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PURIFICATION, PARTIAL CHARACTERIZATION AND ACTIVITY PROFILING OF BROMELAIN PROTEASE

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ABSTRACT

This study aims at the purification and comparative analysis of active bromelain protease present in pineapple (Ananas comosus) (L.Merr.) fruit and its other wastes (core, crown, skin, and stem). The extracted enzyme was precipitated by ammonium sulphate (40%), dialysed and concentrated by TFF (tangential flow filtration) and further purified using IEX (ion exchange chromatography). The purified fractions were analysed by SDS-PAGE and MALDI ToF to confirm the molecular weight (23kDa) of the eluted protein. The fold of purification for Bromelain proteases in the core, crown, fruit, skin and stem was 5.54, 3.04, 0.41, 12.58 and 18.49 respectively. The activity of purified enzyme was confirmed by activity assay as well as Zymography. Statistical analysis suggested specific activity optimum at 45^{o}C temperature while the pH range to be 6.0-7.0. The presence of a glycan moiety in the bromelain protease was detected using novel proteoglycan staining.

KEY WORDS

Protease, Purification, Specific Activity, Zymography, Proteoglycan.

INTRODUCTION

Bromelain is obtained from Ananas comosus (L.Merr.) which belongs to the Bromoliaceace family and the genus of perennial epiphytic herbs. It is a mixture of different thiol endopeptidases, phosphatases, glucosidases, peroxidases, cellulases, glycoproteins, carbohydrates, and several protease inhibitors. [1] Bromelain is known since 1876 and used as a phytomedical compound in several native cultures. [2] India is the fifth largest producer of pineapple (Ananas comosus) contributing an annual output of 1.2 million tons to the total annual world production of 14.6 million tons. The pineapple fruit is the only marketable part of the plant but Bromelain is distributed almost in the entire plant. [3] Post juice extraction, significant portions of the fruit parts such as the core, crown, stem and skin are considered as agricultural residues and when disposed off create environmental problems. [4] Pineapple wastes have proven its uses as raw materials which can be converted to valuable products.Bromelain has wide range of applications in meat tenderization, baking industry, protein hydrolysates, enzymatic browning inhibition agent, animal feed, textile industry, tooth whitening as well as cosmetic industry. [5] Bromelain is also applicable in a wide range of pharmacological activities like fibrinolysis, anti-dermatous, antithrombotic, anti-inflammatory and anticancer *in vitro* as well as *in vivo.*[6]

Owing to the vast range of applications, acquiring active bromelain from waste parts of pineapple remains the major focus of the study. To adopt efficient purification methods, [7] approaches like centrifugation, ultrafiltration and lyophilization are most frequently used for bromelain purification as per research reports. Modern techniques such as gel filtration, ion exchange chromatography, affinity chromatography, aqueous two phase extraction and extraction via reversed micelles, RMS (Reverse Micellar System), ATPS (Aqueous Two Phase System) Ethanol, Acetone and PEG (Poly ethylene glycol) precipitation,

HSCCC (High speed counter current chromatography)have also been used for bromelain purification. Pardhi *et al*., 2016 reviewed that the use of ammonium sulphate precipitation followed by anion exchange chromatography for the purification of Bromelain yielded a high recovery and activity.[8] Thus, for efficient purification and maximum recovery of bromelain, implementation of purification methods that allows maximum protein concentration, recovery and high activity need to be applied.

MATERIALS AND METHODS

Chemicals and reagents:

Annanas Comosus L.Merr Species was used for bromelain extraction. Standard bromelain and glutardialdehyde was procured from Sigma Aldrich (Germany), Analytical Reagent (AR) grade cysteine, tyrosine, trichloroacetic acid (TCA), Tris, silver nitrate, casein, EDTA, Alcian Blue was procured from Loba Chemicals. Na2HPO4, NaH2PO4, EDTA, ammonium sulphate and NaCl was procured from Merck, India. All reagents for polyacryalamide gel electrophoresis were manufactured by Bio-Rad (USA). DEAE sepharose was obtained from GE healthcare, Protein ladder was procured from Thermo Fischer. All other chemicals used were of AR grade.

Methods:

2.1. Preparation of bromelain extract

The chopped pieces of core, crown, fruit, skin and stem were homogenized in mortar and pestle and then ground with 100 mM sodium acetate buffer pH 7.0. The juice obtained was filtered and centrifuged at 3500g for 10mins at 4°C.This extract was used as crude bromelain preparation from the respective parts for further purification. [9]

2.2. Ammonium Sulphate ($NH_4(SO_4)_2$) Precipitation:

The crude core, crown, fruit, skin and stem proteins were precipitated separately by using ammonium sulphate at 40% saturation. To each sample, solid ammonium sulphate salt was added with continuous stirring. The samples were kept overnight at 4°C to ensure better precipitation. These were centrifuged at 9000g for 10min and the precipitate obtained was dissolved in 10 mM tris, pH 7.0. [10]

2.3. Dialysis:

To remove salt, the ammonium sulphate precipitated samples were dialyzed using the TFF (tangential flow filtration) system. Initially the samples were diluted with the exchange buffer (10 mM tris, pH 8.0) and subsequently passed through the TFF system. The Centramate cassette of nominal molecular weight cut-off (NMWC) of 5 kDa was used to dialyze the protein with 10 exchanges of buffer. The feed flow pressure was maintained at 75kPa, and the retentate flow pressure was kept below 50 kPa.

2.4. Ion Exchange Chromatography (IEX/FPLC):

DEAE sepharose fast flow was packed in 1.75 X 3 cm column and Samples were loaded at a flow rate of 4mL/min maintaining the pH at 8.0 with the binding buffer (20mM tris). Elution was carried out using a linear gradient of NaCl from 1 mM to 1000 mM in presence of 20mM tris, pH 8.0. The detector response was measured at 280nm and the fractions were collected and stored at 4^0C for further studies.[11]

2.5. Protein Quantitation and SDS PAGE analysis:

Target protein was quantified on UV visible Spectrophotometer (UV-Vis 1800, Lab India) using extinction coefficient of bromelain. The absorbance at 276 nm was used to calculate protein concentration. Crude samples along with IEX purified fractions were analysed on 10% SDS PAGE, under non-reducing conditions (Biorad apparatus). After separation, the purified fractions were detected with Coomassie brilliant blue staining.

2.6. MALDI ToF analysis:

IEX fractions were analyzed by Bruker Daltonics MALDI-ToF using the LP30 210 kDa method for mass identification, under positive ion mode. The pH of the samples were lowered below pH 3.0 with 10% Trifluoroacetic acid (TFA), after which the samples were mixed with matrix (Sinapinic acid+ Acetonitrile) in 1:1 ratio and spotted on the MALDI plate. [12]

2.7. Proteolytic Activity Assay

The protease activity was assayed using the Casein Digestion Assay method[13] by monitoring the rate of tyrosine release (µM) per min. The reaction mixture contained 1% casein (w/v) and 0.1 mL enzyme appropriately diluted in 50 mM potassium phosphate buffer. Post 10 mins of incubation, the reaction was stopped by adding 0.3 mL of 5% TCA (w/v). The test mixture was centrifuged at 9000g for 10 min and the supernatant was collected. The absorbance of the supernatant at 275 nm was measured against the

corresponding blank minus the enzyme. From the standard curve, the activity of protease samples were determined in terms of Casein Digestion Units (CDU), which is the amount in micromoles of tyrosine equivalents released from casein per minute.

2.8 Calculation of fold purification and yield –

The fold of purification was calculated to determine the number of purification times compared with the crude extract. Percentage yield was calculated to display the highest enzyme activity that can be achieved.

Fold purification = Specific activity of fraction/Specific activity of crude fraction

Yield (%) = Total enzyme activity of fraction/ Total enzyme activity of crude fraction*100

2.9. pH and temperature profiling:

The pH and temperature profiling was performed at pH 3.0(citrate buffer), 6.0 (phosphate buffer), 7.0 (phosphate buffer), 8.0 (phosphate buffer), and 9.0 (carbonate buffer), at incubation temperatures 25^{o}C, 37^{o}C, 45^{o}C, 65^{o}C, 75^{o}C and 85^{o}C. The protease activity was determined using Casein Digestion Assay method.

2.10. Activity staining:

Zymography was performed to check in-gel activity of the bromelain protease. [14] Four µg of purified bromelain protease was loaded on 10% PAGE with 0.05% casein and stained with Coomassie brilliant blue

2.11. Proteoglycan staining:

The purified fractions were loaded on 10% SDS-PAGE. After electrophoresis proteoglycan staining was carried out using [15] method. The gel was washed and subjected to Alcian blue staining, glutardialdehyde sensitization and silver staining.

2.12. Statistical Analysis:

The obtained value for pH, Temperature profiling were analysed statistically using Hour Stats Software. Mean value and the Standard deviation was estimated and represented in graphical format.

3. RESULTS

3.1 Purification and molecular weight determination of bromelain protease:

Bromelain protease was extracted and purified from core, crown, fruit, skin and stem of Ananas Comonus L.Merr species using ammonium sulphate precipitation and further purified by IEX chromatography using DEAE sepharose. The purification profile is summarized in (Fig 1).

The molecular weights observed after SDS-PAGE electrophoresis were ~23 kDa for all the fractions. To confirm the obtained molecular weights, IEX purified samples were analysed by MALDI ToF. As seen in Fig. 2 the molecular weights for core, crown, fruit and skin obtained were 23.67,23.62, 23.67 and 23.68 kDa respectively. 3 peaks were obtained for Stem at 13.11, 20.95, 27.84 kDa (Fig. 2).

3.2 Effect of pH and Temperature on bromelain activity:

Bromelain protease purified from core, crown, fruit, skin and stem exhibited maximum mean enzyme activity at pH 7.0. The enzyme extracted from core, crown and fruit were active up to pH 9.0 whereas the activity of skin and stem decreased after pH 7.0. The effect of pH on bromelain protease can be seen in Fig. 3 As observed from fig. 4 the mean activity at various temperatures was studied for the purified enzyme from fruit, core, crown, skin and stem was maximum at 45°C and shows activity up to 65°C after which the activity started to decrease. It was observed that the bromelain protease was most stable and active at pH 7.0 and 45°C.

3.3 Activity profile of bromelain protease purified from various parts:

From Table No.1 it is observed that the highest activity was obtained in the order of core, crown, fruit, skin and stem.

Table no. 1: Summary of Bromelain protease purification studies.

Samples	Protein amount purified(mg)	Crude Activity (U/mg)	Purified Activity (U/mg)	%Yield	Fold of Purification
Core	30.68	792.52	4403.6	25.85	5.54
Crown	80.41	404.76	1959.3	32.51	3.04
Fruit	127.4	833.12	1477.45	40.65	0.41
Skin	58.63	255.41	1049.9	12.01	12.58
Stem	43.11	171.22	555.13	30.56	18.49

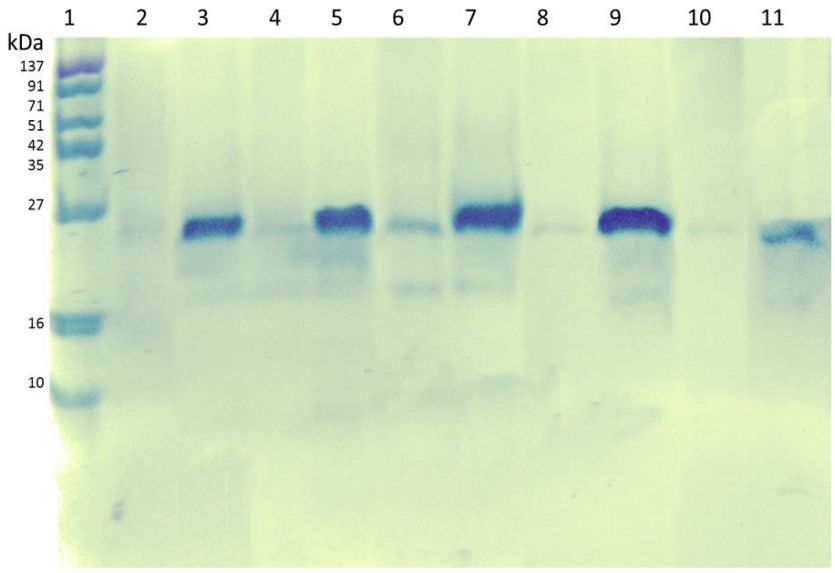

Fig1.10% SDS-PAGE, under non- reducing condition, stained with 0.25% Coomassie Brilliant blue R250 shows crude and purified fractions.

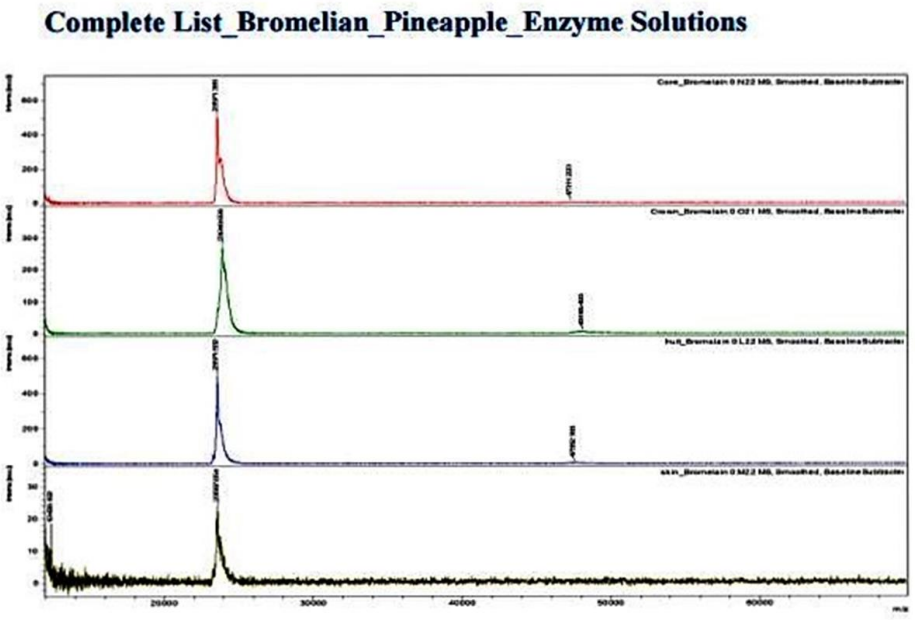

Fig 2: MALDI ToF analysis of eluted IEX fractions in core, crown, fruit, skin

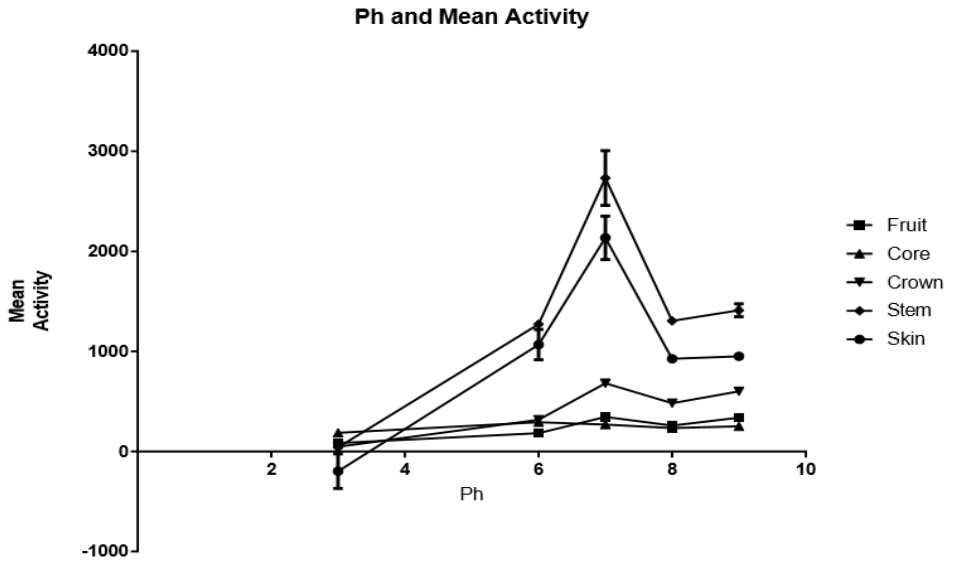

Fig 3. pH profile

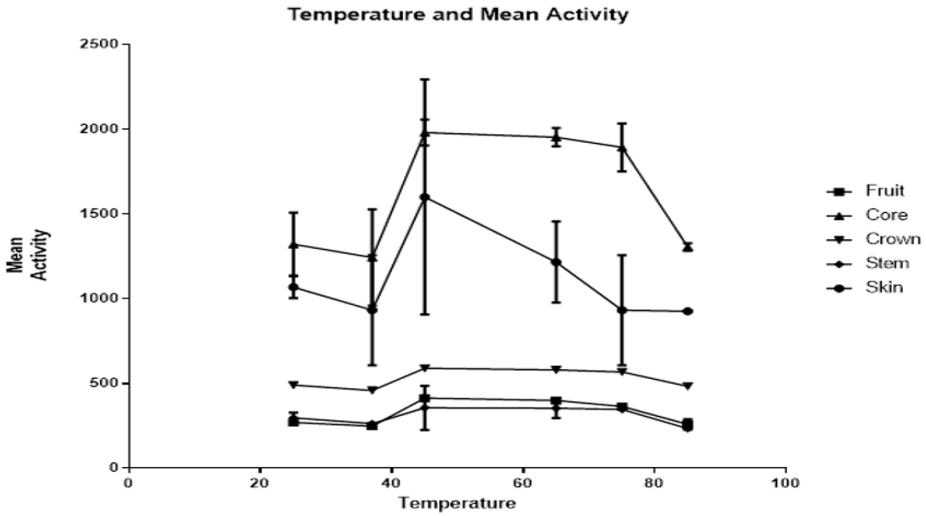

Fig 4. Temperature profile

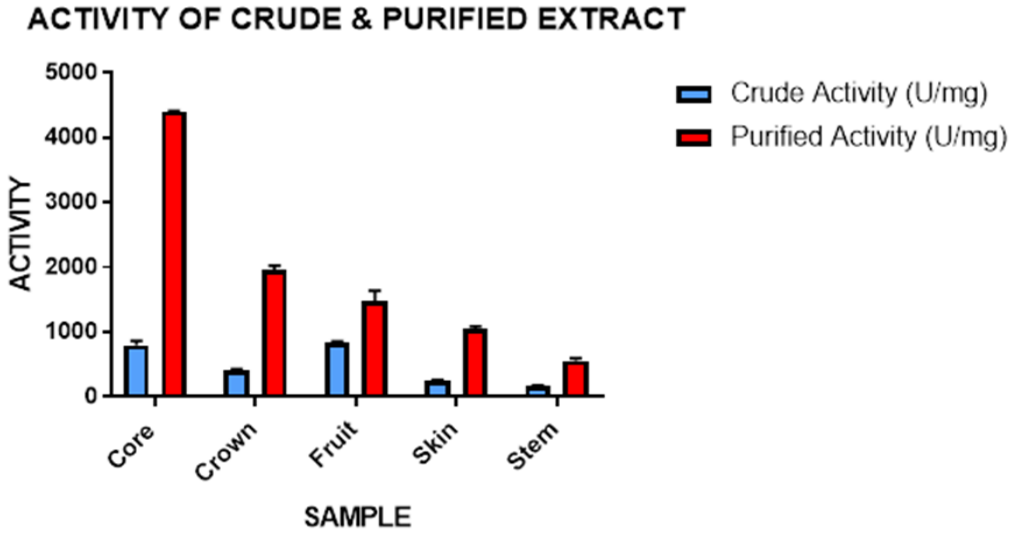

Fig. 5: Activity profile of Crude and purified Bromelain protease in CDU

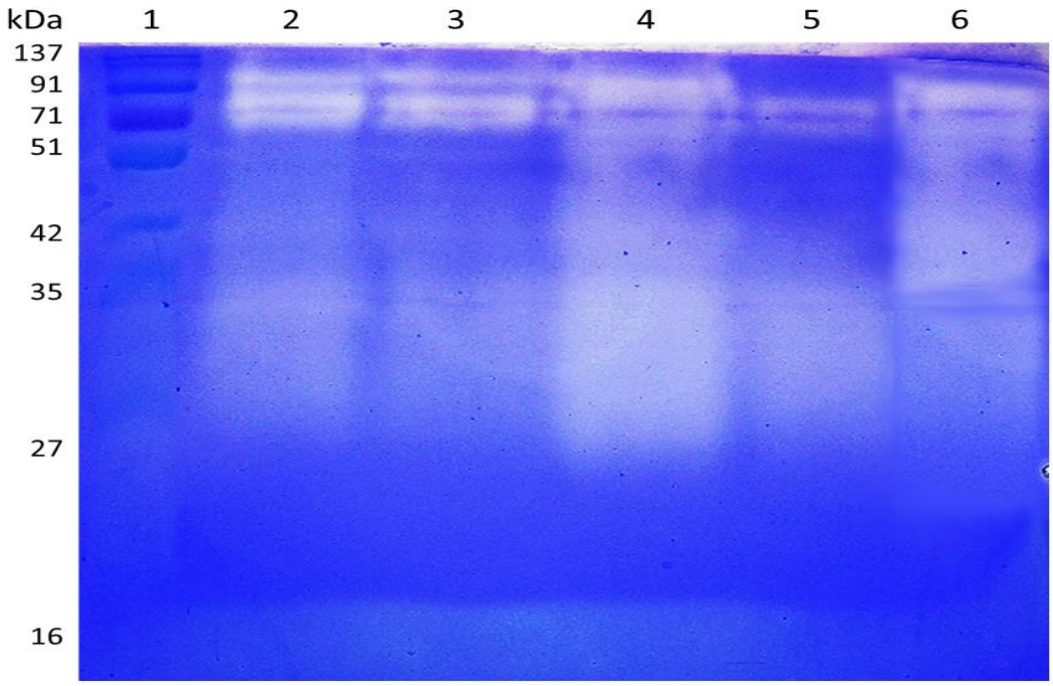

Fig.6.10% PAGE, Stained with 0.1% Coomassie Brilliant blue R250, in presence of 0.5% substrate. Lane 1-6 contains Prestained molecular weight marker, Core, Crown, Fruit, Skin and stem fractions respectively.

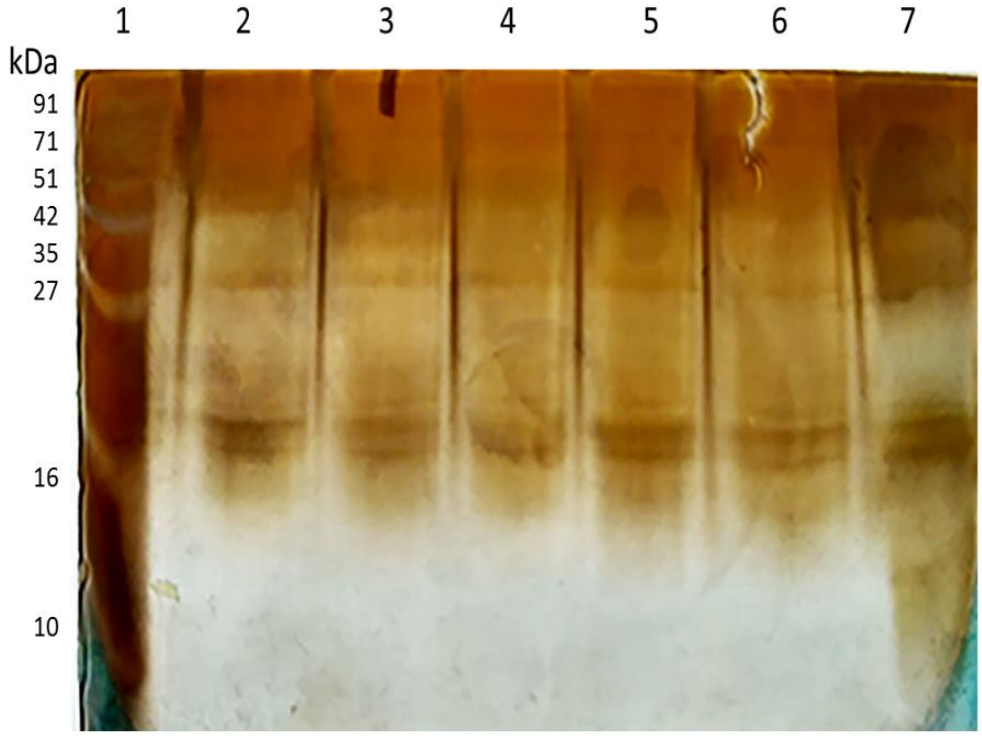

Fig. 7: 10% SDS-PAGE, showing, stained with Proteoglycan staining method, showing glycan moiety present with the Bromelain. Lane 1 contains Prestained Protein marker. Lane 2-6 includes core, crown, fruit, skin and stem fractions respectively.

A significant increase in the specific activity of the fractions after chromatographic purification was obtained as shown in Fig. 5. Percent yield was reduced as more purification methods were employed to obtain pure bromelain proteases. The fold of purification depicts how pure is the enzyme preparation compared to crude. An increase in fold of purification suggests that most of the impurities have been removed. (Fig 5).

3.4. Activity staining

In-gel activity of bromelain protease was studied using zymography. Clear zone on the gel for all samples indicated substrate digestion by the enzyme. (Fig. 6).

3.5. Proteoglycan analysis using PAGE and staining method:

Proteoglycan staining of IEX fractions after electrophoresis was done with Alcian Blue, a developing reagent to detect glycans in the proteins. Band at ~23 kDa was observed after staining which confirmed the presence of glycan moiety in Bromelain. (Fig.7).

DISCUSSION:

Bromelain proteases are known for its medicinal qualities with wide range of pharmacological actions, the mechanism of which is unknown yet. In order to study the mechanism behind the action of bromelain protease, it is important to isolate and purify it from the Pineapple plant. Different parts possess bromelain protease with different concentration and activity. As per early accounts, the bromelain that has been purified to homogeneity and characterized was reported from fruit and stem of pineapple. [16] [11] Purifying bromelain protease from pineapple fruit and its waste parts with enhanced specific activity was the major finding of our study. We report the better fold of purification of active bromelain protease. Moreover, studying the kinetic parameters of the purified enzyme and its statistical analysis facilitated a better understanding of the part wise distribution of bromelain proteases. This would help the researchers to explore the pineapple characteristics to validate its potential. Also, the structural characterization of bromelain protease which we would be targeting further will provide us a deeper insight towards its mechanism of action as fibrinolytic, antithrombotic, anti-inflammatory and anticancer agent.

The IEX chromatography could separate and purify Bromelain from the crude extracts of various parts. The requirement of high concentration of NaCl to elute Bromelain protease suggests the strong binding of it to the anion exchanger matrix (DEAE Sepharose FF). This indicates that the bromelain protease exhibits high negative charge density at experimental pH value. The purified Bromelain protease thus shows acidic nature. The bromelain is a complex of different enzymes ranging from ~19-35 kDa [5], while the enzyme purified in this study was of 23kDa as described in Fig. 1 And 2. Percent yield depicts the efficiency of the method to purify the target protein is described in table 1.0 and its high for Fruit. The fold of purification was estimated by enzyme quantitation and activity assay. The fold of purification for Bromelain proteases in core, crown, fruit, skin and stem was 5.54, 3.04, 0.41, 12.58 and 18.49 respectively (Table 1.0). Optimization of activity parameters were done post purification using the purified fractions which were then compared with the activity of the crude sample in order to generate accurate specific activity profile of bromelain protease. The statistical analysis of values obtained for specific activity of bromelain protease at various pH and Temperature range shows that there is significant difference in the activity of bromelain protease. The deviation in the mean activity in some parts at few pH values might be the result of atypical behaviour of Bromelain protease. However, it works best within the range of pH 6.0-7.0 in all parts of plant.

Zymography confirms the bromelain protease activity which supports the activity assay data. The core, crown and fruit showed higher activities in assay as well as in Zymography. However, stem showed lesser activity in both. It has been reported that the bromelain is glycoprotein [17] and the presence of glycan moiety and its structural characterisation was studied using mass spectrometry based techniques. However, in this study, we adapted a basic method for glycoprotein characterization of bromelain proteases. Electrophoresis method followed by proteoglycan specific Alcian blue staining could reveal the presence of glycan moiety. The Alcian blue is a cationic dye which binds to the negatively charged glycosaminoglycan side chains. As neutral glycoproteins requires some modifications of Schiff base reaction which involves initial oxidation of carbohydrates(glycan) by periodic

acid, the associated glycosaminoglycan in bromelain protease are acidic in nature. [15]

CONCLUSION

In the above study, an attempt was made to develop a method to purify bromelain protease, one of the constituents of bromelain complex from pineapple fruit and its waste parts. The confirmation of molecular weight of bromelain protease and its specific activity at different pH, temperature and substrate concentration was studied. The estimation of molecular weights by MALDI-Tof method and visualisation of glycan moiety by Proteoglycan staining for bromelain protease is reported for the first time in the current study. Moreover, the molecular characterisation and its structural elucidation would give more insights towards the mechanism of action of bromelain protease.

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