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Physicochemical studies of bromelain precipitated by ammonium sulphate from pineapple (*Ananas comosus*)

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ABSTRACT

Bromelain, a protease enzyme found in pineapple, was recovered from the different parts of the fruit; crown, flesh and peel of pineapple (*Ananas comosus*).Each part of pineapple after separation was weighed blended filtered and then filtrate was precipitated with ammonium sulphate and centrifuged. All extracts from each stage were collected and assayed for bromelain activity. The concentration of protein was also measured by using bovine serum albumin as standard while proteolytic activity was determined using azocasein as substrate at standard conditions. The effects of temperature and pH on bromelain were evaluated as well as the effect of activators and the enzymes proteoytic activity on different substrates. Results showed that bromelain was precipitated successfully in the 20-40% ammonium sulphate precipitation, its optimum temperature and pH being obtained at 40 ºC and 7.0 respectively. Also, cysteine was a better activator than Ethylenediamine tetraacetic acid (EDTA) and bromelain's activity was highest when bovine serum albumin (BSA) was used as substrate. The bromelain recovery from this purification method is a viable process which can be applied for possible industrial purposes especially from pineapple which are discarded as waste.

Keyword: Bromelain, pineapple, azocasein, ammonium sulphate, protease, enzyme
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INTRODUCTION

Bromelain is a complex mixture of different thiol endopeptidases and other components likephosphatase, glucosidase, peroxidase, cellulase, escharase, and several protease inhibitors (Ramalingam et al*.,* 2012).*In vitro* and *in vivo* studies demonstrate that bromelain exhibits various fibrinolytic, antiedematous, antithrombotic, and anti-inflammatory activities as bromelain is considerably absorbable in the body without losing its proteolytic activity and without producing any major side effects (Rajendra et al*.,* 2012). Biochemical studies have shown that bromelain could be obtained from all parts of pineapple such as fruit, stem and peel with different methods of protein precipitation (Ramalingam et al*.,* 2012; Bresolin et al*.,* 2013; Omotoyinbo and Sanni, 2017). Clinical studies have also shown that bromelain may help in the treatment of several disorders such as angina pectoris, bronchitis, sinusitis, surgical trauma, and thrombophlebitis, debridement of wounds, and enhanced absorption of drugs, particularly antibiotics (Lakshminarasimaiah et al., 2014; Ali et al*.,* 2015). It also relieves osteoarthritis, diarrhea, and various cardiovascular disorders (Lakshminarasimaiah et al., 2014), while studies on its possible anticancer activities are not fully proven although it has been seen to promote apoptotic cell death. Bromelain has also been widely used in food, pharmaceutical, cosmetic and other industries as well (Mohan et al., 2016). In this study, some physicochemical parameters of partially purified bromelain obtained from different parts of *Ananas comosus* pineapple fruit variety were studied.

MATERIALS AND METHODS

Pineapple fruits *Ananas comosus* were bought from a local fruit market at the Federal University of Technology Akure (FUTA) in Akure, Nigeria. The pineapple fruits

Table 1. Bromelain purification from three parts of pineapple using ammonium sulphate precipitation.

Variety	Parts	Vol. (ml)	Enzyme activity (U/ml)	Protein conc. (µg/ml)	Specific activity (U/µg)	Yield	Fold
Ananas comosus	CROWN	7	0.0074	1.32	0.006	43.08	0.43
	FLESH	7	0.0102	1.66	0.006	31.58	0.32
	PEEL	5	0.0099	1.39	0.007	70.00	0.70

were identified at the Department of Crop Science and Production in FUTA. The pineapple fruits were then washed thoroughly with distilled water and then weighed before been peeled and sliced to separate different parts; crown, flesh and peel were weighed to ascertain percentage part weights. One hundred (100 g) of each part was blended with sodium phosphate buffer 0.1 M pH 7.0 (w/v). The mixture obtained was then filtered using cheesecloth, to obtain the juice which was further centrifuged at 6000 rpm for 20 min at 4°C. The supernatant obtained (crude bromelain) was used for further experiments.

Bromelain activity

The enzyme activity was assayed by the azocasein method as described by Oliveira et al., (2006). The assay mixture contained 125 µL of substrate and 125 µL of extracted enzyme was incubated for 10 min at 37°C and the reaction was stopped by addition of 750 µL of 5% trichloroacetic acid (w/v). The samples were centrifuged at 6000 rpm for 10 min at 4°C.

Ammonium sulphate precipitation

The precipitation of bromelain was carried out using 50 ml of the crude bromelain for all the parts considered. Ammonium sulphate was added to get 20% saturation (obtained using encorbio ammonium sulphate calculator) with constant stirring using a magnetic stirrer at 4°C. The stirring was continued for 10 min after complete addition of the ammonium sulphate to allow attainment of equilibrium between the dissolved and aggregated protein. The salt enriched solution was then subjected to centrifugation at 6000 rpm for 20 min and the precipitate was collected. The supernatant was recollected and the volume obtained was then brought to 40% saturation with ammonium sulphate.

Dialysis

The dialysis membrane was filled with bromelain extract obtainedfrom 20 – 40% ammonium sulphate fraction and sealed. The dialysis membrane was kept in a beaker containing cold sodium phosphate buffer (0.01 M at pH 7). The dialysis bag along with the buffer was continually stirred using magnetic stirrer in a cold room, while the buffer was changed every 6 h over a 48 h period.

Protein estimation assay

The protein concentration as determined according to Lowry's method (Lowry et al., 1951) using bovine serum albumin (BSA) as standard. The protein concentration of the each variety was estimated from the standard graph.

Effect of pH on bromelain activity

The effect of pH was evaluated using buffers of different pH ranges: glycine/HCl buffer (pH 3), sodium acetate/acetic acid buffer (pH 4 – 6) and sodium phosphate buffer (pH 7 - 8). The bromelain activity was carried out according to earlier assay procedure.

Effect of temperature on bromelain activity

The effect of temperature on bromelain was assayed between 30 and 80°C at an 10°C interval.

Activation of bromelain with different activators

The percentage enzyme activation of ammonium sulphate fraction by different activators: EDTA and Cysteine. Activation of bromelain with EDTA was subjected at concentrations; 0.1, 0.2 and 0.5 mM while for cysteine was at concentrations 5.0, 2.5 and 1.0 mM.

RESULTS AND DISCUSSION

The crude extract from three different parts of the pineapple extracted showed the cysteine protease bromelain activity (Table 1). Protein precipitation involved the addition of salts, polar solvents, non-polar solvents, and organic polymers into cell extracts or by varying the temperature or pH. They are salted out as co-precipitate by ammonium sulfate because the saturation concentration provides high molarity that causes precipitation of most proteins. Hence, when the solubility of proteins in solution is reduced by increasing the concentration of precipitating agents, salting out will occur (Ashrad et al., 2014). Bromelain was precipitated by ammonium sulphate between 20-40 % saturation with the pineapple peel having the best yield of 70 % and specific activity of 0.007 U/µg. The result of this study showed similar purification fold of 0.70 to that bromelain from pineapple peel, but slightly lower yield when

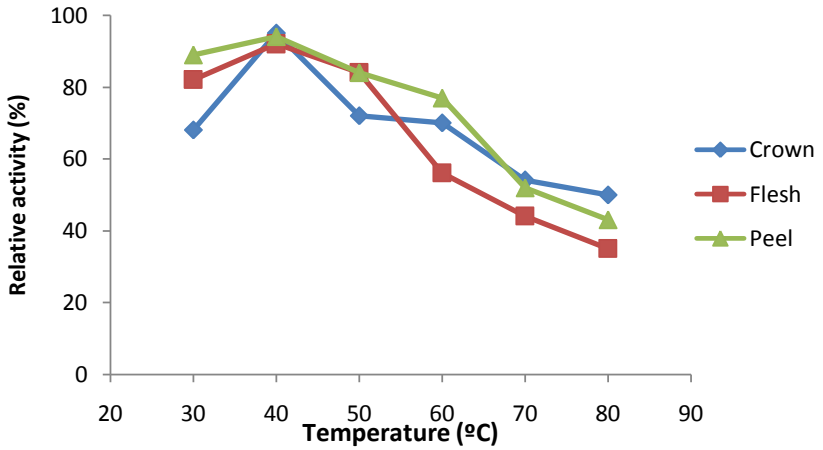

Figure 1. Effect of temperature on bromelain activity obtained from *Ananas comosus.*

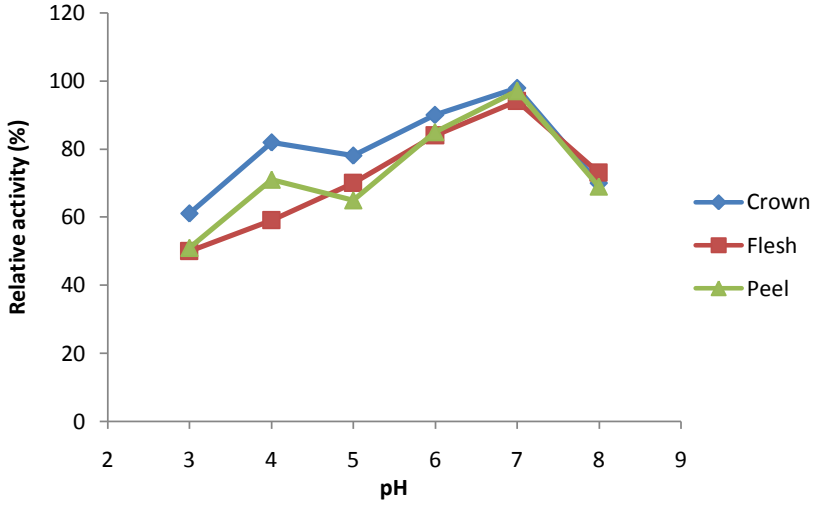

Figure 2. Effect of pH on bromelain activity obtained from *Ananas comosus.*

compared to the report of Omotoyinbo and Sanni (2017), using 70 % ethanolic precipitation. Bromelain generally function as bacteriostatic and protect most proteins from denaturation in solution state, which makes it important in biotechnological and pharmaceutical applications. The decrease of repulsive forces and the dominance of attractive forces at the isoelectric point cause protein aggregates to reduce their solubility (Ashrad et al., 2014).

The effect of temperature on the activity of bromelain for this variety of the pineapple shows a gradual increase in activity of enzyme as temperature increased and shown in (Figure 1).The optimum temperature obtained for bromelain optimal activity from all the three parts of the pineapple was 40°C. This was similar to the optimum temperature range of 30°C and 40°C for bromelain from pineapple stem bark (Ferreira et al., 2011). Although, gradual decrease was observed in bromelain activity from the peal and flesh part of the pineapple between 40 to 50°C. This result is also comparable to the characteristics of pineapple bromelain with good activity between 40 and 60ºC (Okino et al., 2010).

Furthermore, bromelain activity was observed to be stable with approximately 50% enzyme activity at temperature range of 30-60ºC until it finally began to reduce in activity at 70ºC. 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).

The effect of pH on the activity of bromelain is shown in (Figure 2**)**. Optimal pH for bromelain activity was at pH 7.0 for all parts of the pineapple considered in this study. The enzyme was more active in the pH of 4, 6, and 7. This result supports the bromelain activity as reported by

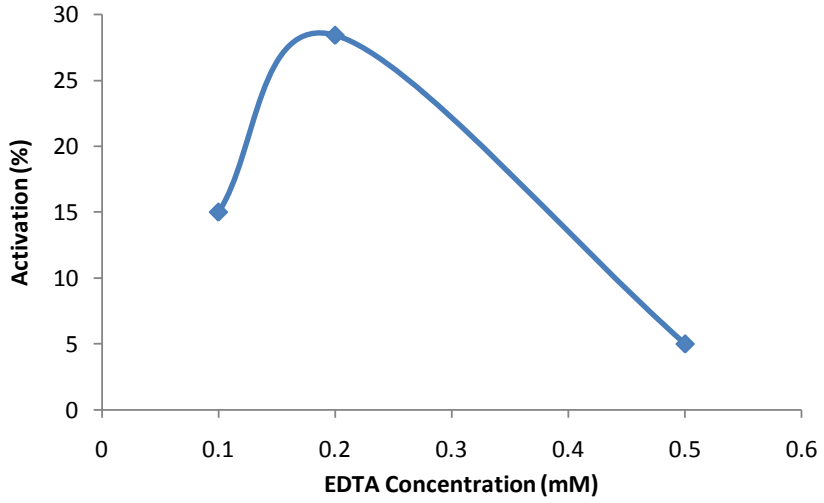

Figure 3. Activation of bromelain in the presence of different concentrations of EDTA.

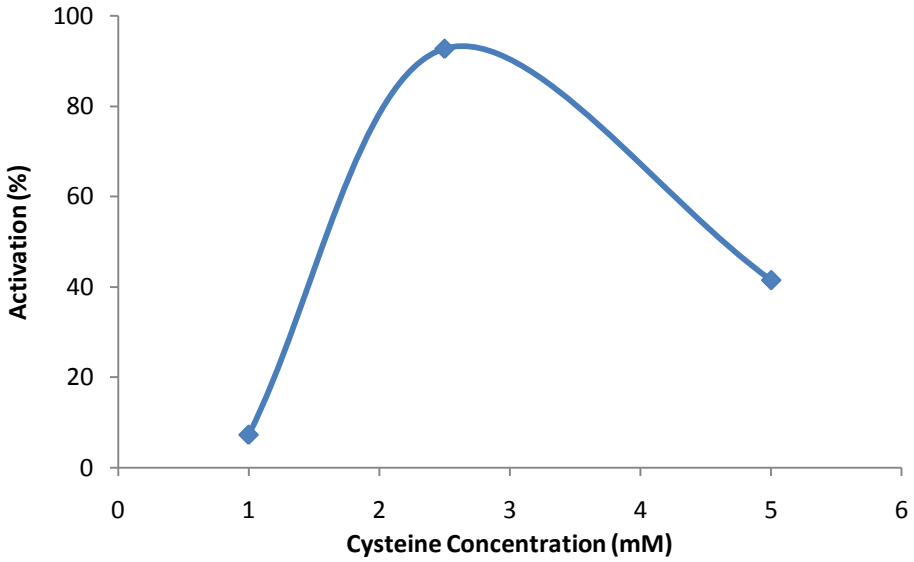

Figure 4. Activation of bromelain in the presence of different concentrations of cysteine.

Martowibowo et al., (2009) and Omotoyinbo and Sanni (2017). However, beyond this pH value, the activity declined. The effect of pH can be explained considering the surface charge on the adsorbent material. Enzymes are generally sensitive to pH changes in their environment and have optimum pH 7.0 at which they have their maximum activity, beyond which their activity decreases (Afiukwa et al., 2010).At low pH, due to the high positive charge density, electrostatic repulsion will be high, resulting in lower uptake of positively charged bromelain. The isoelectric point of bromelain is 9.55 (Wharton, 1974), hence the adsorption decreases at a higher pH. Some proteins contain acid labile groups and even relatively mild acid treatment may cause irreversible loss of function.

The percentage of bromelain activation in the presence of EDTA and cysteine (Figures 3 and 4) showed that the maximum percentage activation of bromelain was 28.4 % at 0.2 mM EDTA, while the presence of cysteine showed better activation of bromelain with maximum percentage activation of 92.6 % at 2.5 mM of cysteine. This support the hypothesis that EDTA is capable of chelating heavy metal inhibitors such as silver by forming ring metal-ligand complex making the active site available for catalysis by preventing the action of such metals on the enzyme (Roger, 2005). Cysteine is present at the active

site of bromelain (Godfred and Reichelt, 1983) which aids it catalytic activity. Therefore, using cysteine as an activator will prevent the oxidation of cysteinyl-residue at the active site, making the site available for activity. However, when activation was carried out with a mixture of cysteine and EDTA at concentrations of maximum activity observed earlier showed that 8.9 % activation of bromelain was obtained with mixture of 2.5 mM cysteine and 0.2 mM EDTA, while 22.7% activation was obtained with mixture of 2.5 mM EDTA and 0.2 mM cysteine.

Conclusion

Crude bromelain extracted and partially purified by ammonium sulphate precipitation from pineapple parts of *Ananas comosus* that were investigated in this study, showed that pineapple peel gave best bromelain yield, while the optimal temperature and pH for enzyme activity of 40^{o}C and 7.0 respectively for all the parts, and that better activation of bromelain in industrial process will be obtained using cysteine as an addictive.

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