observe the same two-peak profile.

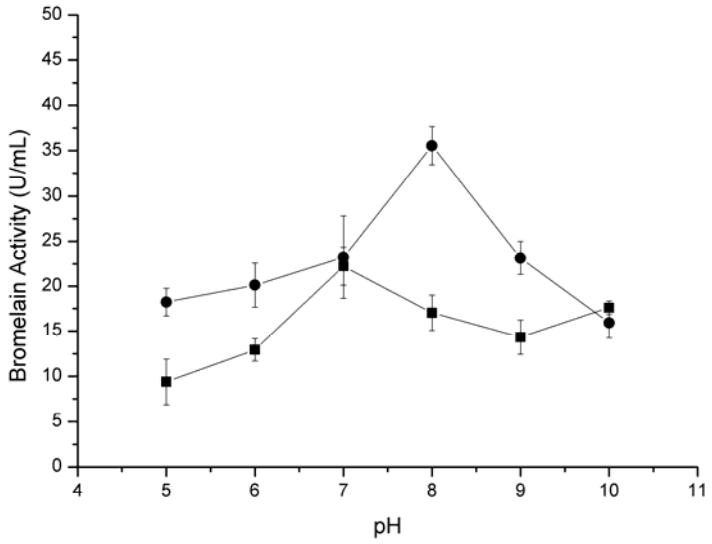

Figure 1: Effect of pH on enzyme activity. ● - Crude Extract; ■ - Ethanol Purified

The temperature influence on crude and purified bromelain is shown in Figure 2. The enzyme activity increased with increasing temperature until it reached 50 °C, where it began to decline rapidly. After purification, the increase of temperature showed a more abrupt increment at the enzymatic activity until it reached 60 ºC, where it began to decrease.

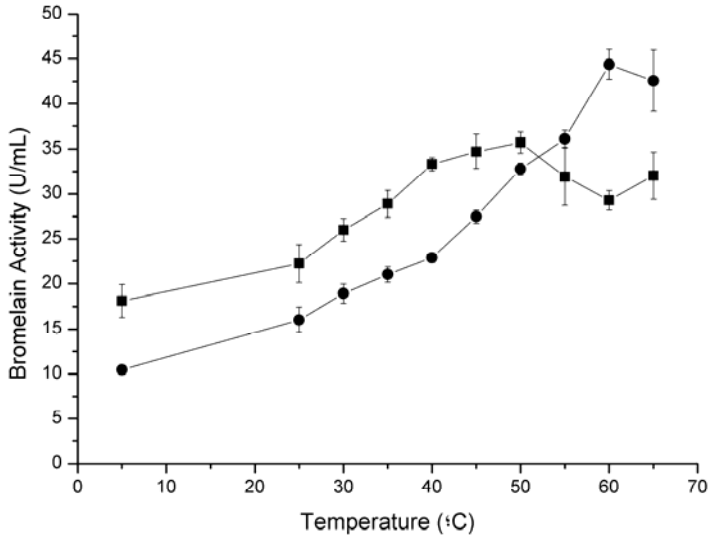

Figure 2: Effect of Temperature on enzyme activity. ● - Crude Extract; ■ - Ethanol Purified

These results are in accordance with the ones described by Ketnawa et al. (2012), Vallés et al. (2007) and Koh et al. (2006). At those studies, the maximum activity was reached at 60, 63 and 60 ºC, respectively. As the temperature increases, more molecules have kinetic energy to undergo the reaction. After the temperature is raised above the optimum temperature, a biochemical threshold, the systems energy is so high that peptide bonds and disulfide bonds are disrupted, therefore inactivating the enzymes.

The optimum temperature and pH for crude bromelain was 50 °C and pH 7.0, respectively, in which was observed the better enzymatic activity. On the other hand, optimum temperature and pH for purified bromelain was 60 °C and pH 8.0, respectively, in which was observed the better enzymatic activity. Bromelain is remarkably heat stable, retaining proteolytic activity up to 60 ºC where most enzymes are destroyed or denatured.

3.3 Stability at pH on simple extraction and precipitation by ethanol

The stability at different pH is shown in Figure 3. In crude bromelain showed higher stability at pH 8 and slightly stable at all pH studied (Figure 3a). In contrast, ethanol precipitation the enzyme showed poorer stability at pH 5, 6 and 10 over the time and higher stability at the others (Figure 3b). These results are in accordance with those described by Vallés et al. (2007), in which the enzyme was declared unaffected at a broad pH range (4-9). The purified bromelain showed a less stable enzyme probably due to removal of thermal stabilizing components once presented at the crude extract.

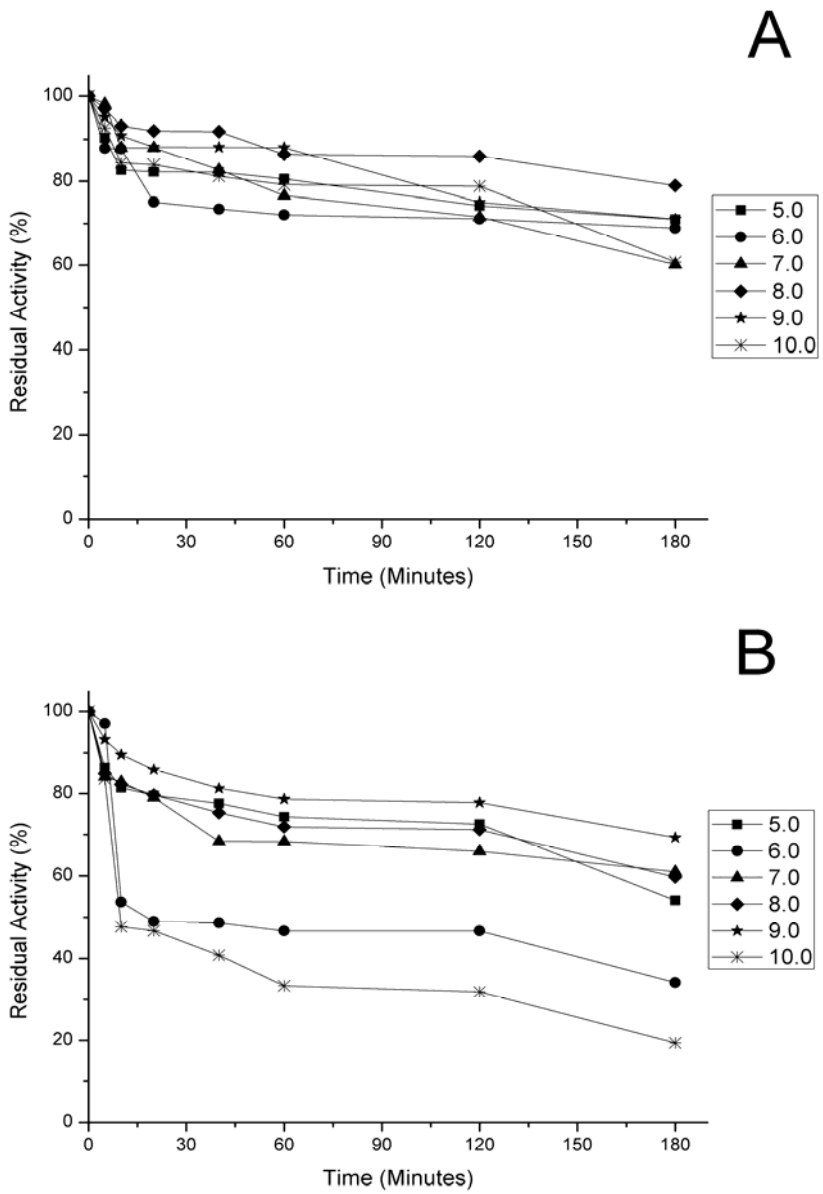

Figure 3: Stability at pH on crude bromelain (A) and ethanol purified (B)

4. Conclusion

The bromelain showed a maximum activity at pH 7 and 50 °C at the simple extraction and most proteolytic activity at pH=8 to 60 °C when being precipitated by ethanol. In relation to enzyme stability over time is noted that for simple extraction the pH that remained stable over was the pH=10, however, to the ethanol precipitate broth pH 5 and 6 were better than the stabilized bromelain.

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