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Extraction and Preliminary Characterization of Bromelain from Curaua (*Ananas erectifolius* L.B.SMITH) Purple and White

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The characterization and determination of kinetic parameters of bromelain extracted from curaua (Ananas erectifolius) white and purple varieties were studied. Optimal pH and temperature conditions for proteolytic activity were determined by azocasein method. The enzyme has shown optimum activity at pH 6.0 for white and 6.0 and 7.0 for purple curaua, and at 40 °C and 60 °C for white curaua and at 40°C for the purple curaua. The lowest result of Km and the highest result of Vmax/Km were found for purple curaua, but the two varieties showed affinity by the substrate, with K_m value of 185.18 μmol·L^{-1}min, V_{max} of 158.73 μmol·L^{-1} and activation energy of 13,903 for white curaua and K_m of 192.0 μmol·L^{-1}min, V_{max} of 188.68 μmol·L^{-1} and activation energy of 14,941 cal/mol for purple curaua. This enzyme showed lower activation energy (26,000~28,000 cal/mol) than those of other coexisting proteinases.

1. Introduction

Environmental and economical concerns are stimulating research in the development of new materials for construction, furniture, packaging and automotive industries. Particularly attractive are the new materials in which a good part is based on natural renewable resources, preventing further stresses on the environment by depleting dwindling wood resources from forests (Leão et al., 1998). Examples of such raw material sources are annual growth native crops/plants/fibers, which are abundantly available in tropical regions. These plants/fibers have been used for hundreds of years for many applications such as ropes, beds, bags etc. These renewable, non-timber based materials could reduce the use of traditional materials such as wood, minerals and plastics for some applications (Rowell et al., 2000). There is a tremendous interest by the pharmaceutical industry in exploring the rain forest for new drugs, but so far there has been little interest in exploring the rain forest for fast growing native plants as a fiber source (Leão et al., 2000).

Among the species in the Amazonia region with potential for the production of fibers, we highlight the curaua or Ananas erectifolius. In Brazil and outside, this plant fiber is frequently researched and has shown some significant results, making this species the most promising among those produced in Brazilian Amazonia for this purpose (Oliveira et al., 2008).

Ananas erectifolius is a plant species belonging to the botanical family Bromeliaceae, from which few data are available in the literature. As a medicinal plant, it is traditionally employed to heal wounds (Fujihashi and Barbosa, 2002).

As with every plant of the Bromeliaceae family, curaua contains as a constituent the enzyme bromelain. The purpose of this study was to characterize the proteolytic activity extracted from curaua white and

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purple species, using azocasein as substrate (pH 4.0 – 9.0 at 5 – 100 ºC), to obtain the best conditions of utilization for this enzyme, followed by a stability study to facilitate the enzymatic preparation for analytical, medical and industrial applications.

2. Material and methods

2.1 Plant material

The leaves of curaua (Ananas erectifolius L.B.SMITH) varieties “purple” and “white” used in this study were kindly provided by the company PEMATEC, (State of Pará – Brasil). The leaves were washed and used for the bromelain extraction.

2.2 Extraction and Lyophilization of Bromelain from leaves of curaua

The leaves of curaua (white and purple) were processed using a domestic juicer without water or buffer addition. For extraction of fibers, the solid material separation, was performed by centrifugation at 8,000×g for 30 min at 4 ºC then frozen in liquid nitrogen and lyophilized according to the procedure described by the manufacture (Lioalfa 6-80 Telstar, Spain) and stored in a freezer at -18 °C.

2.3 Optimal pH and temperature determination

The effect of the pH on the bromelain activity from curaua was investigated with azocasein as substrate, at pH values ranging between 4.0 and 9.0 with a constant temperature of 25 ºC. 100 mM sodium phosphate (pH 4.0 – 7.0) and 100 mM Tris-HCl (pH7.0 – 9.0) was used. The buffers used were adjusted to the desired pH to provide the specific reaction conditions for the enzyme.The effect of temperature on the bromelain activity from curaua was investigated with azocasein as substrate, between 5 ºC and 100 ºC.

2.4 Determination of kinetic parameters

The Michaelis-Menten constant, maximum reaction velocity, and the reaction specificity for azocasein were determined by plotting the activity data at optimum pH and temperature as a function of substrate concentration, according to the method of Lineweaver-Burk.

2.5 Bromelain activity

The protease activity was assayed by the method of azocasein (Sarath et al., 1989) by monitoring the rate of release of TCA soluble-azo-coupled peptides from azocasein at 440 nm. The reaction mixture contained 0.2 mL 2 % azocasein and 0.2 mL enzyme appropriately diluted in 25 $mmol \cdot L^{-1}$ potassium phosphate buffer at pH 7.0. The reaction was started by the addition of the enzyme aliquot. After incubating for 10 min at 37 ºC, the reaction was arrested by adding 1.2 mL of 5 % TCA. The test mixture was centrifuged at 6,000×g for 10 min to collect the supernatant containing TCA-soluble azo-coupled peptides. The absorbance of the supernatant at 440 nm was measured against the corresponding blank run side by side in the absence of enzyme. One protease unit (U) is defined as the amount of enzyme required to hydrolyse (and TCA solubilise) one micromole of tyrosine equivalents per minute from soluble casein under standard assay conditions.

2.6 Protein determination

The total protein content was analyzed by the Coomassie blue dye technique according to the procedure described by Bradford (1976). The intensity of the color was measured at 595 nm.

3. Results and discussion

3.1 Effect of pH on the enzymatic activity of curaua bromelain

The effect of pH on the enzyme activity was examined for the pH range 4.0 to 9.0. Activity was maximal at pH 6.0 for both, white and purple curaua. However, a lower intensity peak was observed at pH 7.0 for purple curaua, and suggests that there may be more than one protease in leaves curaua (Figure 2).

Ferreira et al. (2011) studied the two curaua varieties with BSA as the substrate and found values of optimal bromelain activity at pH 8.5 for both, white and purple curaua and a lower intensity peak at pH 5.5. Corzo et al. (2012) found optimal activity with azocasein as substrate, at pH 6.5 and described that there is not much research about how the optimum pH and temperature values change according to the substrate used. Azoalbumin and azocasein appear to be the most recommended substrates due to their lower Km. This data may be very useful in future studies on bromelain stability. All endopeptidases extracted from pineaple (*Ananas comosus*) display a wide profile of maximum activity around neutral pH (Rowan et al., 1990).

Commercial proteolytic enzyme preparations are evaluated according to their proteolytic activity, which should be measured within the optimal conditions of enzymatic reaction. Such data are extremely important in the strategy of purification of the enzyme bromelain and its industrial application, by avoiding

loss of activity through using inappropriate pH ranges and thereby reducing the efficiency of the method employed.

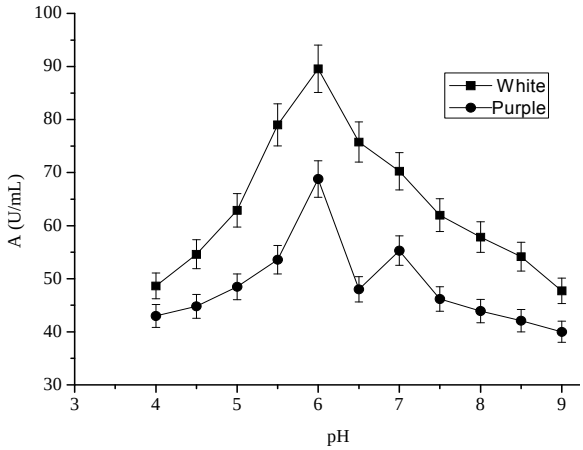

Figure 1. Effect of pH on the proteolytic activity of bromelain extracted from curaua leaves, white and purple species. Assays performed at 25 ºC, with azocasein as substrate

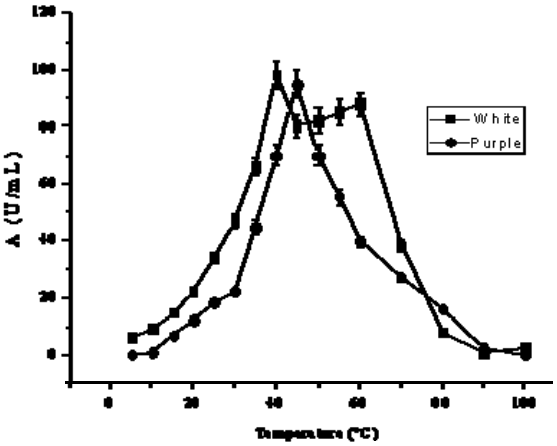

Figure 2. Effect of temperature on the proteolytic activity of bromelain extracted from curaua leaves, white and purple species. Assays performed at pH 6.0, 5 ºC to 100 ºC, with azocasein as substrate

3.2 Effect of temperature on the enzymatic activity of curaua bromelain

The temperature effect under tested experimental conditions showed a maximum at 40 ºC and 60 ºC for white curaua, and 40 ºC for purple curaua (Figure 3). The presence of two or more peaks, where activity was high, indicates again the presence of other proteases in curaua leaves. Ferreira et al.(2011) found maximal activity at 30 °C and another peak was observed at 10 °C for white curaua. For purple curaua, three peaks were observed where activity was maximum (10, 20 and 35 °C). Amid et al. (2011) used a purified recombinant bromelain and this exhibited the highest hydrolytic activity at 45 ºC under routine assay conditions. The activity of recombinant bromelain was moderate between 15 ºC and 35 ºC. The enzyme was devoid of detectable activity at 65 ºC. These results are comparable to the characteristics of pineaple bromelain, which is active between 40 ºC and 60 ºC (Okino et al., 2010).

Temperature is a critical agent on the enzyme activity. When the temperature rises, the activity initially increases, however the process thereafter declines due to the denaturing action of heat (Halpern, 1997). This effect can be seen also in the current results (Figure 3). Ferreira (2007) noted that the ideal temperature range for the characterization of bromelain from pineapple stem bark is between 30 °C and 40 °C, which reinforces the similar behaviour between bromelain from pineapple and from curaua. According to Sriwatanapongs et al. (2000), the different values for optimal activity of bromelain from pineapple, in the different studies, are probably due to the different substrates and methods used for determining enzyme activity.

3.3 Determination of kinetic constants and Activation energy

Determination of kinetic parameter characteristics of enzymes, such as kinetic constants, activation energy, temperature, pH, water activity profiles, etc, determine the usability and productivity of enzymes. For that reason, these characteristics should be determined for a newly discovered enzyme, an enzyme used in different reaction mediums or an enzyme used in different forms (such as free or immobilized). In developing an enzyme-based process, kinetic constants are the most important information which has to be determined. The Michaelis-Menten constant and maximal velocity of the enzyme for the hydrolysis of whole azocasein for both white and purple curaua are depicted as a Lineweaver-Burk plot (Lineweaver and Burk, 1934) in Figures 3 and 4.

The Michaelis-Menten constant was estimated to be 185.18 $\mu mol.L^{-1}min$ for white curaua and 158.73 $\mu mol.L^{-1}min$ for purple curaua. The maximal velocity was found to be 192 $\mu mol.L^{-1}$and 188.68 $\mu mol.L^{-1}$ for the white and purple curaua, respectively (Table 1).

Ferreira et al. (2011) determined K_m values of 0.0095 µmol/mL and V_{max} of 0.03757 µmol/mL.min, using BSA as substrate. Different kinetic models or constants can be obtained from the reaction of the same enzyme, which confirms the different results found by Ferreira et al. (2011) and this work. Typical examples of this situation are the V_{max} and K_m values of glucose oxidase (GOX) enzyme determined in different studies. V_{max} and K_m values for the free form of GOX were reported varying from 1.0 to 66.0 µmol/min·mg enzyme and from 2.9 to 30.0 mM, respectively (Blandio et al., 2001).

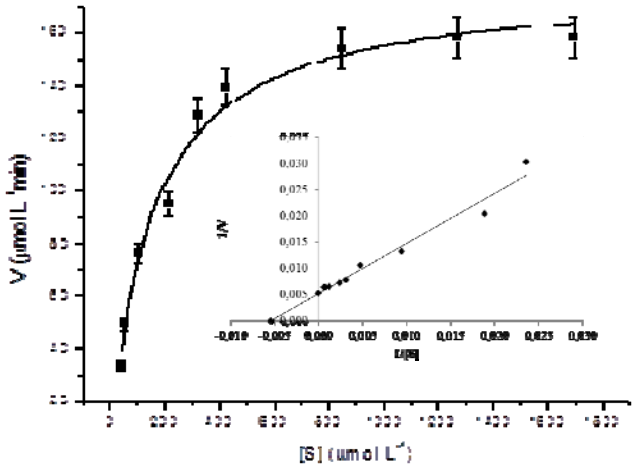

Figure 3. External Chart: Kinetics curve of hydrolysis of bromelain from the leaves of white curaua by azocasein at pH 6.0 and 25 °C. Internal Chart: Linearization of the kinetic data of hydrolysis of azocasein to determine the constants by the method of Lineweaver-Burk – white curaua at pH 6.0 and 25 °C

The primary reason is that the kinetic constants were determined at different or uncontrolled reaction parameters. Generally, it is not possible to compare V_{max} and K_m values of the same enzyme when obtained from different studies. The kinetic constants depend on the reaction parameters and should be determined for each reaction. Furthermore, the optimum reaction parameters are affected by substrate concentration not only at the extreme values, but also in the moderate concentration range (Boyacl, 2010). V_{max}/K_m is called the enzyme catalytic power parameter and, in this research project, was calculated to find the most effective substrate. It was found to be azocasein wth values of 1.036 and 1.180 for white and purple curaua (Table 1).

These results show that both white and purple curaua have affinity to the azocasein. Corzo et al. (2012) studied bromelain activity for different substrates and found that azocasein was the most suitable substrate for the fruit bromelain and highly recommended for the determination of the fruit bromelain activity at optimal reaction conditions

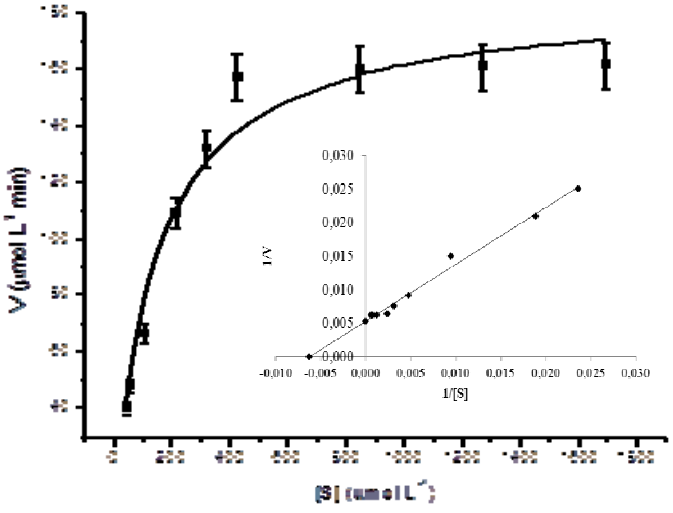

Figure 4. External Chart: Kinetics curve of hydrolysis of bromelain from the leaves of pruple curaua by azocasein at pH 6.0 and 25 °C. Internal Chart: Linearization of the kinetic data of hydrolysis of azocasein to determine the constants by the method of Lineweaver-Burk – purple curaua at pH 6.0 and 25 °C

Table 1. Kinetic parameters of bromelain extracted from white and purple curaua

Bromelain of curaua	K_m (µmol.L^{-1}min)	V_{Max} (µmol.L^{-1})	R^2	Specificity constant (V_{Max}/ K_m)
White	185.18	192.00	0.9753	1.036
Purple	158.73	188.68	0.9921	1.180

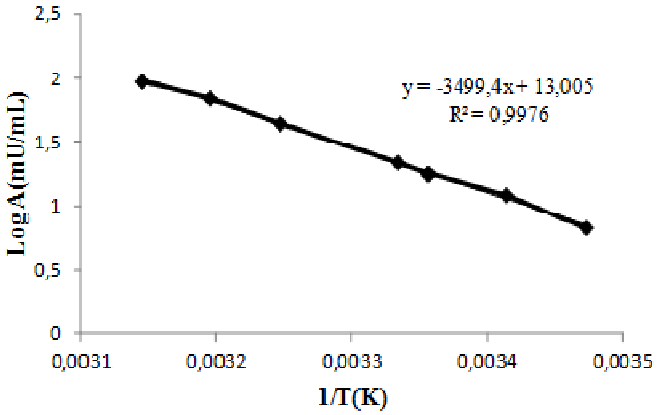

Figure 5. Determination of activation energy, through the effect of temperature on the enzymatic activity of bromelain extracted from purple curaua, based on the Arrhenius equation

The activation energy of the enzyme was measured for the activity against azocasein at 10 ºC to 60 ºC, pH=6.0. Activation energy was estimated according to the Arrhenius formula, where R is the universal gas constant, T - the absolute temperature and k - velocity constant (Nishizawa et al., 1972). The slope was determined by the linear regression method. The activation energy was estimated to be 13,913 cal/mol and 14,941 cal/mol for white and purple curaua, respectively, demonstrating that the white curaua had a lower activation energy compared to purple curaua.This shows that bromelain from this former species contains an enzyme with greater catalytic power (Figures 5 and 6).

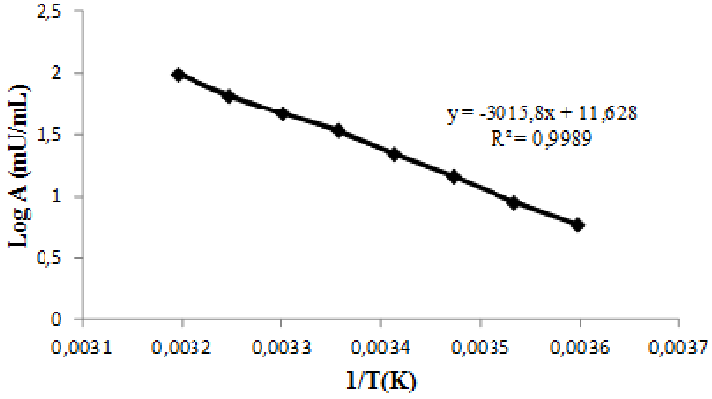

Figure 6. Determination of activation energy, through the effect of temperature on the enzymatic activity of bromelain extracted from white curaua, based on the Arrhenius equationThe curaua bromelain showed lower activation energy than those of other coexisting proteinases (26,000 ~ 28,000 cal/mol)

The results obtained were close to those observed by Akuzawa et al. (2010), when they studied the characterization of a cysteine proteinase from *Lactococcus lactis* and measured an activation energy of 11,500 cal/mol.

4. Conclusions

The leaves of curaua bromelain enzyme had its optimum activity at pH 6.0 for white and pH 6.0 to 7.0 for purple varieties, with azocasein as the substrate, at 25 ºC. The optimal temperature was 40 ºC and 60 ºC

for white and 40 ºC for purple curaua. The lowest value for Km and the highest value for was found for purple curaua, however both varieties of curaua showed affinity to the substrate, azocasein. The use of leaves extracted from curauá by industry could be maximized by extracting not only the fibers but also these proteolytic enzymes of great economic importance in the pharmaceutical industry, thereby reducing the environmental impact and waste of natural resources, as these are currently discarded.

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