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Research Article

Bromelain: Utilization of Pineapple Fruit Waste for Enzyme Extraction, Purification and Applications

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Abstract

Bromelain is a common name for a family of sulfhydryl proteolytic enzymes obtained from Ananas comosus, the pineapple plant. It is a potential proteolytic enzyme present in various parts of pineapple, having industrial significance with a wide range of applications. The objective of the present study focused on extraction, isolation, and purification of bromelain from pineapple fruit waste as a feedstock. The crude Bromelain was extracted, and activity of enzyme was measured using tyrosine as standard and expressed in Units/mL of enzyme. Thus, several commercially available buffers were explored for the extraction of bromelain from pineapple fruit waste. Of the tested buffers, sodium phosphate buffer showed the highest bromelain activity (4.5U/mL) and protein concentration (25.1mg/mL). The effect of pH (3.0-12.0) was studied using suitable pH buffer solutions. The kinetics of the proteolytic activity of the enzyme was explored using casein as substrate. It was found that the optimum pH of the crude extract was 7.0 and temperature (55°C). The enzyme showed the highest activity and purification when precipitated at 0-80% ammonium sulphate and dialysis effectively improved specific activity of enzyme. It provides an increase in bromelain activity by 2-folds. Molecular weight was found as 35kDa. Thus, the present study provides an efficient strategy for valorization of pineapple fruit waste via extraction and purification of bromelain as a potential product.

Keywords: Pineapple, Bromelain, Protein, Enzyme activity, Purification, ammonium sulphate precipitation; thermostability; pH optima

Introduction

The growth in the worldwide population caused an exponential increase in food and energy resources. Thus, the agro-industrial segment has been extended quickly for rapid industrialization and modernization to meet the current demands in terms of quality and quantity. There is an urgent need of novel technologies for sustainable treatment as well as vaporization of agro-industrial waste in population dense country. Several agro based feedstock industries producing mega tons of solid residues. The fruit processing industry is one of the important and leading segments where the circular economy can be integrated to boost a revenue-generating sustainable model. Amongst various fruits cultivated across the country such as mango, orange, grapes, pomegranate, apple, etc., pineapple (Ananas comosus) is one of the chief fruits which is cultivated in most of the states due to the diverse tropical and geographical aspects; India stands as fifth-largest pineapple producer in the world, producing about 1.2 million tons per annum [20]. Utilizing agricultural residues for production of value-added products is providing environmental comfort as well as economy to the agro-processing industry, which is beneficial, as it addresses issues related to residue accumulation. Thus, the upcycling and circular economy approach to utilize pineapple fruit waste for production of bromelain from the pineapple processing industry can provide a techno-economically and environmentally beneficial platform.

Bromelain is a proteolytic enzyme found in the pineapple stem, fruit, eye, peel, and crown [6]. Many studies have shown that pineapple wastes have great potential for bromelain production [14]. Bromelain finds various commercial applications in the food industry [15], pharmaceuticals [22], and skin care [1]. Despite its high

applicability and potential advantages, the high efficiency purification of bromelain enzyme from pineapple by-products on a pilot scale have not been extensively studied. It was estimated that the bromelain market accounts for USD of 40.16 billion in 2020 which can rise to 82.59 billion by 2030 [18] Thus, targeting agro-industrial residues for production of value-added products such as bromelain is a revenue-generating entrepreneurship as well as sustainable utilization of solid waste. There are several reports available in prior articles to obtain bromelain from pineapple leaf, stem, fruit, crown, and peels. There are many advanced techniques such as microwave extraction, ultrasound assisted extraction, and reverse micellar extraction, which are also explored widely [23].

Keeping in view of the extensive use of Bromelain enzyme in several industries, our objective in the present work is to extract Bromelain from pineapple fruits and peels and studies were conducted on its activity at different pH and temperature. Moreover, studies were conducted on its kinetics and stability of the enzyme. The obtained bromelain molecular weight was identified by SDS-PAGE analysis. The effects of parameters for purified bromelain in different processes were also investigated using the specific activity, enzyme recovery, and purification factor. The objective of this study demonstrates the potential for developing an effective process for producing bromelain from pineapple by-products and to produce value added products.

Material and Methods

Material and Chemical

Pineapple fruit were purchased from the fruit shop located in Ras Al Khaimah, United Arab Emirates and stored at 4°C. Ammonium sulphate, tyrosine, Trichloroacetic acid, Casein, Phosphate buffer pH-7 was prepared in laboratory. Sodium Carbonate and Sodium hydroxide were purchased from Merck and Himedia Laboratories Pvt. Ltd. The equipment used for the present study are Centrifuge (REMI), Magnetic stirrer, UV Visible spectrometer (Jenway Make) and Weighing balance (Shimadzu).

Crude Bromelain Enzyme Extraction

Extraction and separation of Bromelain enzyme from the pineapple fruit material is essential. The pineapple fruit and peel were separated and cut into small pieces and was processed separately for the extraction of enzyme. Fruit pieces and peel were weighed and grinded in mortar and pestle to extract the juice. After extracting the juice, crude enzyme from the fruit remaining was further extracted by adding 20 mL distilled water was added to it. Then the juice was filtered using Whatman filter paper (150mm). The filtrate was centrifuged at 10,000 rpm at 4°C for 10 min to remove insoluble materials. The supernatant was collected and stored at 4°C as shown in Fig. 1. It was used as crude extract of bromelain enzyme from pineapple fruit and peel [5]. Total four samples were extracted.

Bromelain Enzyme Activity

Total Bromelain protease activity was measured using a casein substrate by a modification of the Anson Method [11]. A 1 ml of the culture supernatant was mixed with 1 ml 0.05 M phosphate buffer-0.1M NaOH (pH 7.0 adjusted with phosphoric acid) containing 2% casein, and incubated for 10min at 37°C. The reaction was stopped by adding 2mL 0.4 M Trichloroacetic acid. After 30 min stand at room temperature, the precipitate was removed by centrifugation and the optical density of the assays was measured at 660 nm. A standard curve was generated using solutions of 0-60µg/mL tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1µg/mL tyrosine under the experimental conditions used.

International Units (IU)

One protease unit was defined as the amount of enzyme that released 1µg of tyrosine per mL per minute under the above assay conditions.

Protein Content

The protein content of the crude enzyme extract was determined by the method of Lowry et al. [12] using bovine serum albumin as a standard.

Partial Purification

The crude extract was fractionated by using ammonium sulphate at saturation level of 0-20%, 20-40%, 40-60%, and 0-80%. Protein concentration of each fraction was determined by Lowry method using bovine serum albumin (BSA) solution as a standard [3,5]. Molecular weight determination of bromelain enzyme was done by SDS- PAGE assembly (BioRad SDS-PAGE).

Enzyme Kinetic Method (Stability of Crude Enzyme Extract)

pH Optima: Since any activity of enzyme is dependent on the pH, it is essential to find out the pH at which the Bromelain enzyme is most active. The effect of pH on the activity of crude Bromelain enzyme was estimated by using casein as substrate following the procedure as explained earlier. The pH optimum of the bromelain protease enzyme was determined by preparing the casein substrate in various buffer solutions (0.2M HCl-KCl buffer of pH 2.0, 0.2M citrate phosphate buffer of pH 3.0-7.0 and 0.2M Tris-HCl buffer of pH 7.0-12.0) and applying the enzyme extract to the substrate to assay the enzyme activity [19].

pH Stability: The influence of pH on the stability of the Bromelain protease was determined by pre-incubating the enzyme in the above-mentioned buffer solutions for 30 min at room temperature (25±1°C) then determined the remaining activity [2].

Temperature Optima: The influence of temperature on the activity of the bromelain enzyme was determined at various temperature intervals (25-65°C).

Thermostability: The enzyme solution was incubated at various temperatures (25-60°C) for 3 hrs. Samples were removed at intervals of 30min and residual activities of bromelain protease was examined.

Statistical Analysis

Experimental error was determined for triplicate assays and expressed as Standard Deviation (SD).

Results

In the present investigation the juice of the pineapple was extracted, which contains the cysteine protease Bromelain. The crude extract of Bromelain was separated from the juice after filtration and centrifugation Fig 1. Then crude extract was stored at 4°C for further studies. The volume of pineapple juice was noted (Table 1) (Figure 1).

Table 1: Volume of Pineapple Juices.

S No.	Sample	Volume(mL)
1	PJ1	300
2	PJ2	250
3	PJ3	320
4	PJ4	280

Figure 1: Crude extract of Bromelain protease enzyme from Pineapple juice.

Partial Purification of Crude Bromelain Enzyme

Several methods have been developed for the purification and separation of crude bromelain extracted from pineapple waste. One common approach is partial purification using ammonium sulfate precipitation [10] (Table 2).

The purification steps, protein concentration, specific activity and yield of bromelain protease are shown in Table 2. The specific activity and purification fold were 0.37IU/mg protein and 2, respectively, when 0-80% ammonium sulphate used Table 2.

Table 2: Purification steps of protease from pineapple juice.

Purification step	Volume (mL)	Protein (mg/mL)	Enzyme activity (U/mL)	Total protease units (IU)	Total Protein (mg)	Specific activity (IU/mg)	Purification fold	% yield
Crude enzyme	195	25.1	4.5	877.5	4894.5	0.18	1	100
0-80% Ammonium sulphate fractionation	212	14.5	5.4	1144.8	3074	0.37	2	130.42

Enzyme kinetics

pH Optima: The partially purified protease had the highest activity at pH 7.0 and it then decreased with increasing of pH (Fig 2). Over pH 7.0, more than 50% of the relative activity was lost at pH 9.0. There was near complete loss of protease activity at pH values less than 3.0 and more than 11.0. This result was very close to those reported for bromelain protease, Crude bromelains have an optimum temperature of 55°C for the peel and core and 35°C for the crown. All crude bromelains have an optimum pH of pH 7 [17] (Figure 2).

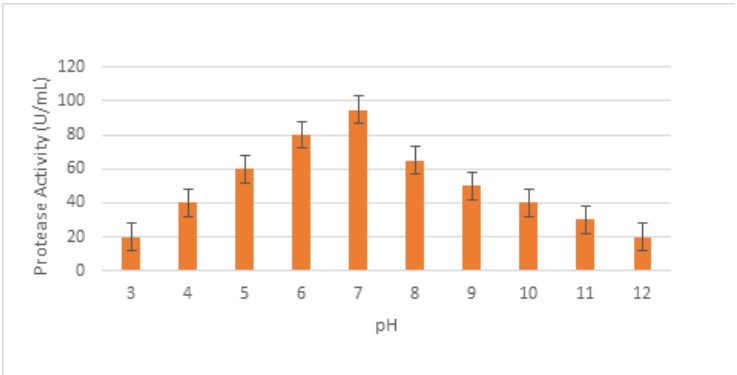

Figure 2: pH Optima of Bromelain Protease.

pH Stability: Fig 3. illustrates pH stability of the bromelain protease. The protease retained more than 98% of its original activity in the pH range 6.0-8.0 and then decreased with increasing pH and reached its lowest relative activity at pH 12.0. These data clearly indicate that the protease was most stable in the pH range 6.0-8.0 and least stable within the pH range 8.0-12.0. Generally, these data are similar with that reported by *Nyi, et al.,* [17] (Figure 3).

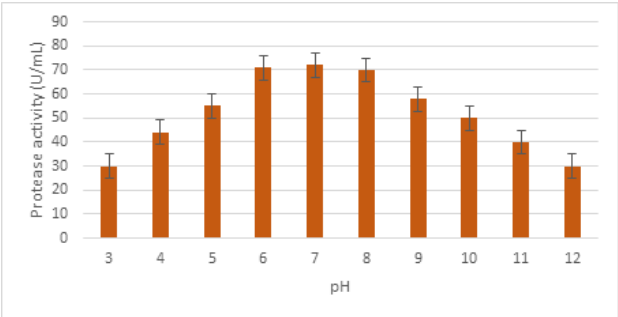

Figure 3: pH Stability of Bromelain protease.

Temperature Optima: The temperature stability profile of protease activity revealed that the enzyme is maximally active at moderately high temperatures ranging from 40°C to 55°C (Fig 4) with highest activity at 55°C (Fig 4) incubation temperature for 1h. The relative activity increased with increasing the temperature from 25°C to 55°C and then decreased; however very less activity was detected at 65°C. Generally, these are similar with those reported by *Simpson, et al., and Dimes, et al.,* [7,21]. Similar results were reported by *Gharge, et al.,* [9] which stated that the maximum bromelain activity of 0.84CDU/mL with a highest protein concentration of 0.147mg/mL was achieved at 55°C, which could be due to saturation stage, at which optimum enzyme activity is reached at optimum temperature. At a temperature of 60°C and above, the bromelain enzyme undergoes denaturation. As the temperature increases, more molecules gain sufficient kinetic energy to participate in the reaction. However, once the temperature exceeds the optimal level, a biochemical threshold is reached where the system's energy becomes so high that peptide bonds and disulfide bonds are disrupted, leading to enzyme inactivation [13] Therefore, a reaction temperature of 55°C was selected to obtain maximum bromalin activity and protein concentration (Figure 4).

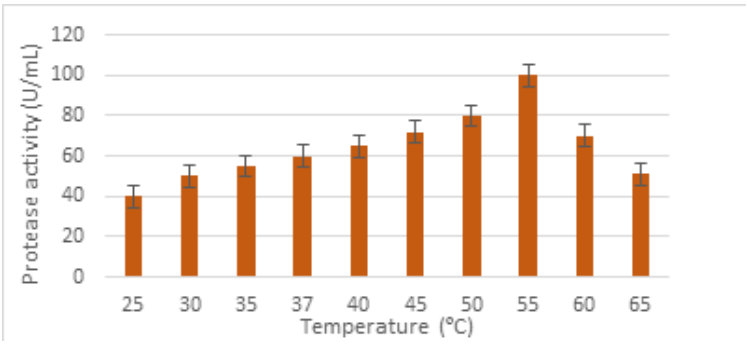

Figure 4: Temperature Optima of Bromelain Protease.

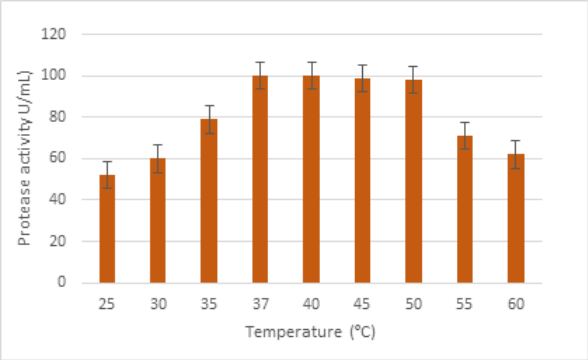

Figure 5: Temperature Stability of Bromelain Protease.

Thermostability: Thermostability of the protease is shown in Fig 5. The protease retained more than 50% of its activity after heating at 37°C and 50°C for 60min, it lost 20% after heating at the same temperature for 120min. A further increase in the reaction temperature caused significant drop in the protease activity. These results are in similar with those reported by *Dimes, et al., and Garcia-Carreno, et al.,* [7,8] (Figure 5).

SDS-PAGE

Molecular weight of partially purified bromelain protease enzyme was determined by SDS-PAGE (Fig 6). Photographic interpretation of the SDS-PAGE (7.5% gel) of different fractions of pineapple bromelain protease obtained during purification steps and standard proteins (Staining reagent 1% Amido black). Lane 1: standard solution Lane 2: F-1a fraction from partial purification Lane 3: F-1 fraction obtained from partial purification Lane 4: Crude protein extracts. The more intense band at 16-50kDa in lane 4 depicts the concentration of crude enzyme in retentate. Single band observed in Lane 2 and Lane 3 at 34kDa and 35kDa. Prior literature reports the molecular weight of bromelain from 24 to 37 [4].

Differences in the electrophoretic pattern may arise from differences in posttranslational modifications and/or proteolytic maturation between the proteins in our extracts and those in the commercial sample. Factors such as the source of bromelain (e.g., fruit, core, stem), the specific pineapple variety, and the stages of harvesting and ripening must also be considered [16] (Figure 6).

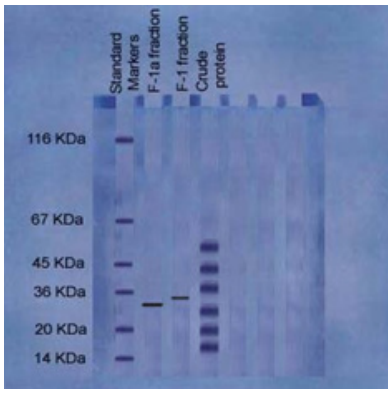

Figure 6: SDS-PAGE.

Conclusion

The objective of the present study is to perform extraction and separation of Bromelain enzyme from the pineapple fruit waste material and finding out optimum conditions for production of value added products for industrial application. For this purpose, experiments such as enzyme activity assay for crude extract of Bromelain, effect of pH on enzyme activity, Kinetic studies on activity, stability of the crude Bromelain enzyme extract, partial purification by ammonium sulphate precipitation and molecular weight determination was performed. The results indicated that the activity of crude Bromelain enzyme from fruit was found to be 4.5U/mL of enzyme was found to be at pH-7.0 and 55ºC and after partial purification activity was found 5.4U/mL. The crude bromelain protease showed maximum activity at 0-80% ammonium sulphate fractionation. It was observed that molecular weight of bromelain protease enzyme was 35kDa. Thus, the developed protocol contributes to establish a platform for development of an industrially feasible process for utilization of pineapple fruit waste for production of bromelain enzyme.

Conflicts of Interest

The authors declare no conflict of interest pertaining to the research report in this manuscript.

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Not applicable.

Availability of Data and Materials

The relevant data and materials are available in the present study.

Competing Interests

The authors declare that they have no competing interests. All procedures followed were in accordance with the ethical standards (institutional and national).

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