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Comparative Study on Characterization of Bromelain Extracted from the Stem and Fruit of Pineapple (*Ananas comosus*)

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Abstract

Bromelain is a group of cysteine proteases that is found in pineapple (*Ananas comosus*) and has a wide range of medical and industrial applications. The present research work characterized bromelain extracted from the stem and fruit of fully ripened pineapple plants by homogenization in an aqueous buffer and then purified by ammonium sulfate precipitation and DEAE-cellulose chromatography. Enzyme activities were spectrophotometrically determined using N-α-Carbobenzoxy-Lysine p-Nitrophenyl Ester (LNPE) as substrate. The purified fractions of fruit bromelain (FBM) and stem bromelain (SBM) from the DEAE-cellulose chromatography (both at 0.05 mM NaCl conc.) were subjected to an evaluation of their temperature and pH optima, and kinetic parameters (K_m and V_{max}) determinations. The results obtained revealed that FBM had higher optimum temperature (45ºC) than SBM (35ºC). Both enzymes showed the same pH optima of 8.0. In addition, FBM (K_m = 0.39 mM and V_{max} = 0.38 µmol/min) was found to be proteolytically more active with greater affinity towards the substrate used than SBM (K_m = 1.17 mM and V_{max} = 0.25 µmol/min). This could serve as possible criteria for quality control/standardization protocols for both enzymes for specific applications and paves the way for exploring their potential usefulness.

Keywords: Cysteine Proteases, Bromelain, N-α-Carbobenzoxy-Lysine p-Nitrophenyl Ester, Optimum Temperature, Optimum pH, Kinetic Parameters.

1. Introduction

Bromelain is the collective name for a group of closely related (cysteine) proteolytic enzymes found in the tissue of the plant family of *Bromeliaceae*, of which pineapple, *Ananas comosus*, is the best known (Benucci *et al.*, 2011). Cysteine proteases in general are involved in a myriad of homeostatic as well as pathological processes in physiological conditions (Manoury *et al.*, 2011). However, it is not yet clear why pineapple plant synthesis and accumulate large quantity of bromelain apart from maybe it is an inherited trait from its evolutionary ancestors (Maurer, 2001). Nevertheless, it has been exploited in various industrial and food industries as well as in medicine (Grzonka *et al.*, 2007) since its discovery in 1875 (Kelly, 1996). Particularly in its therapeutic application where it is believed that bromelain and some other proteases (papain, trypsin and chymotrypsin) are absorbed from the gastrointestinal tract in a functionally intact form, even thought there are still skepticisms to accept such phenomenon by some people (Maurer, 2001).

Bromelain is accumulated in the entire plant and in all variety of pineapples to a different extent and properties (Gautam, *et al.*, 2010). The major endopeptidase present in extracts of plant stem is termed ‘stem bromelain’ (SBM), whereas the major enzyme fraction found in the juice of the pineapple fruit is named ‘fruit bromelain’ (FBM) (Maurer, 2001). Some other minor cysteine endopeptidases (ananain, comosain) are also found in the pineapple stem [Maurer, 2001; Grudkowska and Zagdanska, 2004].

Bromelain has a broad substrate specificity and hydrolyses a great variety of natural and synthetic substrates showing varying temperature and pH optimum conditions. Casein and haemoglobin are the most widely used natural substrates for this protease. Synthetic substrates are also very useful for assaying bromelain. The most commonly used are N-benzoyl-l-Arg-ethyl ester (BAEE) and N-benzoyl-l-Arg-amide (BAA) [Benucci *et al.,* 2011; Corzo *et al.,* 2011].

In a study using crude FBM on several natural substrates of bromelain conducted by Corzo *et al* (2011), azocasein had shown maximum activity at 50 ºC and pH optimum of 6.5, while azoalbumin showed it's at 55 ºC and pH 7.5, respectively. For casein, the activity increased over 59 ºC at optimum pH of 7.7 but afterword produced a sharp loss of enzyme activity. The optimum pH for sodium caseinate in the study was 6.5. But using haemoglobin shows a shape contrast, with an optimum pH of 2.9 (and optimum temperature 37ºC). The author(s) also reported, the optimum conditions for enzymatic hydrolysis using casein as a substrate were pH 8.0 at 50 ºC; pH 7.6 at 40 ºC and pH 6.0 at 30 ºC for FBM.

Similarly, the temperature optimum for SBM range from 50 ºC to 60 ºC and pH optimum of 6 to 8.5 as reported by Grzonka, *et al.* (2007). Stem bromelain (SBM) rapidly digests casein over the pH range 7.0–8.5, while the optimum for hydrolysing haemoglobin was around pH 5.0 and pH 5.0–6.0 and 5.0–8.0 for BAEE and BAA, respectively (Benucci *et al.,* 2011). Other substrates (and their Optimum pH at 25ºC) for SBM include: Suc-Phe-Leu-Phe-pNA (4.0), Suc-Ala-Pro-Leu-Phe-pNA (5.0), (H-Cys-pNA)2 (4.0), Z-Arg-Arg-pNA (8.0) and Bz-Phe-Val-Arg-pNA (8.0) (Benucci *et al.,* 2011).

It was also reported that the optimum temperature for maximum cumulated activity over time as 35–45 °C for both FBM and SBM. At room temperature, the enzyme can survive at least a week even under multiple freeze-thaw cycles (Hale *et al.,* 2005). Moreover, SBM presents isoelectric point at 9.5 (Wharton, 1974) while FBM presents isoelectric point at 4.6 (Nadzirah *et al.,* 2013).

The value of K_m and V_{max} varied considerably for FBM with different substrates. Azoalbumin: 0.026mM and 2.5 µMol/mins; azocasein: 0.037mM and 3.8µMol/mins; sodium caseinate: 0.088mM and 1.8µMol/mins; casein: 0.138mM and 3.0µMol/min and haemoglobin 0.165mM and 2.3 µMol/min, respectively for K_m and V_{max} (Corzo *et al.,* 2011).

Similarly, using Bz-Phe-Val-Arg-pNA substrate at varying pH for SBM gave the following results; at pH 3.2: 0.44 IU/mg and 0.343 mM; at pH 4.0: 0.42 IU/mg and 0.246 mM; at pH 5.0: 0.403 IU/mg and 0.046 mM and at pH 7.0: 0.41 IU/mg and 0.059 mM respectively for K_m and V_{max} as reported by Benucci *et al* (2011).

Enzyme production industry is virtually non-existing in Nigeria. However, enzymes are being used extensively in the country (hospitals, industries, schools, clinical and research laboratories). Bromelain production can actually pioneer a enzyme industry in Nigeria by choosing a cost effective production process and its characterization to enable standardization of the enzyme product(s). So, Bromelain production has the potential to boosts the non-existing enzyme industry. The present research was aimed at assessing and comparing the level of enzymatic activities of SBM and FBM extracted from pineapple plant.

2. Material and Methods

2.1 Material

2.1.1 Chemical Reagents and Equipments

All the chemical reagents and equipments are obtained from reliable sources and are of highest purity.

2.1.2 Sample Collection

The samples used in the research were fully ripened pineapple plants obtained from Uhi village, Uhunmwunde Local Government Area, Edo State, Nigeria. The samples were authenticated by a Botanist in Plant Science Department, Bayero University, Kano.

2.2 Methods

2.2.1 Extraction and Purification of Bromelain

Both SBM and FBM were extracted from the stem and fruit of fully ripened pineapple plants by homogenization in an aqueous sodium acetate buffer (pH 7.0; 100mM). They were purified by ammonium sulfate precipitation (45% saturation) and DEAE-cellulose chromatography with the fraction with highest activity at 0.05M NaCl concentration in 0.025 M Tris HCl buffer (pH: 8.1) (Babagana and Bala, 2015).

2.2.2 Measurement of Enzyme Activity

This was achieved by continuous spectrophotometric rate determination using the method described by Arnon (1976). The assay was carried in 30 mM Sodium Acetate Buffer with 100 mM Potassium Chloride and 1.0 mM L-Cysteine, pH 4.6 at 25°C using N α-Carbobenzoxy-Lysine p-Nitrophenyl Ester (LNPE) as a substrate. The change in absorbance per minute was determined with the unit definition of one unit of bromelain activity is equivalent to 1.0 µmol of p-Nitrophenol released from LNPE at pH 4.6 and 25ºC.

2.2.3 Determination of Optimum Temperature

The optimum temperatures of both enzymes were determined by measuring their activities at different temperature ranges (4, 15, 25, 35, 45, 55, 65, 75 and 85 ºC) using LNPE as a substrate. The result obtained was used to plot a graph of enzyme activity against temperature and the optimum temperatures were determined.

2.2.4 Determination of Optimum pH

The optimum pH of both enzymes were determined by measuring their activities at different pH with 0.1 M Glycine-HCl Buffer (pH: 2, 3 & 4), 0.1 M Sodium Phosphate Buffer (pH: 5, 6, 7 & 8), 0.1 M Tris-HCl Buffer (pH: 9 & 10) and 0.1 M Sodium Bicarbonate Buffer (pH: 11, 12 & 13). The optimum pHs of both enzymes were determined by plotting a graph of enzyme activity against pH.

2.2.5 Determination of Kinetic Parameters

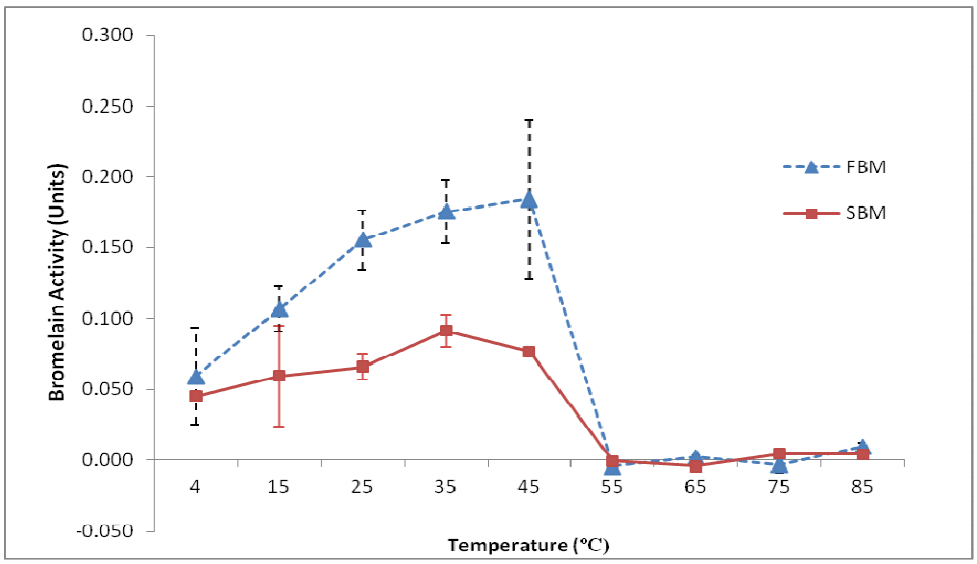
The kinetic parameters determination were carried out on both enzymes by determining the enzyme activity at varying substrate (LNPE) concentration (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mM). A double-reciprocal (Lineweaver-Burk) plot was used to determine the K_m and V_{max} values of both SBM and FBM.

3. Results and Discussion

3.1 Results

3.1.1 Results for Optimum Temperature of FBM and SBM

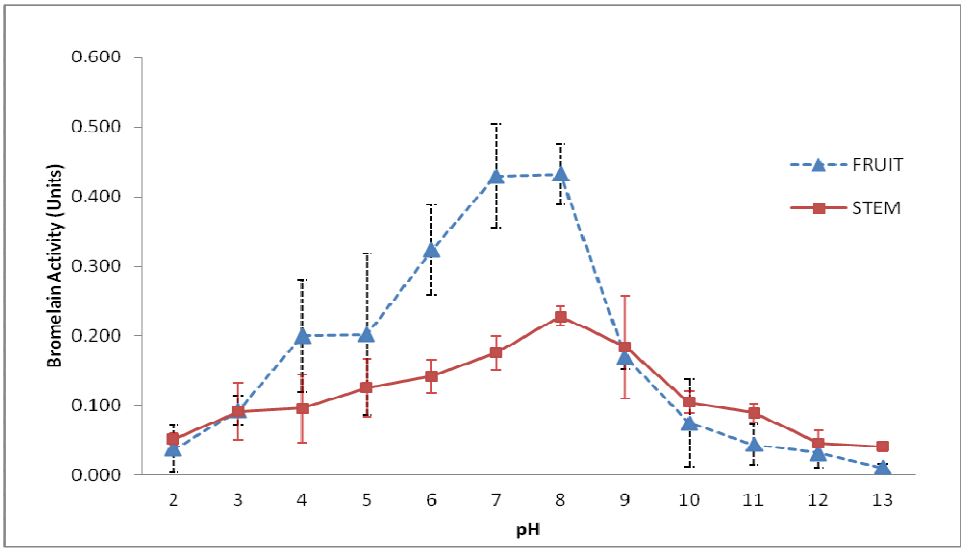
A graphical presentation of the effect of temperature on the activity of FBM and SBM are shown in Figure 1. Fruit bromelain (FBM) appears to have higher activity at optimum temperature of 45ºC than SBM (optimum temperature of 35 ºC). The activities of both enzymes started raising steadily, until each attained their optimum temperature, which the activities then plunges downwards (with recorded activity at 45ºC for stem bromelain) to near zero activity both at 55ºC. There was no significant recorded activities till the last temperature of 85ºC reading for both enzymes.



Values are presented as 'mean ± standard deviation' and 'n = 3'.
Figure 2: Effect of Temperature on FBM and SBM Activities

3.1.2 Optimum pH Result for Fruit and Stem Bromelain

The effect of pH on the activities of FBM and SBM is presented in Figure 2. The optimum pH for both enzymes was observed to be (pH) 8. Both enzymes' activity appears to raise steadily, until each attains its optimum pH, then started falling steadily till the last recorded pH of 13. FBM showed higher peaks and steeper clips then SBM.



Values are presented as 'mean ± standard deviation' and 'n = 3'.
Figure 3: Effect of pH on FBM and SBM Activities

3.1.3 Estimations of Kinetic Parameters for FBM and SBM

The results for kinetic parameter determinations for FBM and SBM are presented in Table 1. FBM appeared to have lower K_m (0.39 mM) and higher V_{max} (0.38) compared with SBM (K_m of 1.17 mM and V_{max} of 0.25).

Table 1: Kinetic Constants Km and Vmax for FBM and SBM

Enzymes	K_m (mM)	V_{max} (µmol/min)
FBM	0.39	0.38
SBM	1.17	0.25

3.2 Discussion

3.2.1 Optimum Temperature Analysis

From the results presented in Figure 1, it could be seen that the optimum temperature were 35 ºC and 45 ºC, for SBM and FRM, respectively. The reason for such a pattern was probably due to the fact that, as the temperature increases, more molecules gain enough kinetic energy to undergo the reaction. If the temperature is raised above the optimum point, the kinetic energy of the enzyme and water molecules is so great that the structure of the enzyme molecule starts to be disrupted. Therefore, a decrease in activity was detected when the temperature increased beyond the optimum (Ketnawa *et al.,* 2011). This pattern shows a resembles (more so with FBM) with results obtained by Bala *et al* (2013) which compares commercial (stem) bromelain with recombinant bromelan with both optimum temperature at 45ºC. Similarly, Amid *et al* (2011) reported a similar optimum temperature of 45ºC with recombinant bromelan. Knowing the optimum temperature would help to explore the application of the enzyme (Ketnawa *et al.,* 2011).

3.2.2 Optimum pH Analysis

The results for optimum pH analysis for FBM and SBM presented in Figure 2 showed that both enzymes have an optimum of pH 8.0. This finding is in accordance with many literatures [Maurer, 2001; Grudkowska and Zagdanska, 2004; Corzo *et al.*, 2011; Bala *et al.,* 2012; Bala *et al.,* 2013] that all reported it at 8.0 or about. However, Amid *et al* (2011) reported the pH optimum of 4.6 for recombinant bromelain. The pattern observed in Figure 2 was likely to be due to fact that the catalytical dyad of Cys25 and His159 of bromelain are in an ion-pair **–S^-...H^+Im–** at/about this pH range [Grzonka1 *et al.*, 2001; Soares *et al.*, 2012]. The higher peaks and steeper clips for FBM observed is due its higher proteolytic activity and broader pH range over SBM [Grudkowska and Zagdanska, 2004; Corzo *et al.*, 2011].

3.2.3 Kinetic Parameters Analysis

The results for Kinetic parameters estimation was shown in Table 1. The observed lower K_m (0.39 mM) of FBM over SBM's K_m (1.17 mM) suggested that former has higher affinity towards the substrate used in this research over the later. However, FBM's V_{max} (0.38 µMol/min) was observed to be higher than SBM's (0.25 µMol/min). These two factors could explained why FBM exhibibited higher proteolytic activity over SBM (Grudkowska and Zagdanska, 2004). The K_m for SBM (2.8 mM) and the V_{max} (0.7 µMol/min) reported by Yodoya *et al* (2003) were higher than the ones reported in this research using *N*-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) as substrate. This suggests that bromelain has different activity and affinity on various substrates.

4. Conclusion

The findings of this study showed that both FBM and SBM had the same optimum pH using LNPE as a substrate. However, FBM had higher optimum temperature and higher catalytic activity over SBM using the same substrate. This could serve as possible criteria for quality control/standardization protocols for both enzymes for specific applications and paves the way for exploring their potential uses.

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