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EXTRACTION, PURIICATION AND KINETIC STUDIES OF BROMELAIN ENZYME FROM ANANUS COMOSUS

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ABSTRACT: Bromelain (EC 3.4.22.32) is a crude extract from the pineapple (Ananas comosus) plant that contains, among other components various closely related proteinases (stem bromelain, fruit bromelain, comosain and ananain) demonstrating both in vitro and in vivo several therapeutic properties including malignant cell growth, thrombus formation, inflammation, control of diarrhoea, dermatological and skin debridement among others. Bromelain also contains peroxidase, acid phosphatase, and several protease inhibitors and organically bound calcium and remains stable over a wide range of pH 2 to 9. Available evidence indicates bromelain is well absorbed orally with its therapeutic effects being enhanced in a dose dependent manner. It has been demonstrated to be safe and an effective food supplement. However, all the mechanisms of its action remain unresolved.
In this work the comparative study of stem , pulp , leaf bromelain has been parformed. Juice were extracted from stem , pulp , leaf of pineapple plant which contains the enzyme stem bromelain , pulp bromelain , leaf bromelain respectively. The juice extracted was called as crude extract of the enzyme and the activity of the crude extract on the hydrolysis of gelatin was estimated and represented in the form of gelatin digestion unit (GDU). From the result, it was determined that the pulp bromelain showing more enzymatic activity then stem bromelain and leaf bromelain after that crude extract as subjected to ammonium sulfate pricipitation to pricipitate the enzyme. The pellet was dissolved in 10 mM Tris HCL buffer and subjected to dialysis to remove the salt and other ions bound to the enzyme. Then enzymes purified by ion exchange chromatography, were the resin used as DEAE and CMC cellulose respectively.
Key words: Bromelain, Ananas comosus, enzyme immobilization, enzyme assay

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INTRODUCTION

Bromelain is one of the protease enzymes found in the pineapple plant (Ananas comosus) and given a lot of attention recently [1-3]. Stem bromelain (EC 3.4.22.32) is the major protease present in extracts of pineapple stem while fruit bromelain (EC 3.4.22.3) is the major enzyme fraction present in the juice of the pineapple fruit [4]. Some other minor cysteine endopeptidases (ananain, comosain) are also present in the protease mixture extracted from pineapple stem. Although the fruit bromelain was discovered much earlier than stem bromelain, the biochemical characterization of the latter enzyme has been described in more detail [5, 6].

Stem bromelain is widely used in industry and medicine, but fruit bromelain is not commercially available, even if it could be easily obtained from pineapple juice by simple ultrafiltration [7-10]. Stem bromelain preparation contains a complex mixture of different thiol-endopeptidases and other partially characterized components such as phosphates, glucosidases, peroxidases, cellulases, glycoproteins and carbohydrates, among others [11]. The entire extracts of stem bromelain exhibit its activity over a wide pH range of 5.5 to 8.0 [12-14]. Bromelain are widely attributed from pineapple and accepted as therapeutic qualities as a traditions in South America, China and Southeast Asia [15]. The use of bromelain in medicine is gaining more recognition owing to the fact that several clinical studies indicates its applications in oncology, inflammatory conditions, skin debridement, immune regulation, etc. Consequently, there is growing demand for the proteases of both eukaryotic and prokaryotic microorganisms. Bromelain acts systemically, affecting directly several cellular and molecular targets and the relevant therapeutic applications. Recently, recombinant bromelain was produced by recombinant DNA method [16], characterized, the fermentation condition and downstream processing were also optimized. One of the important downstream processing for recombinant enzyme is the powder preparation where the production cost will be affected mush at this stage, thus spray drying technique may be chosen to obtain lowest production cost. Spray drying efficiently removes initial moisture resulting in a large throughput and low to medium operating cost, 30 to 50 times less than the freeze drying [17]. Spray drying offers the direct formation of droplets undergoing chemical reaction during drying which makes 38 Azura Amid et al, this technique widely used for drying heat sensitive foods, pharmaceuticals active ingredients and many under sensitive substance. In addition, spray dried product can be transported at low cost and stored in a stable form. However, dehydration process itself may cause protein inactivation. Removal of the aqueous medium surrounding the enzyme may also lead to breakage of the hydrogen bonds. Further, the interactions between the hydrophilic groups may change. These factors lead to enzyme denaturation and inactivation as mentioned above. Thus, in order to obtain the desired quality of recombinant bromelain powder, optimization of operational parameters for spray drying is needed. Therefore, in this study, recombinant bromelain powder will be produced by using the spray drying technique.

METHODOLOGY

Collection of sample

The sample was collected from super market of ECIL, SECUNDRABAD. Wash in tap water and prepare the extract with the help of mortar and pestle. For further activities.

Extraction method of Bromelain from pineapple (Ananas Comasus)

Leaf

The leaves are washed and dried. 50 grams of leaves are cut into small pieces. The leaves are homogenized in a mortar and pestle by adding 75ml of pre cooled sodium citrate buffer.

Stem

The stem are washed and dried. 50 grams of stems are cut into small pieces. The stems are homogenized in a mortar and pestle by adding 75ml of pre cooled sodium citrate buffer.

Pulp

The 50 grams of pulp are cut into small pieces. The pulp is homogenized in a mortar and pestle by adding 75ml of pre cooled sodium citrate buffer. Both are homogenized filtered using a muslin cloth to remove fiber. The homogenates are centrifuged at 5000 rpm for 15 minutes. The pellet are discarded and the crude protease was stored at $4^{o}C$.

Enzyme Assay for crud Extract

2.5ml of Gelatin solution was incubated at $45^{0}C$ for 10 minutes.0.1ml of crude enzyme was added to all the tubes except blank and incubated at $45^{0}C$ for 20 min. 0.1 ml of water was added to blank tube.0.01 microliter of hydrogen peroxide was added to all the tubes and PH was adjusted to 6.9 with 0.05N NaOH. To this 1.0ml formaldehyde was added to all the tubes. Titration was done with 0.05N NaOH and volume of NaOH runned down.

Purification Method of Crude Enzyme of Bromelain from Pineapple

Purification of Bromelain enzyme was done by three methods:

1. Salt precipitation method
2. Dialysis method
3. Ion-exchange chromatographic method

Salt Precipitation by Ammonium Sulphate

Procedure

Weigh 44 grams of Ammonium sulfate (for 100ml of suspension. Add pinch by pinch Ammonium sulfate into the enzyme suspension in ice cold conditions, on magnetic stirrer for 1 hour. Keep it in ice-cold conditions for overnight. After Ammonium sulfate precipitation, take the enzyme suspension into centrifuge tubes. Centrifuge at 10000rpm for 10minutes.Collect the pellet and dissolve it in 10 ml of Tris-HCl buffer.

2. Dialysis Method

Procedure

100 ml of distilled water was boiled and the dialysis membrane was added into the boiling water. After boiling it for 10 min2% sodium bicarbonate was added and boiled it for 10 minutes. 100 ml of distilled water was added and boiled it. The dialysis membrane was transferred into this boiling water and boiled it for 10 minutes.10ml of enzyme suspension was added into the dialysis membrane. Another side of the dialysis membrane was tied. Placed in distilled water containing beaker on magnetic stirrer for 2-3 hours or keep it in refrigerator for overnight.

3. Ion- Exchange Chromatography

Procedure

Six elutes with 25mM Tris- HCl, 25mM, 50mM, 75mM, 100mM,125mM,150mM NaCl were prepared in 6 different test tubes. Column preparation was done by washing the column with ethanol, then with distilled water. DEAE cellulose was added to the column leaves it for settling. Enzymes were added into the column leave it for settling and Collected into the sample into the test tube.

Protein Estimation by Lowery Methods

Procedure

Pipetted out each 0.1ml of crude enzyme, salt precipitation, dialysis, elutes and different volume of standards samples 0.2, 0.4, 0.6, 0.8, 1.0 into a series of different test tubes. Incubated the tubes at dark room temperature for 30 minutes and observed the OD values at 660nm.

Enzyme Kinetics

Effect of Different pH Enzyme Activity

Procedure

2.5ml of gelatin solution of was taken in each test tube and then maintained the different $_{P}H$ 3.5, 4.0, 4.5, 5.0, 5.5.0.1ml of enzyme was added to all the test tubes except blank and Incubated for 45^0C for 10 minutes. 0.1ml of distilled water of blank test tubes was added and 0.01 micro liter of hydrogen peroxide was added to all the test tubes. Adjusted the PH 6.9 with 0.05 N NaOH.8 and then add 1.0ml of formaldehyde to all the test tubes. Titration was done until PH 7.8 was seen with 0.05N NaOH and volume of NaOH is run down.

b) Effect of Different Temperature on Enzyme Activity

2.5ml of gelatin solution of was taken in each test tube and then maintained the different temperature 35, 40, 45, 50, 55.0.1ml of enzyme was added to all the test tubes except blank and Incubated for 45^0C for 10 minutes. 0.1ml of distilled water of blank test tubes was added and 0.01 micro liter of hydrogen peroxide was added to all the test tubes. Adjusted the PH 6.9 with 0.05 N NaOH.8 and then add 1.0ml of formaldehyde to all the test tubes. Titration was done until PH 7.8 was seen with 0.05N NaOH and volume of NaOH is run down.

C) Effect of Activator on Enzyme Activity

2.5ml of gelatin solution of was taken in each test tube and then maintained the different volume of $MgCl_2$ inhibitor 10, 20, 40, 60 100 micro liter.0.1ml of enzyme was added to all the test tubes except blank and Incubated for 45^0C for 10 minutes. 0.1ml of distilled water of blank test tubes was added and 0.01 micro liter of hydrogen peroxide was added to all the test tubes. Adjusted the PH 6.9 with 0.05 N NaOH.8 and then add 1.0ml of formaldehyde to all the test tubes. Titration was done until PH 7.8 was seen with 0.05N NaOH and volume of NaOH is run down.

D) Effect of Inhibitor on Enzyme Activity

2.5ml of gelatin solution of was taken in each test tube and then maintained the different volume of $HgCl_2$ inhibitor 10, 20, 40, 60 100 microliter.0.1ml of enzyme was added to all the test tubes except blank and Incubated for 45^0C for 10 minutes. 0.1ml of distilled water of blank test tubes was added and 0.01 micro liter of hydrogen peroxide was added to all the test tubes. Adjusted the PH 6.9 with 0.05 N NaOH.8 and then add 1.0ml of formaldehyde to all the test tubes. Titration was done until PH 7.8 was seen with 0.05N NaOH and volume of NaOH is run down.

SDS-PAGE Electrophoresis

Procedure

Buffer (staking, separating and electrode buffer)was prepared and glass plate, wiped with alcohol and applied silicon to spacer for attaching two plates. Separating gel was prepared by adding 2ml of distilled water, 2.5 ml separating gel buffer, 3.3 ml of stock acrylamide solution, then added 10 micro liter of TEMED (Tetra Methyl Ethylene Di amine), 200 micro liter of SDS (sodium Dodecyl Sulfate)and 100 micro liter APS (Ammonium per Sulfate). Immediately poured in between to plates. Leaved it for some time, for solidification. Stacking gel was prepared by adding 2.5ml of distilled water, 1ml stacking gel buffer, 2ml stock acrylamide solution, then added 10 micro liter of TEMED,100 micro liter of SDS. Immediately poured between plates and place the comb for wells formation leaves it for some time of solidification. Sample was prepared by taking 30 micro liter of protein sample and 10 micro litre in loading buffer in Eppendorf tubes and boiled it for 5 min in boiling water bath. Removed comb carefully and removed bottom spacer also, wiped with alcohol and placed it in SDS-PAGE tank. Poured the electrode buffer in the tank. The loaded sample into the wells with the help of micropipette or syringe. Switched on the power (100v) supply for 1-2 hours continuously. Switched off the power supply take outs the plates from SDS-PAGE tank. Removed thus side spacers separate the two plates with help of spatula and kept into the staining buffer for overnight. After incubation transfer the gel into the distaining solution, for 1 to2 hours. Then observed the bands.

RESULT AND DISCUSSION

Protein Estimation by Lowerys Method

The amount of protein was estimated by lowery's method and the concentration was plotted in Fig 1.

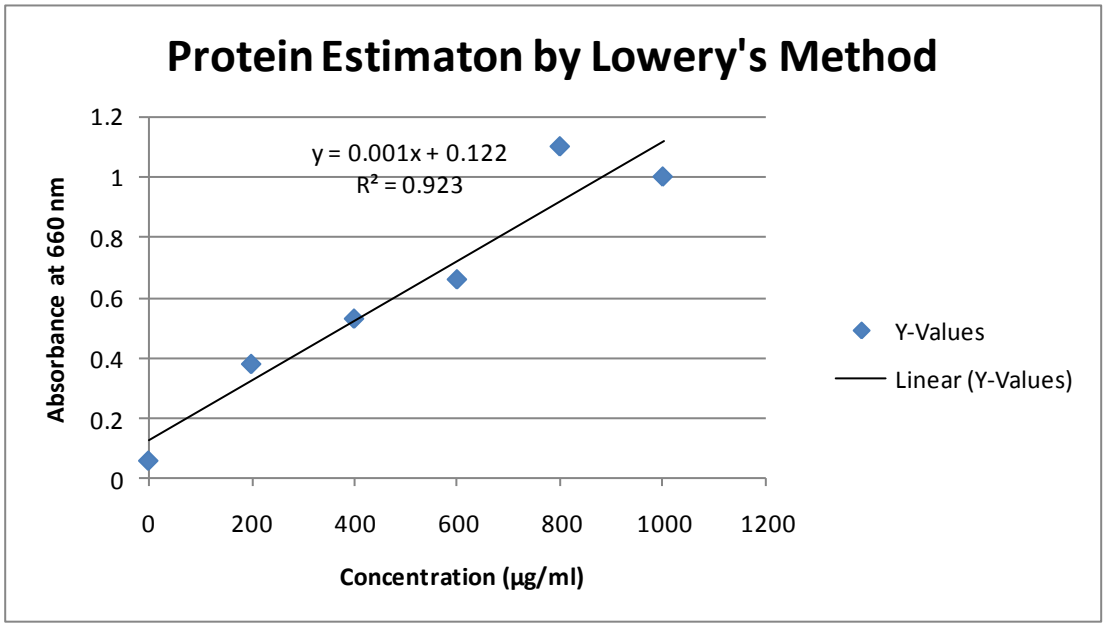

Fig 1: Estimation of protein by Lower's method

Protein Concentration

Protein concentration from pulp, stem and leaf samples were identified and tabulated in Table 1.

Table 1: Protein concentration from pulp, stem and leaf

S.No	Conc. of protein (mg/ml)
Pulp crude	2.5 mg/ml
Stem crude	1.1 mg/ml
Leaf crude	4.8 mg/ml
Pulp salt precipitation	1.3 mg/ml
Stem salt precipitation	0.3 mg/ml
Leaf salt precipitation	1.8 mg/ml
Pulp dialysis	0.3 mg/ml
Stem dialysis	0.4 mg/ml
Leaf dialysis	0.4 mg/ml
Pulp elutes	0.1 mg/ml
Stem elutes	0.1 mg/ml
Leaf elutes	0.4 mg/ml

Among the samples leaf crude has highest protein concentration with 4.8mg/ml. pulp and stem elutes has low protein concentration (Fig 2).

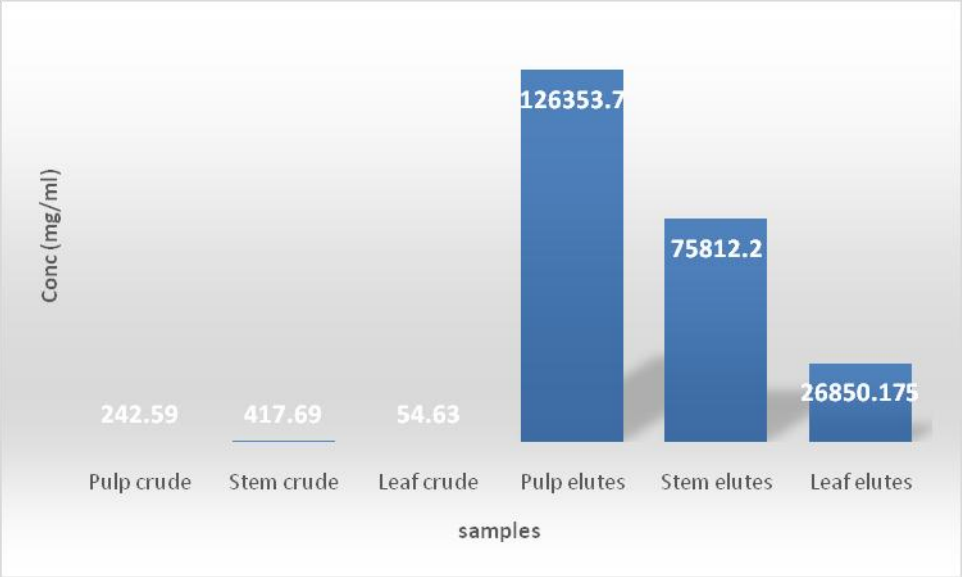

Fig 2: protein concentration in different samples

Enzyme Activity

The enzyme activity was measured by using following formula

Enzyme Activity= (Volume of test – Volume of sample) (N) 14 X 100 /

Mg enzyme/RM

Enzyme activity in pulp, stem and leaf were studied and tabulated in table 2.

Table 2: Enzyme activity in pulp, stem and leaf

S.No	Enzyme activity
Pulp crude	606.498 Unit/mg
Stem crude	459.46 Unit/mg
Leaf crude	263.23 Unit/mg
Pulp elutes	12635.37 Unit/mg
Stem elutes	7581.22 Unit/mg
Leaf elutes	10740.07 Unit/mg

Among the samples tested, pulp elutes showed highest enzyme activity where as leaf crude showed less enzyme activity (fig 3).

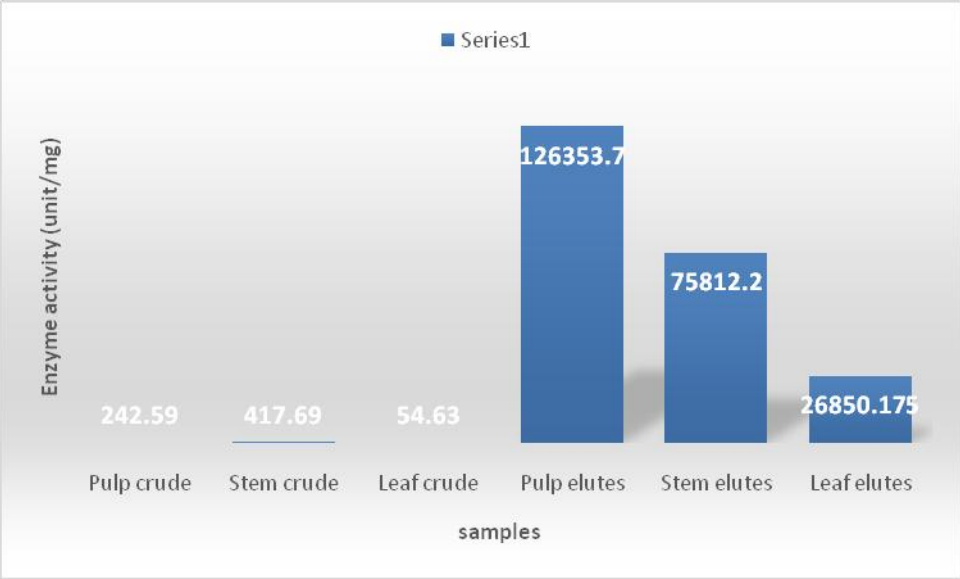

Fig 3: Enzyme activity in pulp, stem and leaf

Specific Activity

Specific activity was measured using following formula

Specific activity =Enzyme activity/protein concentration

The specific activity of the enzyme was measured and tabulated in Table 3.

Table 3: specific activity of enzyme in pulp, stem and leaf

S.No	Unit/mg
PULP CRUDE	242.59 Unit/mg
STEM CRUDE	417.69 Unit/mg
LEAF CRUDE	54.63 Unit/mg
PURIFIED PULP	126353.7 unit/mg
PURIFIED STEM	75812.2 unit/mg
PURIFIED LEAF	26850.175 Unit/mg

Yield

The percentage of yield was measured by following formula

% Yield =Enzyme Activity/Purified Crude

From the studies yield in PULP was4.80 %, STEM was 0.60 % and LEAFwas 2.45 %.

Enzymatic assay of crude extract of bromelain enzyme:

Table 4: Enzymatic assay of Bromelian enzyme

S.No.	Enzymatic specific activity (Unit/mg)	Protein concentration (mg/ml)	Purification fold
1.Pulp crude	606.498	2.5	4.80
1.purified pulp	12635.37	0.1	
2.Stem crude	459.46	1.1	0.60
2.Purified stem	7581.22	0.1	
3.Leaf crude	263.23	4.8	2.45
3.Purified leaf	10740.07	0.4	

CONCLUSION

Bromelain has been used for a variety of clinical applications for more than 35 years. Although its mechanisms of action has not been completed resolved, bromelain has demonstrated a beneficial effect on the kinin system, the coagulation cascade, the cytokine system, and prostaglandin synthesis. Bromelain is believed to enhance the absorption of flavonoids and has been shown to increase absorption of glucosamine, so bromelain supplementation should be considered when these nutrients are given. It may also enhance absorption and utilization of many other substances; however, to date research in this area has focused primarily on antibiotics. Bromelain has been shown to exert a beneficial effect at doses as low as 160 mg/day, however, there is a general consensus among researchers that the best results occur when bromelain is given in doses above 500 mg per day and that results improve in a dose-dependent manner with higher levels of bromelain supplementation. Bromelain has been demonstrated to be well absorbed after an oral dose and has been shown to be safe at high doses for prolonged periods of time. For the conditions discussed in this review, bromelain has shown itself to be an effective supplement.

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