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Isolation and Characterization of Bromelain (BML) Proteases from *Ananas comosus* an asset to Cancer Chemotherapy

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Abstract

Chemo impediment impels the quest for moreover single targeted or brew of multi-targeted agents. Bromelain, potential agent in this regard, is a pharmacologically active compound, present in stems and fruits of *Ananas comosus*, endowed with anti-inflammatory, anti-invasive and anti-metastatic properties. Bromelain is a complex or proteolytic enzymes. These proteolytic enzymes are paraphernalia that promise an impressive number of medical and therapeutic uses, particularly as anti-tumor agents. The purpose of this study was to develop a lead range of BML extraction process. The purification of the enzyme was carried out by precipitation and by chromatography techniques. Further, the drying of chromatographically purified fraction was carried out in freeze dryer and spray dryer. The activity was expressed in terms of casein digesting units (CDU). The BML extract of higher purity was obtained from the fruit and stem using ion exchange chromatography. The elution competence in chromatographic purification was obtained in the range of 90%. Bromelain thus obtained was subjected to spray and freeze drying. The BML was extracted using stem yielded a total protein content of 242.6 mg/mL with the proteolytic activity of 878.14 GDU/mL. The extracted Bromelain was characterized using the standard spectroscopic techniques such as IR Spectroscopy, UV-Spectroscopy, LCMS and PXRD to understand the structure of the lead molecule acting on the cancer cells

Keywords: *Ananas cosmosus, BML Proteases, IR-Spectra, LCMS, PXRD, UV-Spectra.*

1 Introduction

Bromelain is a collective name for proteolytic enzymes or proteases found in tissues including stem and fruit of the pineapple plant family Bromeliaceae [1]. The isolation of enzymes from pineapple fruit and its study have been investigated since 1894. The enzymes occurring in the stem and the fruit of *Ananas comosus* are the most studied. Unlike crude stem BML, which is used widely in industry, fruit BML is not commercially available despite the large quantities of waste pineapple fruit portions at pineapple canneries [2]. The continued interest in BML, for its numerous applications in the food industry as well as in medicine and pharmacology make this enzyme one of the best vegetal proteases. The potential therapeutic value of BML is due to its biochemical and pharmacological properties and hence, it is desired to obtain Bromelain in its highest purified form [3]. The main ingredient in crude BML is a proteolytic enzyme named glycoprotein, besides substances such as insoluble materials, e.g. colored pigments, organic acids, minerals, protease inhibitors, organic solvents and excipient used for enzyme recovery have been reported [4]. The present study examines the extract of fresh pineapple fruit for the presence of cysteine proteinases, besides protein degradation kinetics.

These enzymes offer a wide spectrum of therapeutic efficacies: they demonstrate, in vitro and in vivo, antiedemateous, antiinflammatory, antithrombotic and fibrinolytic activities. They modulate the functions of adhesion molecules on blood and endothelial cells, and also regulate and activate various immune cells and their cytokine production. Indeed, these enzymes are used in the United States and Europe as an alternative or complementary. Medication to glucocorticoids, nonsteroidal antirheumatics and immunomodulatory agents. Their very low toxicity makes them suitable tools for controlling chronic inflammatory diseases [5], [13].

For the therapy of inflammatory and malignant disorders, these proteinases are employed as additives for chemotherapy (to reduce side effects of drugs and to improve quality of life); additives for radiotherapy (to reduce inflammation and edema); additives in surgery (to reduce edema and to improve wound healing); additives to prevent lymphedema by

reducing lymphocongestion, detritus, viscosity of the exudates and stimulation of phagocytosis of associated leukocytes. It should be added that clinical studies support these recommended indications only to a limited extent. Yet the large body of preclinical, pharmacological and daily experience offers an important and worthwhile field for well-designed clinical studies in order to evaluate evidence- based medical indications [13].

2 Materials and methods

2.1 Clarification of fruit crude juice

The stalk (central core) and stem of ripe pineapple fruit was separated from fleshy fruit and was washed thoroughly with distilled water. The fruit portion was then cut into small pieces and crushed in a laboratory juicer to get approximately 2000 mL juice. The juice was double filtered through a muslin cloth to remove the fibrous material. The filtrate was then subjected to vacuum filtration to remove ruptured plant cells. The filtered crude juice was kept for centrifugation at 10,000×g for 20 min at 4°C to remove any insoluble materials. The clear supernatant obtained as a clarified extract was stored in aliquots at 4°C. The protein content in the supernatant was determined before further purification using precipitation and ion exchange chromatography [14].

2.2 Purification of BML proteases from clarified juice

Clarified pineapple juice was subjected to salt precipitation and ion exchange chromatography for the separation of BML enzyme. These methods are described briefly as follows.

2.2.1 Precipitation

The precipitation of BML from clarified pineapple fruit juice (2000 mL) was carried out by slow addition of ammonium sulfate [$(NH_4)_2SO_4$] at 4°C under constant stirring. Initially $(NH_4)_2SO_4$ was added to get 10-100% saturation. The stirring was continued for 30 min after the complete addition of salt to allow equilibration between the dissolved and aggregated proteins. The salt-enriched solution was centrifuged at 10,000×g for 15 min and the precipitate was collected. The supernatant was separated, its volume was noted and the amount of salt required for the next cut was calculated. 40% salt cut was fixed. The amount of $(NH_4)_2SO_4$ added to the solution in order to increase the percentage saturation level from S_1 to S_2 was calculated using following equation [5], [14].

Amount of $(NH_4)_2SO_4$ required (g)

$$\frac{533(S_2 - S_1)}{100 - 0.3S_2} \times \text{volume of solution (lit.)}$$

Where S_1 is initial saturation of solution (%) and S_2 is required saturation of solution (%). The equation is related by the assumption that 100% saturation corresponds to 4.05M salt solution. Ammonium Sulfate precipitate fractions and supernatants were collected at the levels of 20% saturation and assayed for protein content and specific protease activity after dialysis.
The result shows that maximum protein concentration was obtained at 40% Ammonium Sulfate concentration.

2.2.2 Ion exchange chromatography

Chromatographic purification of clarified crude juice was carried out on a preparative scale. DEAE cellulose bed, of 1cm thickness, was prepared in a chromatography column and equilibrated with 0.5M Sodium phosphate buffer solution (pH 8.0) followed by eluting buffer 25mM Tris HCl and 25mM NaCl. The dialyzed sample of stem and fruit BML was impregnated onto the column, from the sides, without disturbing the DEAE cellulose bed and allowed to settle. Enzyme was eluted using the first eluting buffer i.e. 25mM Tris HCl and 25mM NaCl. Elutes was collected in test tube. Elution was done at a flow rate of 1 mL/min. The same process of elution was carried out using solutions 2, 3, 4, 5 and 6 containing 50mM, 75mM, 100mM, 125mM and 150mM NaCl, respectively. Finally, ion-exchange elutes of stem and fruit bromelain were assayed for their activity.

2.3 Dialysis

The salt in the purified bromelain samples as a result of above purification methods was removed by dialysis using ammonium phosphate buffer (50mM, pH 7.0) as a process for buffer exchange. Dialysis membrane (Hi-Media

Laboratory, Bangalore, India) having molecular weight cut-off of 12,000 Da was used for dialysis. The enzyme activity units and protein content after dialysis were checked for the percentage loss in the process [14].

2.3.1 Drying of purified BML

Chromatographically purified bromelain was dried using laboratory spray dryer and vacuum freeze dryer.

2.3.2 Spray drying

A laboratory bench-top spray dryer having 1 Lt/h water evaporation capacity was used for experimental purpose. Fig. 1 shows the schematic diagram of the spray dryer. Peristaltic pump was used to feed BML solution to a two-fluid nozzle atomizer with compressed air from a compressor. The drying chamber and cyclones were made of thick transparent glass. Inlet drying air from HEPA filter after passing through an electric heater flows co-currently with the spray through the main chamber. The dried powder was collected in the receivers from the bottom of the cyclones. The remaining ultra-fine powder was recovered in the filter bag. The parameters such as feed flow rate, drying air temperature and compressed air pressure for atomization were set and controlled through a personal computer. In all experiments, the atomizer pressure and feed flow rate were kept constant at $2kgcm^{-2}$ and 225 mL hr^{-1} respectively. Inlet air temperature was varied in the range from 100 to 170°C. Outlet air temperatures were recorded online continuously. The drying air flow rate and the feed temperature were kept at $41m^3$ hr^{-1} and 27°C, respectively. The duration of each experimental run was 90 min [11], [12], [14].

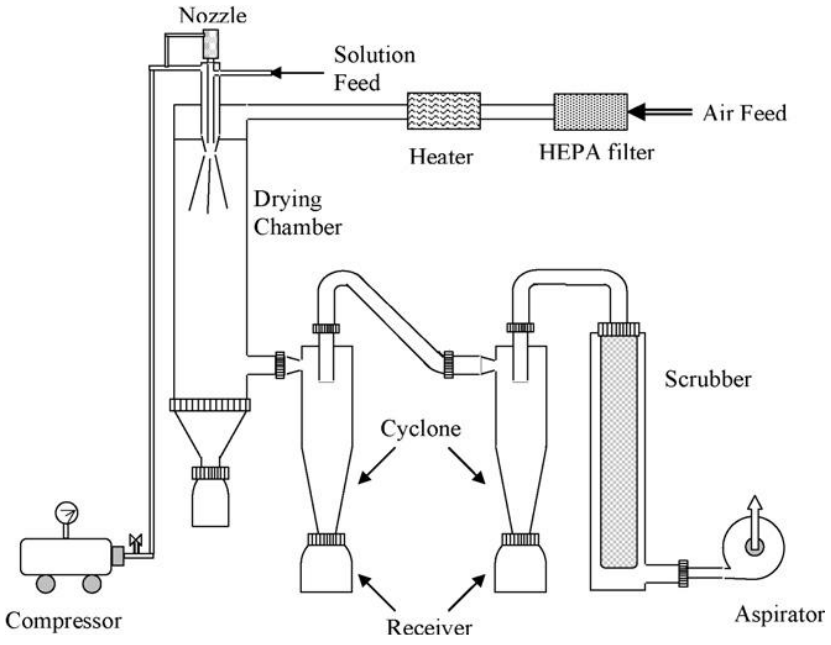


Fig. 1: Schematic diagram of spray dryer [14].

2.4 Measurement of Enzyme activity

The enzyme activity assay was based on estimation of amount of small molecular weight digestion products (Trichloroacetic acid (TCA) soluble material) formed from proteins due to proteolytic action of the enzyme [6]. Proteolytic activity of BML was measured [7]. The assay consisted of 5ml solution of 0.75% casein prepared in anhydrous disodium phosphate buffer (50mM, pH 7). The pH was adjusted using slow addition of 0.1N HCl. The solution was brought to 37°C by pre-incubation for 10 min. To this substrate, a known volume of enzyme was added after diluting it to 1ml with activating buffer. The 30mM cysteine hydrochloride monohydrate in 6mM disodium EDTA was used as an activating buffer. Casein proteolysis was stopped after 10 min by addition of 5mL of 30% (w/v) TCA and was allowed to stand for 30min at 37°C. Thereafter, the solution was cooled to room temperature and filtered twice using Whatman filter paper (No. 42). Absorbance of the filtrate was measured at 280nm using UV-Vis spectrophotometer against the tyrosine standard plot of absorbance versus tyrosine concentration (µg mL^{-1}).

One unit of BML was taken as the amount of enzyme which while acting on the casein substrate under specified conditions, produces one microgram of tyrosine per minute [9], [10], [14].

2.5 Measurement of Protein content

Protein content was measured spectrophotometric ally by using the Lowry's method of protein estimation [8], [9]. Lowry protein assay kit was used. Ten standards of varying concentrations i.e. 0.1 to 1.0 mL were taken and Bovine serum albumin was used as the standard for protein assay. A stock solution of 0.1mg mL^{-1} BSA was prepared in distilled water. Solutions of different concentrations of BSA were prepared by diluting the stock solution with distilled water. 3 samples were taken as unknown. The standards were made up to 1mL with distilled water according to their concentration of standards. Similarly it was done for the samples. To each test tube 5mL of Alkaline copper reagent were added and kept for incubation in the dark for 15 min at room temperature. After the incubation, 1mL of Folin Ciocalteu reagent were added to each test tube and incubated for 30 min in the dark at room temperature. The absorbance of these dilutions was measured at 600nm and a plot of absorbance versus BSA protein concentrations (μgmL^{-1}) was used as a standard [14].

2.6 Effect of Temperature

To investigate the effect of temperature on enzyme, the deactivation of BML proteases was carried out by incubating the sample at various temperatures of 25°C, 35°C, 45°C, 55°C, 65°C, 75°C, 85°C, 95°C and 105°C for 30 min. The heated sample was taken out and cooled immediately in ice water. The Enzyme activity was measured. The temperature of sample solutions was adjusted to the standard enzymatic assays condition after heat deactivation [15].

2.7 Effect of pH

To investigate the effect of pH on enzyme BML was carried out by incubating the sample at various pH ranging from 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0 for 30 min, . The Enzyme activity was measured in terms of casein digesting unit/mL [15].

2.8 Fourier Transforms Infrared (FTIR) Spectroscopy

Fourier transform infrared (FTIR) spectra were measured using a Perkin Elmer FTIR spectrometer (Series 1600) and analyzed using PE-GRAMS/32 1600 software, as described by Liao et al. [9]. Briefly, a dry protein sample (approximately 0.5 mg) was mixed with 1% of potassium bromide and compressed to get a pellet [14].

2.9 Ultra Violet (UV) Spectroscopy

The dry sample of BML was subjected to ultra-violet spectroscopy with 15µg of BML sample in 1mL methanol. Peaks were found at 200 and 230 nm.

2.10 Liquid Chromatography Mass Spectroscopy (LCMS)

LCMS was measured using Agilent Technologies LCMS Spectroscopy. BML proteases were analyzed by LCMS to know the molecular mass profile of the molecule. 50mg of dry BML proteases was dissolved in 0.5mL of distilled water and was analyzed for molecular mass. The chromatogram shows the mother peak at (m-z) = 439.3, BML is a group of proteolytic enzymes shows the splitting of the molecule there by resulting in more number of peaks.

2.11 Powder X-ray Diffraction (PXRD)

PXRD was analyzed using 'X'Pert PRO PANalytical instrument. To investigate the nature of the isolated molecule, BML was subjected for PXRD analysis. 500mg of sample was crushed uniformly and the pellet was subjected to the X-ray diffraction. The Diffractogram shows the peak due to crystallinity nature of the molecule.

3 Results

3.1 Estimation of protein activity of BML proteases

The protein concentration in crude extract is found to be 2.6mg/mL
The protein concentration in stem extracts found to be 4.66mg/mL

The protein concentration in fruit extracts found to be 4.0mg/mL

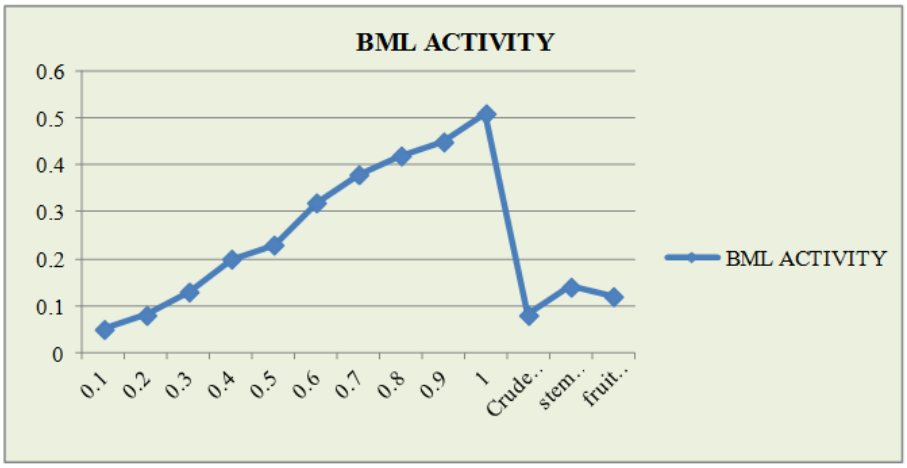


Fig. 2: Protein activity of stem and fruit BML proteases

3.2 Estimation of enzyme activity of BML through Cysteine

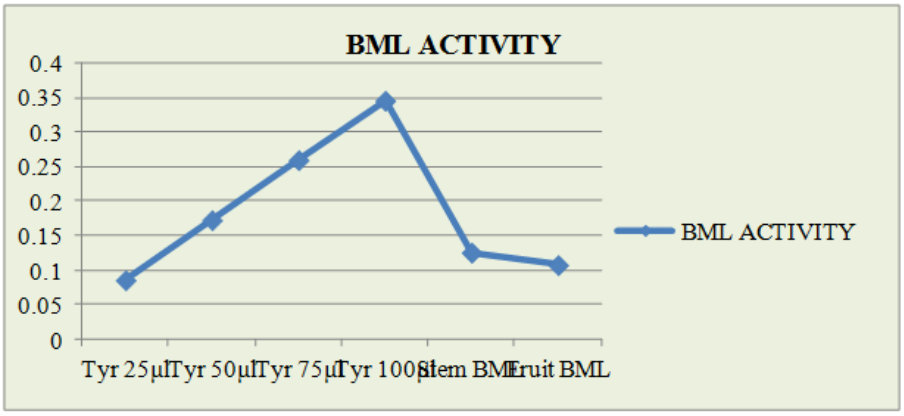


Fig. 3: Activity of stem and fruit BML proteases

The BML Activity in Stem: 878.14 CDU/mL
The BML Activity in Fruit: 753.20 CDU/mL

3.3 Effect of Temperature on BML activity

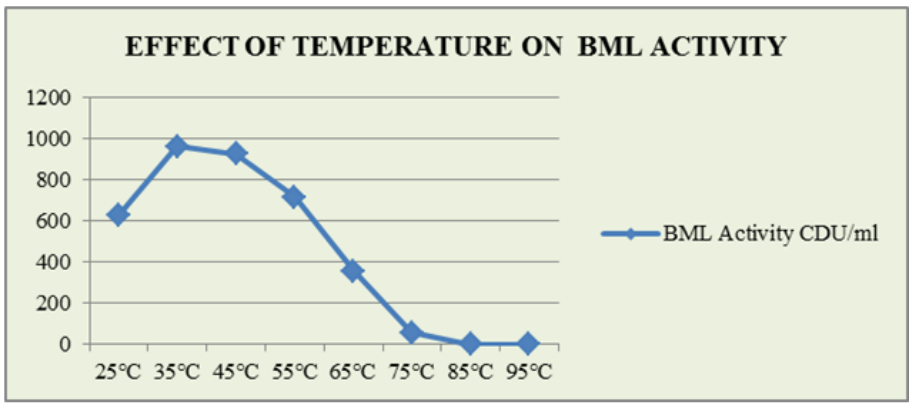


Fig. 4: Effect of Temperature on stem BML Activity

The optimum temperature for BML Activity: 35-45ºC

3.4 Effect of pH on BML activity

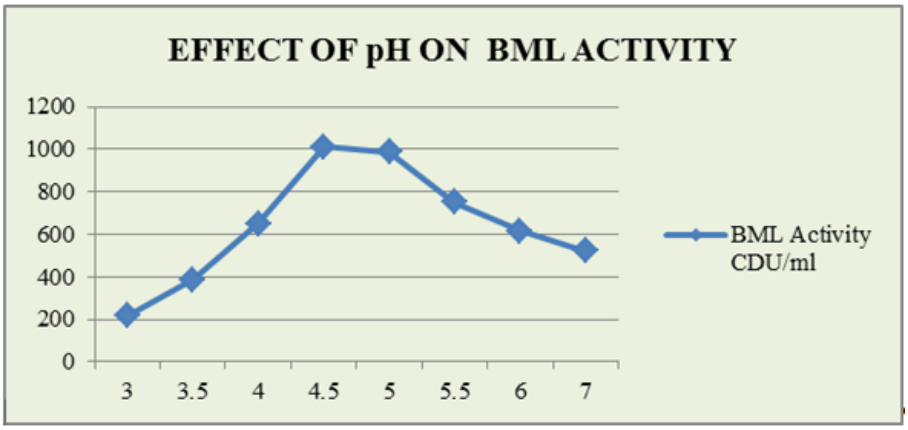


Fig. 5: Effect of pH on stem BML Activity

The optimum pH for BML Activity: 4.5-5.0

3.5 Fourier Transforms Infrared (FTIR) Spectroscopy

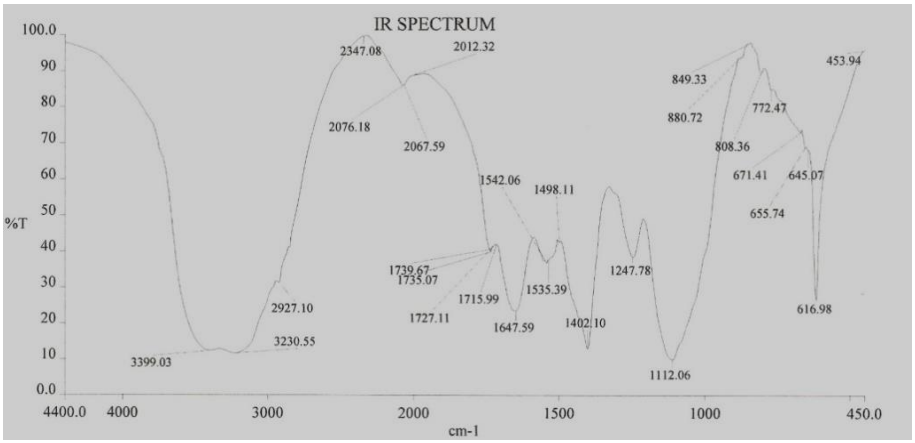


Fig. 6: Infrared spectroscopy of stem BML proteases

IR Frequency (cm^{-1})	Assignment
616.98	NH (1° & 2° amines wag)
1112.06	=CH (Alkenes bend)
1247.78	C-N (Aliphatic amine stretch)
1402.10	C-C (Aromatic stretch)
1542.06	N-O (Symmetric stretch)
1647.59	C=O (Amide stretch)
1727.11	C=O (Aldehyde stretch)
2067.59	C≡C (Alkynyl stretch)
2927.10	CH (Aromatic stretch)
3230.55	COOH (Carboxylic acid stretch)
3399.03	OH (Alcoholic stretch)

3.6 Ultra Violet Spectroscopy

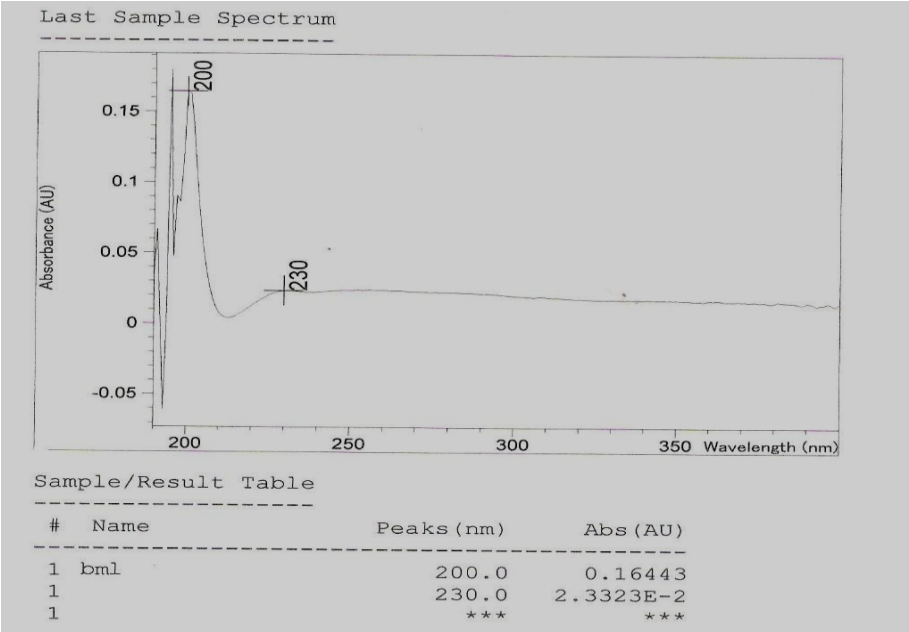


Fig. 7: Ultra-violet spectroscopy of stem BML proteases

3.7 Liquid Chromatography Mass Spectroscopy

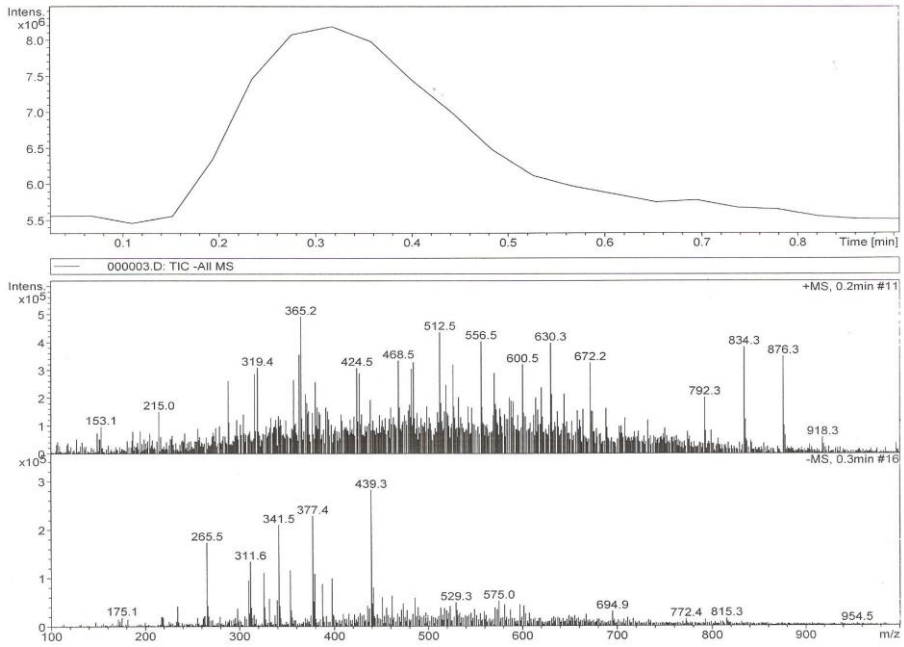


Fig. 8: LCMS of stem BML proteases

3.8 Powder X-ray Diffraction

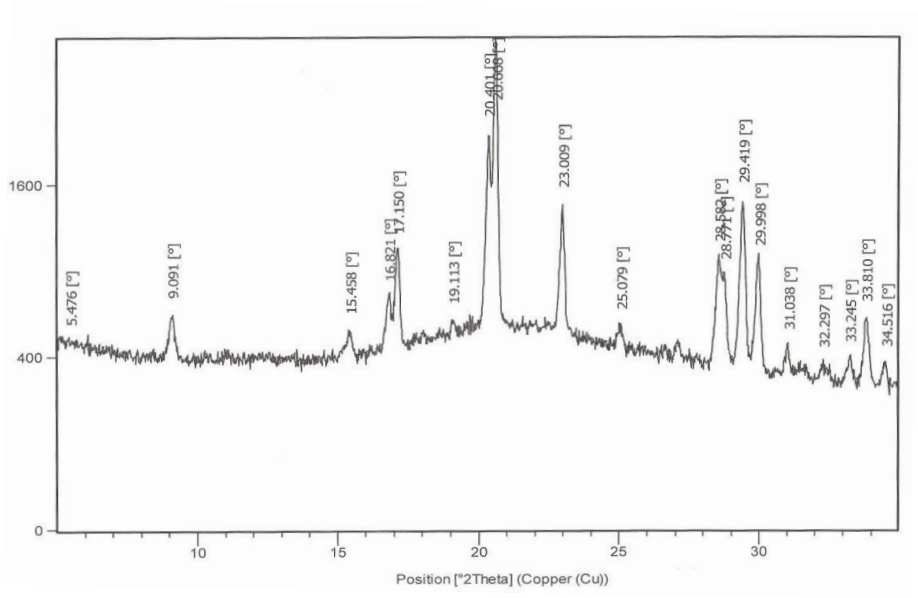


Fig. 9: Diffract gram of stem BML proteases

The Diffractogram shows the peak due to crystallinity of BML proteases

4 Discussion

The BML proteases isolated from the stem of *Ananas comosus* shows the maximum enzyme activity than the BML proteases extracted from fruit. The protein concentration in stem extracts was found to be 4.66mg/mL whereas the protein concentration was less in fruit extracts and the concentration was found to be 4.0mg/mL. The enzyme activity of BML proteases through cysteine was about 878.14 CDU/mL in stem extracts and 753.20 CDU/mL in fruit extracts. The effect of series of temperature modulations was carried out and the optimum temperature for BML Activity was found to be at 35-45ºC. As some enzymes are active at certain pH level, BML was subjected to the effect of pH on its activity. The optimum pH for BML Activity was found to be pH 4.5-5.0 range. As we got the promising results in stem extracts, further spectroscopic analysis for the BML isolated from stem was carried out to characterize their chemical entity.

5 Summary

BML Proteases was isolated to implement the medicinal values of natural product into the integrated medicine. The aim of my research work was to isolate the BML proteases and characterize the purified BML using the different spectroscopic techniques. The BML was extracted using stem yielded a total protein content of 242.6 mg/mL with the proteolytic activity of 878.14 GDU/mL. The analytical evidence shows the structural study of BML proteases and its activity when subjected to different physical parameters such as temperature and pH. Infrared Spectroscopy shows the functional groups present in the isolated molecule. The Diffractogram shows the crystallinity nature of the BML proteases. Further studies on the activity of BML in the treatment of cancer chemotherapy will be discussed in my next publication.

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