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Comparative Study on The Extraction and Purification The Stem and Fruit Bromelain From Pineapple (*Ananas comosus*)

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Abstract
Bromelain is a mixture of enzymes from the pineapple that contains, among other components, various closely related proteases. It has a wide industrial and therapeutic applications. The present research extracted bromelain from the stem and fruit of fully repined pineapple plants cultivated in Nigeria by homogenization in an aqueous buffer and purified by ammonium sulfate precipitation and DEAE-cellulose chromatography. Enzyme activities were determined using N α-Carbobenzoxy-Lysine p-Nitrophenyl Ester (LNPE) as substrate, spectrophotometrically. Total protein was estimated using Bradford method. The results obtained, showed that stem bromelain (SBM) was better suited for the purification procedure adopted with the percentage yield of 463% and purification fold of 4.64 over fruit bromelan (FBM) which showed a percentage yield and purification fold of 0.23 and 23.4%, respectively.
Keywords: cysteine proteases, bromelain, stem bromelain, fruit bromelain, LNPE, bioremediation

1. Introduction

Pineapple (*Ananas comosus*) is one of the most important (sub)tropical plant in the world (Carlier *et al.*, 2007). It is the only source of a cystein proteases, bromelain (Grzonka *et al.*, 2007). Bromelain is a mixture of enzymes from the pineapple that contains, among other components, various closely related proteinases (Maurer, 2001; Pavan *et al.*, 2012). It shows a proteolytic modulation of the cellular matrix in numerous physiologic processes, including (*in vitro* and *in vivo*) antiedematous, antiinflammatory, antithrombotic anticancerous and fibrinolytic activities among other functions. It has numerous medical and industrial applications (Chobotova *et al.*, 2010; Pavan *et al.*, 2012). Bromelain is accumulated in the entire pineapple plant and in all varieties to a different extent and properties (Gautam, *et al.*, 2010). There two chemically distinct *iso*-enzymes of bromelain, the major endopeptidase found in the pineapple stem is known as 'stem bromelain' (SBM) (EC.3.4.22.32) while the major endopeptidase found in the pineapple fruit is termed as 'fruit bromelain' (FBM) (EC.3.4.22.33) (Maurer, 2001). Bromelain is prepared commercially from a cooled pineapple juice by centrifugation, ultrafiltration, and lyophilization. The process yields a yellowish powder, the enzyme activity of which is determined with different substrates such as casein (FIP unit), gelatin (gelatin digestion units), or chromogenic tripeptides (Pavan *et al.,* 2012).

Extraction, isolation, purification of bromelain are carried out by mean of various biochemical techniques, such as centrifugation, Isoelectric Focusing (IEF), Reverse Micellar System (RMS), Aqueous Two-Phase Extraction System (ATPES), Affinity Based Reverse Micellar Extraction and Separation (ARMES), ultrafiltration, lyophilization, ethanol precipitation, ammonium sulphate precipitation, dialysis ultrafiltration, cation exchange chromatography, SDS-PAGE, multicathodal-PAGE, lyophilization (freeze drying and spray drying) etc (Devakate *et al.,* 2009; Pavan *et al.,* 2012; Nadzirah *et al.,* 2013). Each technique (step) has its own advantage and limitations. In the study conducted by Nadzirah *et al* (2013), which compares four techniques; RMS, ATPES, cation exchange chromatography and ammonium sulphate precipitation, cation exchange chromatography had shown the best bromelain purification technique with purification fold of 10.0. This is followed by RMS containing CTAB(Cetyl Trimethylammonium Bromide)/ isooctane/ hexanol/ butanol, ATPE containing polyethylene glycol (PEG) polymer, ammonium sulphate precipitation and ATPE containing PEO-PPO-PEO with purification fold of 5.2, 4.0, 2.81 and 1.25, respectively.

Similarly, a study by Hebbar *et al* (2008) using RMS containing CTAB/isooctane/hexanol/butanol and AOT/isooctane to extract and purify bromelain from crude aqueous extract of pineapple wastes (core, peel, crown and extended stem), A good activity recovery of 106% and purification of 5.2 fold of bromelain was obtained. In the study, the peel, stem and crown resulted in purification folds of 2.1, 3.5, and 1.7, respectively. In another purification of bromelain from pineapple wastes using precipitation by ethanol (30% and 70%), a reported purification factor of 2.28 fold and yielded more than 98% of the total bromelain activity was observed (Soares *et al.,* 2012)

The adsorption of bromelain from an aqueous solution by polyacrylic acid (PAA)-bound iron oxide magnetic nanoparticles was studied by Chen and Huang, (2004). In it, the adsorption and desorption of bromelain were quite fast with 87.4% retention of activity.

Kumar *et al* (2011) pioneered the use ARMES to extract and purify bromelain from pineapple (*Ananas comosus* L. *Merr.*) waste. The extraction resulted in purification of 12.32 fold with an activity recovery of

185.6%, which is higher than that reported for conventional RME.

In an ATPES study by Babu *et al* (2008) on employed for separation and purification of mixture of enzymes (bromelain and polyphenol oxidase) from the pineapple (*Ananas comosus* L. *Merr.*). Bromelain preferentially partitioned to the top (polyethylene glycol) phase, while polyphenol oxidase to the bottom (potassium phosphate) phase. The polyethylene glycol/potassium phosphate system (comprising of 18% PEG 1500 and 14% phosphate) resulted in about 228% activity recovery and 4.0-fold increase in purity in case of bromelain and about 90% activity recovery and 2.7-fold increase in purity of polyphenol oxidase.

A research carried out on the purification and drying of bromelain by precipitation, chromatography followed by drying using freeze dryer (96% yield) and spray dryer (50-70% yield), the purity of the enzyme obtained was 2.8 times more than that of a commercial sample (Devakate *et al.,* 2009). Bromelain was also reported to have been recovered from ground pineapple stem and rind by means of precipitation with alcohol at low temperature. In the end, the processing performance efficiency was enhanced and so was the quality (enzyme activity) of the product (Silva *et al.,* 2010).

Nigeria was ranked as 8th largest producers of pineapple in 2009 (FAOSTAT, 2014). However, large percentage of these pineapples are consumed locally but not exported (Carlier *et al*., 2007). As such pineapple waste presents a pollution problem in the country (Contreras, *et al.*, 2009). Stem bromelain production has the potential to reduce pollution problems in the country. The aim of this research is to assess and compare the level of enzyme purification fold and yield of SBM and FBM obtained from pineapple by extracting and preparing a crude enzyme extracts both from the stem and fruit of the pineapple.

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

Fresh samples used in the research were fully ripened pineapple plant obtained from Uhi village, Uhunmwunde Local Government Area, Edo State, Nigeria. The samples were identified in biological science department, Bayero University Kano.

2.2 Methods

2.2.1 Extraction (method adopted from Gautam *et al* (2010))

a. Stem: Fresh pineapple stems parts were washed with 0.1% hydrogen peroxide solutions. It was then peeled off, cut into small pieces and weighed (77.3g wet weight). The juice were collected from the fresh pineapple stem part by homogenization using Qlink Turinar 2L capacity blender, in the presence of sodium acetate buffer solution (pH: 7.0; Conc.: 100mM) which was then filtered. Sodium benzoate was also added to served as a preservative at a concentration of 1 g per kg of stem. The filtrate was collected as 'Stem Crude Lysate' (SCL).

b. Fruit: Fresh pineapple fruit was cleaned and cut into small slices and weighed (600 g wet weight). The juice was extracted using a homogenizer (Qlink Turinar 2L capacity blender) and then filtered. Sodium benzoate was also added to the filtrate, to served as a preservative at a concentration of 0.2 g for each 100ml of filtrate. The filtrate was then labelled 'Fruit Crude Lysate' (FCL).

2.2.2 Purification (method adopted from Gautam *et al* (2010))

The crude lysates (SCL and FCL) were centrifuged for 10 minutes at 2,000 rpm, 10 minutes at 4,000 rpm and 15 minutes at 4,000 rpm consecutively. The supernatants were then collected and labelled as 'Stem Crude Extract' (SCE) and 'Fruit Crude Extract' (FCE) for stem and fruit, respectively

a. Ammonium sulphate precipitation: Ammonium sulphate precipitations were carried out on both enzymes' crude extract, by adding 13.2 g of ammonium sulphate salt, pinch by pinch, to 30 ml SCE and FCE with continuous stirring for 45 minutes each. The sample solutions were incubated overnight at 4ºC. After the incubation, the precipitated enzymes were centrifuged at 4,000 rpm for 30 minutes. The pellet of both extracts were collected and dissolved in 10 ml of 10 mM Tris HCl buffer (pH: 8.0).

b. Ion exchange chromatography: Diethylaminoethyl (DEAE) cellulose beds, of 15 cm thickness, were prepared in a chromatography column and equilibrated with 0.5 M sodium phosphate buffer solution (pH: 8.0) followed by eluting buffer 1 (pH: 8.1) which contains; 25 mM Tris HCl and 25 mM NaCl. The enzyme sample was poured onto the column, from the sides, without disturbing the DEAE cellulose bed and allowed to settle. The sample was eluted using the first eluting buffer 1 (i.e. 25 mM Tris HCl and 25 mM NaCl), pH: 8.1. The elution was at a flow rate of 6 drops per minutes and the eluate was collected in 5 ml capacity plain container. The same process of elution was carried out using solution buffer 2, 3, 4, 5 and 6 containing 50 mM, 75 mM, 100 mM, 125 mM and 150 mM NaCl, respectively (pH: 8.1). The enzyme samples were poured onto the column again but with the enzyme being eluted using eluting buffer 2 (10 ml of 25 mM Tris HCl and 50 mM NaCl), pH: 8.1. The process of elution was continued using eluting buffers 3, 4, 5 and 6 containing 75 mM, 100 mM, 125 mM and

150 mM of NaCl, respectively. Finally, all ion-exchange eluates (5ml each) were assayed for enzyme activities and total protein concentrations determined by Bradford method.

2.2.3 Determination of Enzyme Activity

Bromelain activity was determined by continuous spectrophotometric rate determination using the method described by Arnon (1976). Two test tubes were set up (Test and Blank). Exactly 2.60 ml of Reagent A (30 mM Sodium Acetate Buffer with 100 mM Potassium Chloride and 1.0 mM L-Cysteine, pH 4.6 at 25°C) was pipetted in the test tube labelled 'Test' while 2.70 ml was pipetted into the test tube labelled 'Blank'. This was followed by addition of 0.10 ml of the enzyme sample to the test tube labelled Test alone. They were mixed by inversion and equilibrate to 25º C. Then 0.10 ml of Reagent B (the substrate: 50 mM Na-CBZ-L-Lysine p-Nitrophenyl Ester) was added to both test tubes. They were immediately mixed by inversion again and the increase in $A_{340\ nm}$ was recorded for approximately 5 minutes. Finally, the ΔA_{340nm}/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.

2.2.4 Total Protein Concentration Determination

Total protein was determined using BioAssay Systems' QuantiChrom^{TM} protein assay kit based on an improved Coomassie Blue G method (Bradford, 1976) using Bovine Serum Albumin (BSA) as standard.

3. Result and Discussion

3.1 Result

The result obtained from bromelain purification for FBM and SBM are presented in Table 1 and Table 2, respectively.

Table 1: FBM Purification Table From Pineapple Fruit

Steps	Volume (ml)	Total Protein (mg)	Bromelain Activity (Units)	Total Activity (Units*ml)	Specific Activity (Units/mg)	Fold Purification	% Yield
Crude Lysate	840.5	0.22±0.01	5.07±3.56	4259.65	23.28	1.00	100.00
Crude Extracted	713.5	0.25±0.04	3.25±1.56	2319.66	13.18	0.57	56.61
Ammonium Sulphate (45% Sat.)	20	0.19±0.03	2.15±0.81	43.02	11.28	0.48	48.45
DEAE	5	0.09±0.01	0.48±0.01	2.42	5.45	0.23	23.40

Total protein and bromelain activity are presented as MEAN±S.D (n=3)

Table 2: SBM Purification Table From Pineapple Stem

Steps	Volume (ml)	Total Protein (mg)	Bromelain Activity (Units)	Total Activity (Units*ml)	Specific Activity (Units/mg)	Fold Purification	% Yield
Crude Lysate	282	0.11±0.01	0.12±0.00	32.76	1.07	1.00	100
Crude Extracted	273	0.07±0.00	0.11±0.01	29.32	1.43	1.34	133
Ammonium Sulphate (45% Sat.)	20	0.05±0.02	0.06±0.00	1.21	1.11	1.03	103
DEAE	5	0.05±0.00	0.24±0.01	1.19	4.97	4.64	463

Total protein and bromelain activity are presented as MEAN±S.D (n=3)

3.2 Discussion

From Tables 1 and 2 it could be observed that the purification fold for FBM and SBM are 0.23 and 4.64 while their percentage yields are 23.4 and 463 respectively. The higher purification fold of SBM could probably due to the structural modification of the enzyme active sites in the presence of an unidentified component in the purification step. A similar case was reported by Ketnawa *et al* (2011) with percentage yield of 206% purification fold of 3.44. In their case, the bromelain's active site (from pineapple peel) was modified by Polyethylene glycol (PEG) used, in a Aqueous Two-Phase Extraction System (ATPS). Similarly, 106% percentage yield and 5.2 purification fold was reported by Hemavathi *et al* (2007), with the bromelian's active site been modified by concanavalin A of reverse micellar system (RMS) used. The greater 'purification fold' and 'percentage yield' in SBM over FBM seen in this research, suggested that SBM is better suited for the purification procedure adopted for this research than FBM (Burgess, 2009). This is in contrast with what Rabelo

et al (2004) observed, where 79.5% and <15% yield for FBM and SBM respectively reported using a Two-Phase System containing PEO-PPO-PEO block copolymer. Higher number of steps are involved in the purification procedure employed in this research which could remove more natural inhibitor(s) of the bromelain and/or activates more zymogens to their mature bromelain enzyme forms (Bala, *et al.,* 2012), compared to what are normally involve using ATPhase systems employed by Rabelo *et al* (2004).

5. Conclusion

Both SBM and FBM had been extracted and purified. However, higher purification fold was seen in SBM than FBM. This means SBM is better suited for the purification procedure adopted for this research. This is promising, because pineapple stem, from which SBM is gotten from, is largely considered as waste in the areas where pineapples were cultivated. This could pave a way for bioremediation of these waste and pioneering an enzyme production industry in Nigeria.

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