



## Research Article – Biochemistry

# Purification, characterization and application of bromelain from *Ananas comosus*

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### Abstract

Bromelain is a major protease, isolated from pineapple (*Ananas comosus*). Bromelain is accumulated in the entire plant to different extent and properties depending on its source. In the present study, Bromelain was extracted from all parts of pineapple using sodium citrate buffer. Bromelain was filtered, centrifuged and used for further studies. After the determining the protease activity and protein content, the Core and Pulp extract of *A.comosus* was chosen using gelatin as the substrate. The Bromelain was purified by precipitation and dialysis. Then SDS-PAGE was performed in order to determine the molecular size of the obtained protein. Then the effectiveness of Bromelain as an anti-browning agent, extracted and purified from pineapple core and pulp was determined. The study showed that Bromelain is a better anti-browning agent when compared with some of the available commercial anti-browning agents. Further the application of Bromelain was tested by stain removal, compared to positive control, the ability of stain removing property for the produced enzyme was observed to be good. Immobilized Bromelain prepared using calcium alginate beads and its stability and characters were noted.

**Keywords:** *Ananas comosus*, Bromelain, Pine apple, SDS-PAGE, Immobilised enzyme

### Introduction

Pineapples widely grow in tropical countries may be cultivated from a crown cutting of the fruit, possibly flowering in 20–24 months and fruiting in the following six months. Pineapple does not ripen significantly post-harvest. Pineapples are consumed fresh, cooked, juiced, and preserved and are found in a wide array of cuisines. In addition to consumption, in the Philippines, the pineapple leaves are used to produce the textile fibre pina employed as a component of wallpaper and furnishings, among other uses. Pineapple (*Ananas comosus*) is one of the tropical plants that have been used as traditional medicines from a long time. It was originated from tropical South America and was discovered by Europeans (Bhattacharya, 2008).

A protease is also termed as peptidase or proteinase is any enzyme that performs proteolysis activity by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein. Proteases have evolved multiple times, and different classes of protease can perform the same reaction by completely different catalytic mechanisms (Oda, 2012). Proteases can be found in animals, plants, bacteria, archaea and viruses. Papaya and pineapple are two of the richest plant sources, as attested by their traditional use as natural "tenderizers" for meat. Papain and Bromelain are the respective names for the proteolytic enzymes found in these fruits (Hale *et al.*, 2005a).

Bromelain has been used widely in food, medical, pharmaceutical and cosmetic industries and other industries as well. In the food industry, it is used for meat tenderization, grain protein solubilisation, beer clarification, baking cookies and protein hydrolysate production (Walsh, 2002). It was studied that pineapple juice was an effective enzymatic browning inhibitor in fresh apple slices and

several important medical applications (Hale *et al.*, 2005b; Hale *et al.*, 2002).

### Materials and Methods

#### Collection

Fresh pineapple was collected from local market, Chennai. The samples were washed, peeled and rinsed with tap water and distilled water to remove any dust particles repeatedly. It was then kept in the refrigerator for experimental studies.

#### Extraction

10g of the sample was weighed and homogenized with 0.1M of Sodium Citrate buffer (pH 5) (cooling condition) (1:1 ratio) using mortar and pestle, it was then filtered. The filtrate was centrifuged at 10,000 rpm for 15 minutes. The supernatant was stored and used as enzyme source.

#### Qualitative Protease assay

Water agar medium (half strength) is to be supplemented with proteinous substrate (1% gelatin, casein and skimmed milk) for the assay of proteolytic enzyme was prepared and autoclaved at 121°C for 15minutes. The plates were allowed to solidify and then, 5 wells (8mm diameter) were made by using a sterile cork borer. The 4 different volumes (25µl, 50µl, 75µl and 100µl) of the supernatant were loaded in the wells and 25µl of phosphate buffer was used as control. The plates were incubated for 24h at room temperature. After 24h of incubation, the plates were flooded with mercuric chloride indicator solution (concentrated HCl-20 ml, Distilled water-80 ml and HgCl<sub>2</sub>-15g) for 5 – 10 minutes. Protease production was visualized by a translucent zone around the wells. The zone of clearance were observed and measured. Based on the results

produced, one test material will be selected for further studies (Dubey *et al.*, 2007).

#### Estimation of protein

1ml of the enzyme source (supernatant) was mixed with 5ml of CBB dye solution (Coomassive Brilliant Blue-G250). The mixture was mixed well and incubated for 5 minutes at room temperature. Simultaneously, control without the enzyme source and with 5ml of CBB-dye solution was maintained. The OD of the solution was measured at 595nm in a spectrophotometer and compared with Bovine Albumin Serum (BSA) to determine the protein content of the sample (Bradford, 1976).

#### Determination of Protease activity

An assay mixture was prepared by mixing 0.5ml of 1% gelatin with 0.5ml of cell free culture filtrate (enzyme source). The mixture was incubated at 37°C for 60 minutes. 1ml of 10% trichloroacetic acid (TCA) was added to the reaction mixture. The reaction mixture was centrifuged at 10,000rpm for 15 minutes and the supernatant was collected. To 0.5ml of supernatant, 2.5ml of alkaline solution (2.9% Na<sub>2</sub>CO<sub>3</sub> and 0.3N NaOH) and 0.75ml Folin phenol reagent (1ml of reagent diluted with 3ml of double distilled water before use was added and incubated at room temperature). After 20 minutes, the absorbance of the solution was measured at 650nm in a spectrophotometer. Simultaneously, control without the enzyme source was maintained. One unit of protease activity is defined as the amount of enzyme required to liberate 1µmol of tyrosine/ml/min/mg of protein was expressed (Mc Donald and Chen, 1965).

#### Production and Purification of Bromelain

The pineapple fruit was chosen, weighed and homogenized with Sodium Citrate buffer using mortar and pestle. It was then filtered. The filtrate was centrifuged at 10,000 rpm for 15 minutes. The supernatant was stored in the refrigerator and used for further purification.

#### Purification of Bromelain

Precipitation of proteins using organic solvent is done usually at 0°C or subzero temperature in order to minimize denaturation of proteins especially enzymes by acetone. Hence the acetone is cooled overnight at -20°C and added slowly with constant stirring to an icecold solution of the enzyme in cool condition. 65% acetone precipitation was preferred for precipitating the proteins. The enzyme source was transferred to clean beaker and the magnetic pellet was dropped inside the beaker and was placed on the magnetic stirrer. While the sample is stirring, required amount of acetone was slowly added until desired saturation level attains. After the total amount of acetone was added, the mixture was incubated over night to ensure complete precipitation. Then the sample was centrifuged 8000rpm for 15minutes. The supernatant was carefully removed and the pellet was dissolved in sodium phosphate buffer and stored in a refrigerator for purification process. The precipitated sample was subjected to the determination of enzyme activity and also for application studies (Dubey *et al.*, 2007).

#### Dialysis

The dialysis membrane is usually treated to remove some undesirable impurities such as glycerol, heavy metals, sulphide. First, the membrane was treated with sterile

distilled water at 65°C for 10 minutes to remove glycerol. Then, the membrane was immersed in 50ml of 10mM EDTA containing half pellet NaOH to remove heavy metals. Then the membrane was transferred to 30ml 2% sodium bicarbonate to remove the sulphur molecules. Finally, it was washed with sterile distilled water. The sample solution was filled up to 2/3 of the tube volume carefully and the open end was closed securely by tying with a thread. The bag was placed in sodium phosphate buffer overnight at required temperature with gentle agitation provided by magnetic stirrer. The dialyzed sample was carefully taken out using sterile syringe and stored in microfuge tubes. This was further used for the determination of molecular weight by SDS-PAGE (Dubey *et al.*, 2007).

#### Characterization of proteins

The protease enzyme was characterized by Sodium Dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

#### Immobilization of Bromelain

Sodium alginate was used as the immobilizing agent for the bead preparation. Various concentrations of aqueous sterilized sodium alginate solution ranging from 1% to 6% were prepared to find out an optimum concentration for stable bead preparation (Choi *et al.*, 2002). The sample was suspended in cooled alginate and the sample-alginate suspension was transferred into a disposable plastic syringe. Then the alginate-sample mixture was extruded drop by drop into a gently stirred 0.2M calcium chloride solution and hardened in this solution for 30 min. The resulting calcium alginate gel beds were thoroughly washed with sterile saline water and used (Rishi *et al.*, 2011).

#### Application studies

##### Anti browning activity

Fresh red apples were purchased from local market and were washed and peeled to remove the outer layer. Apples were crushed using the kitchen blender; the resulting juice was filtered using muslin cloth. The juice was transferred into new eppendorf tubes containing pulp sample and core sample and commercial anti-browning agents such as Acetic acid, Ascorbic acid, Citric acid, Oxalic acid and etc. Apple juice alone serves as a control. Samples were incubated at room temperature for 1hr and the absorbance was read at 420nm in a spectrophotometer for every 10minutes for 1h. The percentage of inhibition was calculated using values coming from control and fractions.

$$\% \text{ of inhibition} = \frac{A_{420} \text{ Control} - A_{420} \text{ Treatment}}{A_{420} \text{ Control}}$$

##### Destaining activity

A clean and dried white cloth was cut into four pieces and placed into four sterile Petri dishes. Little amount of cheese sample was gently applied into each of the cloth placed in petri plates and the plates were labelled as; test, control, positive control and negative control respectively. To the test Petri dish, 20ml distilled water and 2ml purified enzyme solution were added. To the control Petri dish, 20ml distilled water was added. To the positive control Petri dish, 1ml detergent + 20ml distilled water were added. To the negative control Petri dish, 20ml phosphate buffer was

added. All the four plates were stirred gently and incubated at 60°C for 15 minutes. The solutions were poured off, and the plates were dried. The stain removing capability was evaluated on three conditions such as poor removal, moderate removal and complete removal.

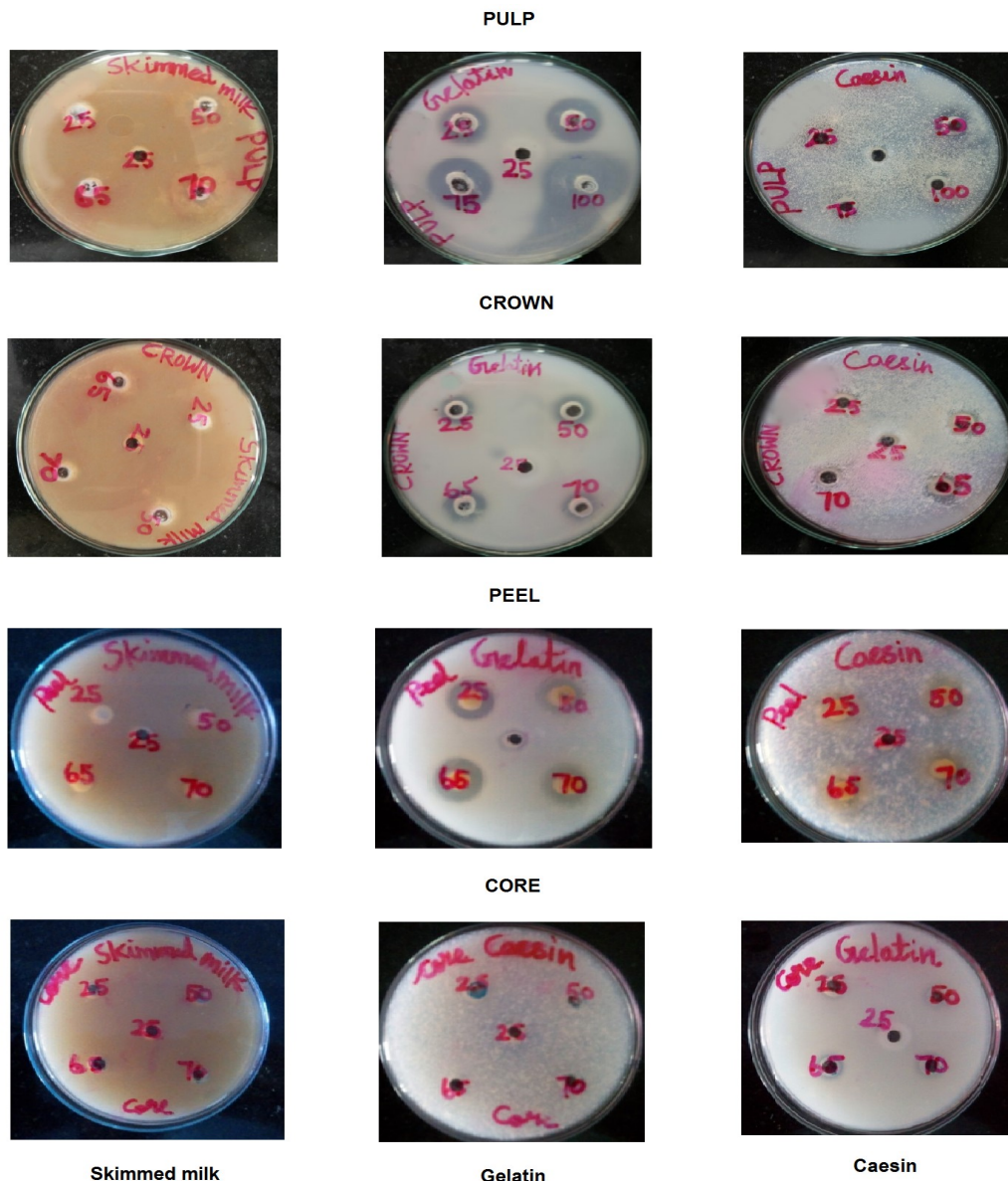
**Results and Discussion**

**Collection and Extraction of Bromelain**

The Crown extract of pineapple were green in colour, this may be due to the presence of chlorophyll. The Pulp and Core extract were yellow in colour, this may be due to the presence of carotenoids and xanthophylls. The peel extract were brown in colour, which may be due to the presence of melanin. On extraction, various volume of crude sample was obtained, Pulp - 25ml, Core - 23ml, Peel - 19ml and Crown - 16ml. The difference in volume of extraction obtained may be due to the amount of water content, fibre and other components present in *A.comosus*. The chemical composition of pineapple it has also to be mentioned the presence of bromelain enzyme and phenol compound.

**Qualitative Protease assay**

The sample of pulp, peel, crown, core extract of *A. comosus* was tested for the presence of protease and to detect the suitable substrate. Screening for protease producing samples was tested on water agar medium with three different substrates Gelatin, Casein and Skimmed milk, based on the zone formation due to protease hydrolysis. The more suitable substrate was selected based on the zone of clearance observed. Casein exhibited zone of clearance in lower amount when compared to gelatin and skimmed milk did not exhibit the zone of clearance. On comparison of all four samples (Pulp, Core, Peel and Crown), the pulp extract showed highest zone of clearance (28mm) when compared to the zone of clearance obtained by Peel (22mm), Crown (17mm), Core(13mm). From the result obtained, it was concluded that the selected four samples has protease activity and gelatin is considered as the most suitable substrate (Table 1, and Fig. 1). Choudhary and Jain (2012), in their study reported that alkaline protease activity of 141 test fungi was determined using 0.5% casein as protein substrate on solid Reese media.



**Fig. 1. Qualitative Protease Assay**

**Table 1.** Qualitative protease assay

Substrate	Control	Zone of Inhibition (mm)															
		Pulp (µl)				Core (µl)				Peel (µl)				Crown (µl)			
		25	50	65	70	25	50	65	70	25	50	65	70	25	50	65	70
Caesin	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	17	19	21	22	Nil	Nil	Nil	Nil
Skimmed milk	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	12	14	15	16	Nil	Nil	Nil	Nil
Gelatin	Nil	14	20	22	28	10	11	12	13	18	19	20	22	12	13	15	17

**Determination of Protease Activity**

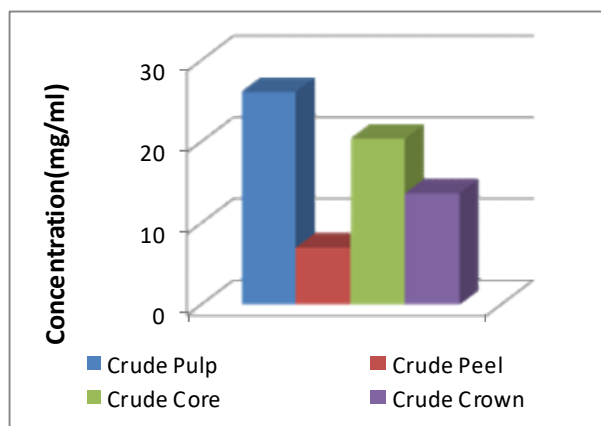
The results showed that the crude pulp sample had higher activity. Followed by it, the peel sample had higher activity than the core and crown sample (Table 2). Mohan *et al.* (2016), in their study reported that the activity of crude Bromelain enzyme from fruit was found to be 4.71 U/mL of enzyme and from peel was found to be 4.52 U/mL. Aravind Krishnan and Gokulakrishnan (2015) reported that the enzyme assay was conducted to determine the activity of the crude extracts and it was found to be the highest in the leaves followed by the peel, stem and was lowest in the pulp.

**Table 2.** Determination of Protease activity

Sample	Protease assay (EU/ml)
Pulp	2802.7
Peel	2016.1
Core	1268.9
Crown	281.5

**Estimation of protein for crude sample**

The protein content of the crude pulp sample was found to be 26.22mg/ml. Next to pulp the crude core sample had higher protein content, which shows 20.46mg/ml. The quantity of protein found in crude crown sample had 13.74mg/ml. The least amount of protein was identified in the crude peel sample 7.08mg/ml. Comparatively within all the selected parts of *A.comosus* the pulp and core were rich in protein content may be reflect in the level of enzymes (Fig. 2). Extracts concluded that on quantitative estimation by Lowry’s method and the concentration of the enzyme in the crude extracts of the peel, pulp, leaves and stem was found to be 11.4 mg/ml, 9 mg/ml, 2.08 mg/ml and 6.2 mg/ml respectively. Hence the concentration of the enzyme in the crude extracts was the highest in the peel and lowest in the leaves.

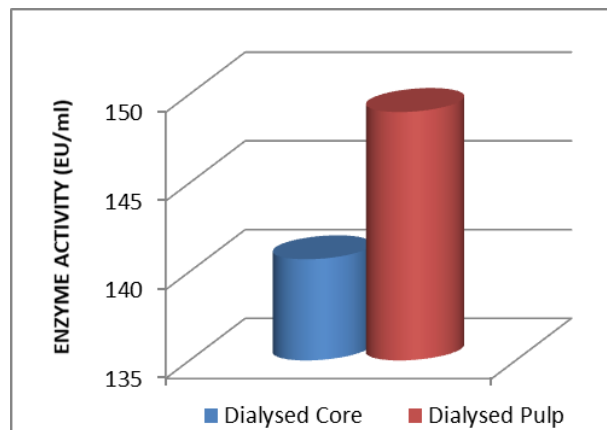


**Fig. 2.** Estimation of protein for crude sample

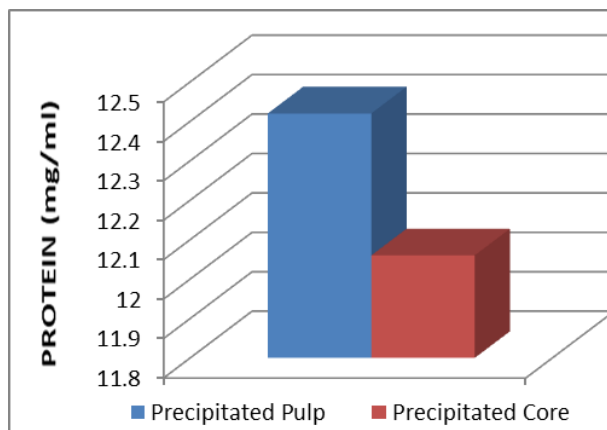
**Purification of Bromelain**

The ammonium sulphate precipitation and purification of protein yields 70ml of pulp and core bromelain with 130ml of acetone. We obtained approximately 15 to 20ml of

precipitated core and pulp bromelain. Then the enzyme activity of the precipitated sample was determined. Fig. 3 shows the Bromelain activity for precipitated samples.



**Fig. 3.** Determination of Bromelain activity on precipitated pulp and core samples



**Fig. 4.** Determination of Bromelain activity on dialysed pulp and core samples

Report says that the bromelain from the pineapple peel was extracted and partially purified by precipitation with ammonium sulfate in the range of 40-80% saturation, in which 75% of bromelain was recovered. This result was in accordance to Devakate *et al.*, (2009) who recovered approximately 80% of bromelain from the pineapple fruit by ammonium sulfate in the same range of saturation. After the precipitation, the re-suspended precipitate was desalted and lyophilized for storage, as recommended by Maurer (2001). The results of enzymatic activity and total protein concentration of bromelain samples after precipitation was found to be 175 U and the total protein was found to be 0.31 mg/ml. Reports supports that bromelain also can be precipitated with Organic solvents such as Ethanol, Acetone etc. Activity profile of Bromelain post-precipitation is showing increasing pattern from low to high Ethanol



concentration, numerically, 0 U/mg at 0-20% to 150 U/mg at 40-60% of Ethanol. But the recovery profile shows a reverse pattern i.e. at low concentration of ethanol recovery is highest and it drops down with slight change from 20-80% of Ethanol. The enzyme activity was quantified for dialyzed sample and the effectiveness was comparatively observed and proved to be in higher purity after dialysis process. Fig. 4 shows the Bromelain activity for precipitated samples.

The enzyme activity was found to be 134.5 EU/ml for precipitated core sample and the enzyme activity of precipitated pulp sample was found to be 260.8 EU/ml. The enzyme activity is 140.7 EU/ml for dialyzed core sample and the enzyme activity is found to be 149.0 EU/ml for dialysed pulp. The activity of Precipitated sample was higher than the activity of the dialysed sample. Of the precipitated samples, the activity of the pulp sample was higher than the core sample. Of the dialysed sample, the activity of the pulp sample was higher than the core sample.

#### Estimation of protein from Purified Bromelain

The amount of protein present in the purified samples before and after dialysis was shown in Fig. 5. Aravind Krishnan and Gokulakrishnan (2015) reported that the purified bromelain from the different parts were subjected to quantitative method and the concentration of bromelain from the peel, pulp, leaves and stem was found to be 0.22 mg/ml, 0.64 mg/ml, 0.12 mg/ml and 0.24 mg/ml respectively. It can be observed that the concentration of the purified bromelain was the highest in the pulp followed by the stem, peel and lowest in the leaves.

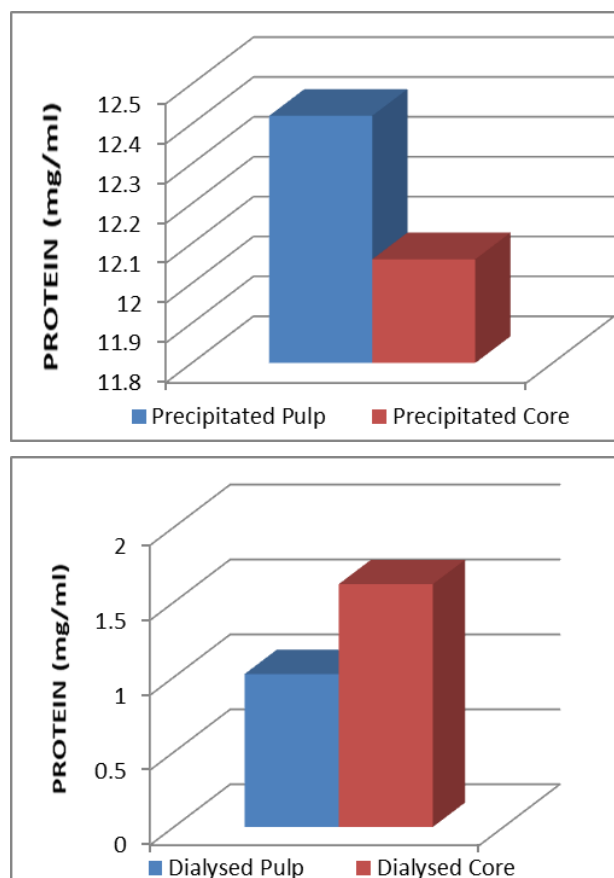


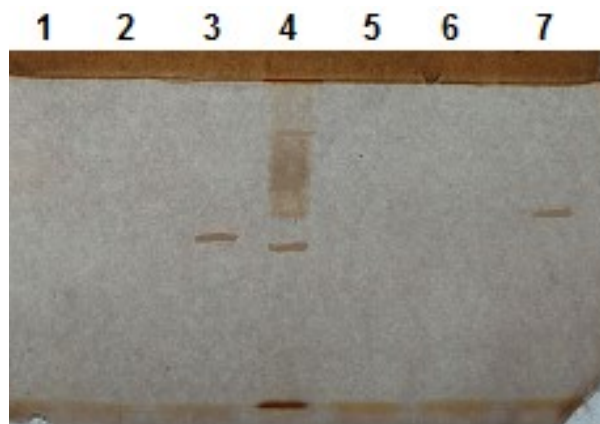
Fig. 5. Estimation of protein from purified Bromelain

The concentration of the purified bromelain was less

when compared to the crude enzyme concentration as the later contains the presence of other proteins. Mohapatra *et al.* (2013) reported that the protein concentration after dialysis of stem extract concentration as 75µg/0.1ml, Pulp extract concentration as 70 µg/0.1ml and Peel extract Concentration as 30µg/0.1ml.

#### Characterization of proteins

The SDS-PAGE was performed in order to determine the molecular size of the obtained protein. The pulp and core bromelain samples were loaded in their respective wells. The molecular weight of the pulp bromelain was found to be 45 kDa and the molecular weight of the core bromelain was found to be 60 kDa when compared with protein marker. Fig. 6 indicates SDS-PAGE for the dialyzed sample. Bromelain is becoming increasingly important in high value applications in the textile industry, medicinal industry and the production of variety chemicals and drugs. Grzonka *et al.*, (2007) and Kumar *et al.*, (2011) reported that the molecular weight range for SBM is 26-37 kDa and for FBM molecular weight range is 24.5-32 kDa. Grzonka *et al.*, (2007), Lopes *et al.*, (2009), Gautam *et al.*, (2010) reported that the molecular weight of the protein in pulp was to be 40 - 60 kDa and crown was to be 30 - 60 kDa respectively.



Lane 1: Core (30µl), Lane 2: Core (35µl), Lane 3: Core (40µl), Lane 4: Molecular Marker (20µl), Lane 5: Pulp (30µl), Lane 6: Core (35µl), Lane 7: Core (40µl)

Fig. 6. SDS-PAGE of Purified Bromelain

#### Immobilization of bromelain

Various concentrations of aqueous sterilized sodium alginate solution ranging from 1% to 6% were prepared to find out an optimum concentration for stable bead preparation. The 6% concentration of sodium alginate is capable of forming a stable bead when compared to the others. As of core, the number of bead was formed more by 5% sodium alginate solution. As of pulp, the number of bead formed was more by 2% sodium alginate solution (Fig. 7 and Table 3).

#### Anti browning activity

The study showed that bromelain is a better anti browning agent when compared with some of the available commercial anti browning agents. All of the commercially available anti-browning agents were taken at a concentration of 0.1gm/100 ml (0.1%). Amongst the anti browning agents taken for studies, bromelain is the most effective agent then followed by it, citric acid, acetic acid and finally ascorbic acid. The bromelain extracted from the pineapple pulp and

**CORE**



**PULP**



**Fig. 7.** Immobilized Bromelain

**Table 3.** Immobilized Bromelain

Concentration (%)	Quantity		Stability		Shape	
	Pulp	Core	Pulp	Core	Pulp	Core
1	30	8	Less Stable		Round	
2	92	41	Less stable		Round	
3	85	53	Less Stable		Round	
4	74	47	Moderately Stable		Round	
5	64	61	Moderately Stable		Round	
6	69	35	More Stable		Round	

**Table 4.** Anti browning activity for Pulp and Core Bromelain

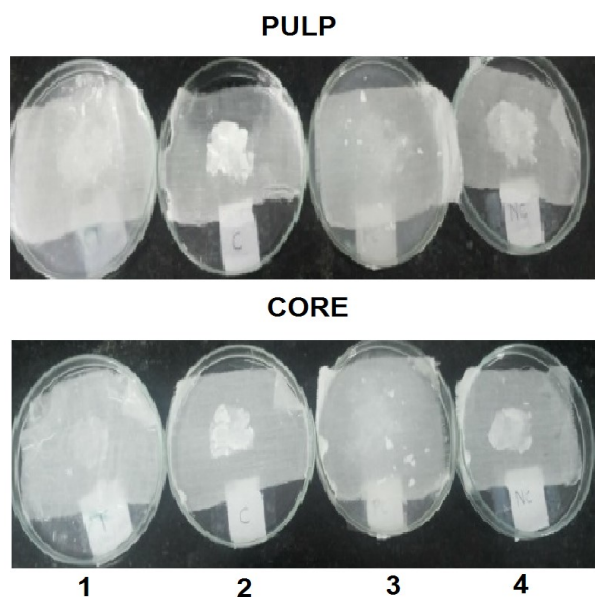
Sample	% of Inhibition					
	Incubation time in minutes					
	10	20	30	40	50	60
Control	1.834	1.937	1.910	1.921	1.932	1.904
Acetic acid	85.7	99.01	94.76	96.0	98.63	94.18
Ascorbic acid	99.06	98.39	97.58	96.54	95.61	93.97
Citric acid	82.30	97.15	92.30	91.94	93.61	89.66
Core	75.87	91.68	84.97	87.88	89.99	87.66
Pulp	73.09	87.14	82.83	84.13	84.60	81.68

core was significantly effective in preventing browning. On comparison of pulp bromelain and core bromelain, the pulp Bromelain showed better activity. This can be explained by the fact that protein content in the fruit was found out to be more, which directly correlates it to the amount of protease present (i.e.) amount of protease is more in the fruit extract (Table 4). Although commercial anti-browning agents are widely used, some are being restricted and banned by FAO such as sulfites. Even with many beneficial effects there are several negative attributes associated with sulfite use which has led to decreased consumer acceptance. In particular, sulfites can induce severe allergic reactions or even anaphylactic shock in a proportion of the asthmatic

population. Hence there is a need for natural anti-browning agents which can be used in the food industry. The inhibitory effect of Stem Bromelain as anti-browning agent was also found to be very minimal (Tochi *et al.*, 2009). The anti browning activity was found out to be higher in the fruit pulp extract in comparison with the crown leaf extract as the former showed higher protease activity almost twice the crown leaf extract. Bromelain within the concentration range of 0.1% to 0.8% hardly proved to be a potent anti browning agent but 1% crown extract bromelain was a better anti browning agent than acetic acid and also fruit pulp bromelain was found to be a much better anti browning agent than ascorbic acid and acetic acid (Ramalingam *et al.*, 2012).

### Destaining Activity

The stain removal activity of the bromelain were been carried out. In stain removal, the bromelain showed good activity on the test sample as compared with positive control which contains only detergent and distilled water as shown in the Fig. 8. Compared to positive control, the ability of stain removing property for the produced enzyme was observed to be good. De staining properties with detergents, after 10min of incubation at 60°C the detergent solution supplemented with the enzyme was able to remove the blood stains completely, while the detergent solution only could not remove it. Bhosale *et al.* (1995) has reported high activity of alkaline proteases of *Conidiobolus coronatus* showing compatibility at 50°C in the presence of 25 mM CaCl<sub>2</sub> with varieties of detergents. Adinarayana *et al.* (2003) worked with *Bacillus subtilis* PE II proteases and reported its compatibility and stability with various locally available detergents at 60°C in the presence of CaCl<sub>2</sub> and glycine as stabilizers.



1 - Test, 2 - Control, 3 - Positive Control, 4 - Negative Control

**Fig. 8.** Destaining activity of Bromelain

### Summary and Conclusion

Protease is one of the major groups of enzyme that plays the important role in the regulation and nutritional value in the living system. Due to the diverse application field of protease enzyme, they are in great demand and are being produced in high amount. Protease can be used in the detergents, food industries, cheese production, meat processing, the medical field so the demand is also increasing day by day. In recent times protease accounts for the 60% of the total enzyme consumed in the market.

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