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ISOLATION, PURIFICATION AND QUANTITATIVE ANALYSIS OF CYSTEINE PROTEASE, BROMELAIN FROM *ANANAS COMOSUS* (PINEAPPLE)

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ABSTRACT

Proteases were extracted and assayed from the fruit of *Ananas comosus* (Pineapple). They were precipitated utilizing variable concentrations of acetone (40%,60%,80%,100%), ammonium sulphate (45%,65%) and Jello (40%,60%,80%,100%). The extracted enzymes exhibited proteolytic activity which was determined using other assay techniques. Activity determined for *Ananas comosus* was 2U/mL. The enzymes were, in their crude state, analyzed using SDS-AGE. Bands were observed between the molecular weight range of 24-45 KDa. The enzymes were purified from the extract by anion exchange chromatography using Silica Gel Column (pH-8.0, Sodium Phosphate Buffer). The elution of *Ananas comosus* extract resulted in two bound fractions. Since the second fraction of the *Ananas comosus* extract exhibited no protease activity, it was ignored. The purified samples were analyzed using SDS-AGE, the purified extract of *Ananas comosus* displays a single band (characteristic of bromelain, a monomeric molecule).

KEY WORDS: **Protease, SDS-PAGE, Silica Gel, *Ananas comosus*.**

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INTRODUCTION

The major cysteine protease of pineapple fruit *Ananas comosus* is Bromelain. Bromelain is a mixture of enzymes found naturally in the juice and stems of pineapples. It is in the same family of thiol proteases as papain. Bromelain is commonly used as a commercial meat tenderizing agent; however, it has been known to show therapeutic properties. Bromelain has been suggested as a complimentary treatment for sinusitis. Preliminary studies suggest that it may help reduce congestion, improve breathing and suppress coughing. It is approved by the Commission E as a complimentary treatment for nasal and sinus swelling and inflammation after ear, nose, throat, surgery. A review of three small but well designed previously published studies found that bromelain may help relieve sinusitis symptoms.[1] Bromelain is a popular natural digestive aid due to it's ability to digest proteins. Bromelain may help with mild pain associated with osteoarthirits. Bromelain and other proteolytic enzymes have been explored as a complimentary treatment for cancer. Although there is some preliminary research, there isn't enough evidence at this time soon the safety or effectiveness of bromelain for cancer. It should never be used in place of conventional treatment.

MATERIALS AND METHODS

REAGENTS

- Acetone
- Ammonium Sulphate
- Jello (Gelatin)

1) LOWRY'S REAGENTS

- 2% Sodium Carbonate in 0.1 N NaOH
- 2% $CuSO_4.5H_2O$
- 2% Sodium Potassium Tartarate (Rochelle Salt)

OR

- Folin's Phenol – 2 N solutions of Folin's Phenol commercially available, yellow in colour and was used for colour generating reactions.

2) BRADFORD'S REAGENTS

- Coomassie Brilliant Blue G
- Phosphoric Acid, 85% *(Caution : It can burn skin)*
- Bovine Serum Albumin (BSA), 10mg/ml in water, from above

3) SDS-PAGE REAGENTS (38:1 w:w ratio of acrylamide to N,N'-methylene bis-acrylamide)

NOTE: Mix ingredients GENTLY! Ensuring no bubbles form. Pour into glass plate assembly CAREFULLY. Overlay gel with iso-propanol to ensure a flat surface and to exclude air. Wash off iso-propanol with water after gel has set (+15 min).

- **RESOLVING GEL: Gels concentrations of 12.5% in 0.25 M Tris-HCl, pH-8.8 (Volume/Volume)**

Reagents	30mL	10mL
40% Acrylamide Mixture	9.4mL	3.1mL
Distilled Water	12.3mL	3.8mL
1 M Tris HCl (pH-8.8)	7.5mL	2.5mL
10% SDS	0.3mL	0.1mL
1% Ammonium Per Sulphate	0.5mL	0.13mL
TEMED	20microL	8microL

- **STACKING GEL : Gels concentrations of 4.5% in 0.125 M Tris-HCl, pH-6.8 (Volume/Volume)**

Reagents	15mL	10mL
40% Acrylamide Mixture	1.7mL	1.1mL
Distilled Water	10.8mL	7.1mL
1 M Tris HCl (pH-8.8)	1.9mL	1.25mL
10% SDS	0.15mL	0.1mL
1% Ammonium Per Sulphate	0.5mL	0.13mL
TEMED	20MicroL	8MicroL

4) SAMPLE BUFFER

4.8 ml of deionised water + 1.2mL of 0.5 M Tris-HCl (pH-6.8) + 20mL of 10% SDS + 1mL of Glycerol + 0.5 mL of 0.5% of Bromophenol Blue (w/v in water) were mixed and were stored at room temperature.

5) REDUCING BUFFER

50 Micro-litre of 2-Mercaptoethanol was dissolved in 950 microlitre of sample buffer.

6) POLYMERIZATION CATALYST

Freshly prepared 10% Ammonium per Sulphate Solution in water TEMED was used directly from the supplied bottle which should be stored in the dark.

7) ELECTRODE BUFFER

15 g Tris + 72 g Glycine + 5g SDS was dissolved in 1 Litre of de- ionised water, pH-8.3.

8) STAINING OF GELS

COOMASSIE BRILLIANT BLUE STAINING

Make up stain: 0.2% CBB in 45:45:10 % methanol: water: glacial acetic acid. .

9) DESTAINING SOLUTION

De-stain with 25:65:10 methanol: water: glacial acetic acid mix, with agitation and observed under white light transilluminator.

10) CHROMATOGRAPHY BUFFER

- **BUFFER A – 0.1 M Sodium Phosphate Buffer, pH-8.5:** 3.1 g of $NaH_2PO_4.H_2O$ and 10.9 g of Na_2HPO_4 (anhydrous) was added to distilled water and the final volume was made to 1 litre with the pH 8.5.
- **BUFFER B – 0.1 M Sodium Phosphate Buffer + 1 M NaCl – Elution Buffer:** Add 3.1 g of $NaH_2PO_4.H2O$ and 10.9 g of Na_2HPO_4 (anhydrous) was dissolved in distilled water and total volume was made to 1 litre and the pH was adjusted to 8.5, in this 40 g NaCl was added.
- **1 M Sodium Phosphate Buffer (pH-7.0):** 1 M Sodium Phosphate Buffer was prepared to dissolve precipitated pellets. It was prepared by mixing 1 M NaH2PO4. That is for pH 7.0 buffer, 390 mL of 1 M NaH2PO4, stock solution and 610 mL of 1M NaHPO4 stock solutions were mixed.

METHODOLOGY

➢ ***ACETONE PRECIPITATION***

- The outer skin of pineapple fruit (*Ananas comosus*) was peeled off and flesh was crushed in mixie to form juice, use little water if required.
- Different concentrations of acetone were prepared – 40%, 60%, 80% & 100% in 10mL.
- 10mL of juice was precipitated with 10mL of above prepared concentrations of acetone.
- Precipitation was performed with ice cold acetone only, at 4 deg. C overnight, in order to prevent the denaturation of enzyme.
- After overnight treatment with acetone, the crude enzyme or juice samples were subjected to centrifugation at 4 deg. C using cooling centrifuge at 10,000 RPM for 10 minutes.
- The supernatant was discarded and the pellet so obtained was dissolve in 0.1 M Sodium Phosphate Buffer.

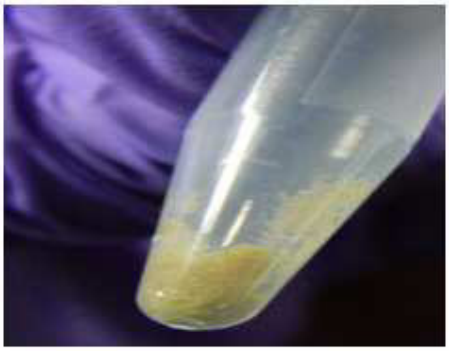

Figure 1
Pellet obtained in one of the eppendorf's after centrifugation (Acetone Precipitation)

➢ ***AMMONIUM SULPHATE PRECIPITATION***

- The outer skin of pineapple fruit was peeled off and flesh was crushed in mixer to form juice, use little water, if required.
- Dissolve 75 g of Ammonium Sulfate salt in 100mL of water to form 100% saturated solution of ammonium sulfate.
- From the above formed saturated solution of ammonium sulfate, final 45% and 65% saturation of ammonium sulfate with fruit juice was obtained by adding 8.181mL and 18.57mL of above 100% saturated solution to 20mL of juice.
- The ammonium sulfate precipitation was performed in cold room at 4 deg. C using magnetic stirrer and magnetic bead overnight.
- After overnight treatment with acetone, the crude enzyme or juice samples were subjected to centrifugation at 4 deg. C using cooling centrifuge at 10,000 RPM for 10 minutes.
- The supernatant was discarded and the pellet so obtained was dissolved in 0.1 M Sodium Phosphate Buffer.

Figure 2
Rings formed after AS Precipitation (OVERNIGHT TREATMENT, 4 Deg. C)

➢ ***JELLO PRECIPITATION : PREPARATION OF THE JELLO***

- Heat about 250mL of deionized water to boiling.
- Weigh about 8.50 g of jell-o in a 100mL beaker.
- Add 23mL of boiling water and stir until the jell-o dissolves
- Cool the jell-o to about 50°C before using it in further experimentation.

Procedure of precipitation

- The outer skin of pineapple fruit (*Ananas comosus*) was peeled off and flesh was crushed in mixer to form juice, use little water if required.
- Different concentrations of gell were prepared – 40%, 60%, 80% & 100% in 10mL.
- Total volume make up – 3.5 mL

- Add different concentrations of jell-o in each of the test tubes with same volume of sample.
- Transfer the obtained sample to 2mL eppendorf and place them for centrifugation.
- Centrifugation should be done at 2,000 RPM for 10 minute at 4°C using cooling centrifuge.
- After centrifugation, obtained pellet is should be dissolved in 0.1 M Sodium Phosphate Buffer.
- Leave it for overnight treatment at 4 deg. C.

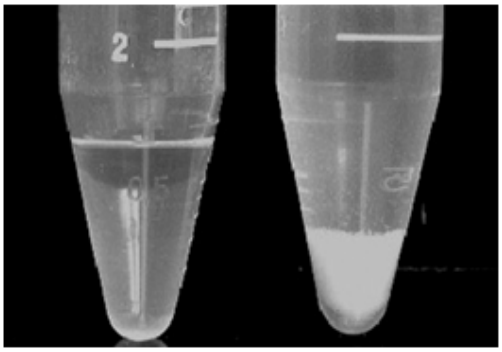

Figure 3
Pellet obtained in different conc. of jello (Comparison)

ENZYMATIC ACTIVITY ANALYSIS
Folin-Ciocalteau Reagent Analysis (LOWRY's ASSAY)
PREPARATION OF BSA STOCK
0.1g of BSA **(Bovine Serum Albumin)** was weighed and was dissolved in 100mL of distilled water. Concentration used was 100 mg/mL.

PREPARATION OF WORKING STANDARD
From the above prepared solution 10mL was taken and was made upto 100mL of distilled water. Concentration - 100 microgram/mL.
NOTE: ALL THE REAGENTS SHOULD BE PREPARED FRESH FOR EXPERIMENTAL ANALYSIS.

LOWRY'S ASSAY PROTOCOL FOR DRAWING STANDARD CURVE AND QUANTIFYING UNKNOWN SAMPLES OF PROTEINS USING STANDARD CURVE
UNKNOWN CONC. OF PROTEIN = O.D./SLOPE

- For unknown sample, i.e., enzyme sample from pineapple fruit 0.5mL of it was taken and 2.5mL of DW was added to it followed by addition of 4.5mL of alkaline $CuSO_4$ solution and followed by addition of 0.5 mL of Folin's Phenol.
- The reading of unknown samples color was taken using spectrophotometer at 550nm & calculated as above mentioned.

(SODIUM DODECYL SULPHATE - POLYACRYLAMIDE GEL ELECTROPHORESIS)
(SDS-PAGE)

ELECTROPHORESIS BUFFER
The final tank buffer composition is –
- 196 mM Glycine
- 0.1% SDS
- 50mM Tris-HCl (pH-8.3)

(Made by diluting a 10X stock solution. This goes in both top and bottom tanks)

PROCEDURE
- Base plates and notched plates were washed and were assembled properly using spacers fixed with the help of petroleum jelly. The assembly was ensured leak proof by pouring water in between them.
- The gel solutions were poured between plates till the level of gel is 2cm below the notched plate.
- 200-250 microlitre of water was added to make surface even.

- After gel was set (approximately 20-30minutes) it was washed with distilled water to drain off the water completely.
- The stacking gel was poured directly into already polymerized separating gel and was left for 30 min. for it to be completely casted.
- The comb was inserted into gel solution without trapping any air bubbles approximately 1 cm above the stacking gel.
- 25microlitre of protein sample (pineapple) and 10microlitre of protein marker were pippeted into individual vial. To each of these vials 15microlitre of gel loading dye was added.
- The vials were placed in boiling water bath for 5 minutes.
- After the stacking gel is set the comb and bottom spacer was removed and the gel was washed thoroughly to remove unpolymerised acrylamide.
- The bottom reservoir was filled with 1 X Reservoir Buffer.
- The plates were carefully fixed to PAGE apparatus without trapping any air bubble between the bottom of the gel and buffer with notched plate facing top reservoir.
- Pineapple fruit samples were loaded in wells.
- The cords were connected to the power supply according to convention red-anode, black-cathode.
- Voltage was set at 100V and power supply was switched on.
- When the dye front reached 0.5cm above the bottom of the gel the power supply, was switched off.
- The plates were removed using spatula to remove them apart.
- The gel was transferred in tray containing water and was washed for 5 minutes.
- 20 mL of CBB Stain was added to the gel and was stained for 30-60 minutes.
- Then the gel was de-stained for about 24 hrs to obtain clear background.

ENZYME PURIFICATION USING ANION EXCHANGE CHROMATOGRAPHY

MATERIALS

1. BUFFER A
2. BUFFER B
3. Distilled water
4. Micropipette (Pippetemen)
5. Tips (Tarson)
6. Beaker (Borosil)
7. Sodium Hydrogen Phosphate (Merck)
8. NaCl (Sodium Chloride) (Merck)
9. Filter Paper
10. Silica Gel
11. Enzyme Sample

PROCEDURE

- The precipitated protein samples were filtered using 0.44nm filter paper.
- The resultant sample was loaded onto a SILICA GEL COLUMN equilibrated with BUFFER A for 1.2 Hrs.
- The major protease of the fruit was eluted from column using BUFFER B. Protein fraction was collected at its peak

Figure 4
CHROMATOGRAPHIC COLUMNS (MATRIX: SILICA GEL)

RESULTS

Figure 5
Peel of *Ananas comosus*

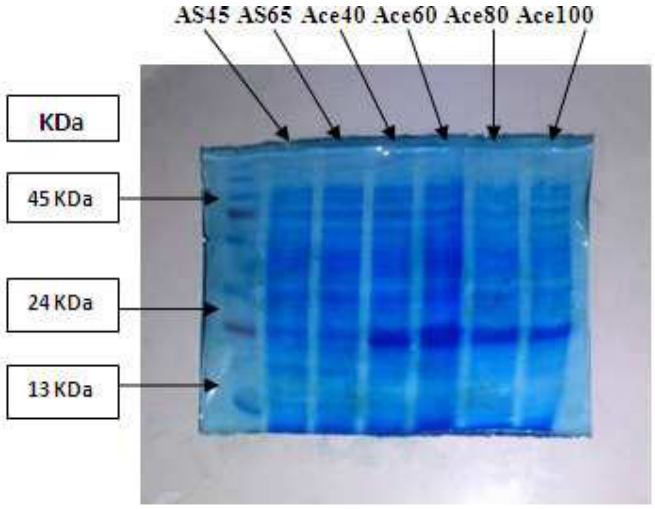

Figure 6
PAGE Gel with loaded sample and marker

TABLES

TABLE 1
LOWRY'S QUANTIFICATION – PINEAPPLE ACETONE PRECIPITATION

Concentration of acetone	Optical density 550 nm	Concentration of protein (ug/ml)
60% (A)	0.54	525
60% (B)	0.51	
80% (A)	0.53	535
80% (B)	0.54	
100% (A)	0.52	480
100% (B)	0.44	

TABLE 2
LOWRY'S QUANTIFICATION – PINEAPPLE AMMONIUM SULPHATE PRECIPITATION

SATURATION OF AMMONIUM SUPLHATE	OPTICAL DENSITY 550 nm	CONCENTRATION OF PROTEIN (ug/mL)
45%	0.50	500
65%	0.42	420

PURIFICATION USING SILICA GEL COLUMN CHROMATOGRAPHY

Four syringes filled with crude enzyme samples of *Ananas comosus* were subjected to Anion Exchange Chromatography; the resulting fractions were collected and stored at –20°C.

mAu ---- UV

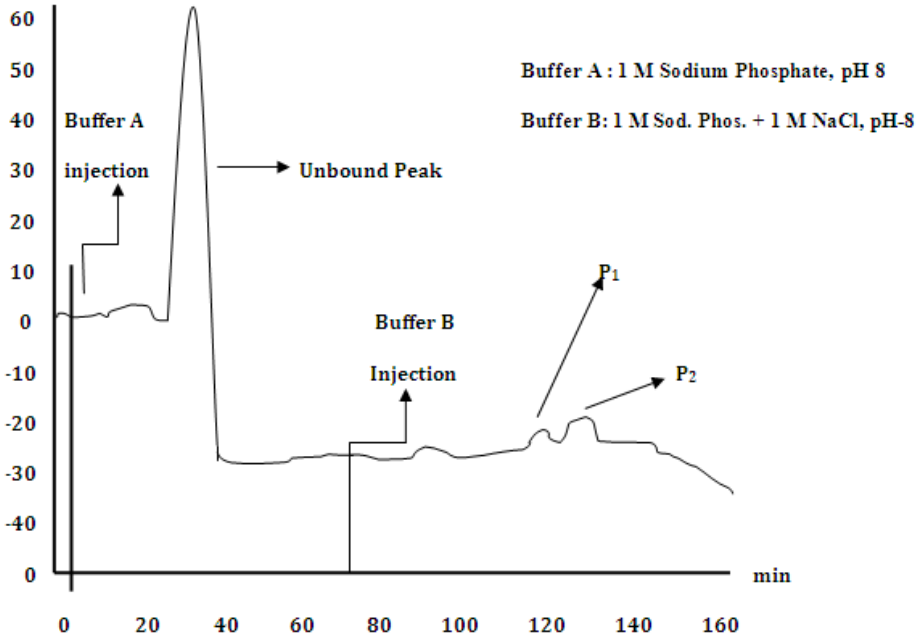

Figure 7
Anionic Exchange Chromatogram above depicting the purified fractions of enzyme from crude enzyme extracts of Ananas comosus

GRAPHS

(STANDARD CURVE-LOWRY'S)

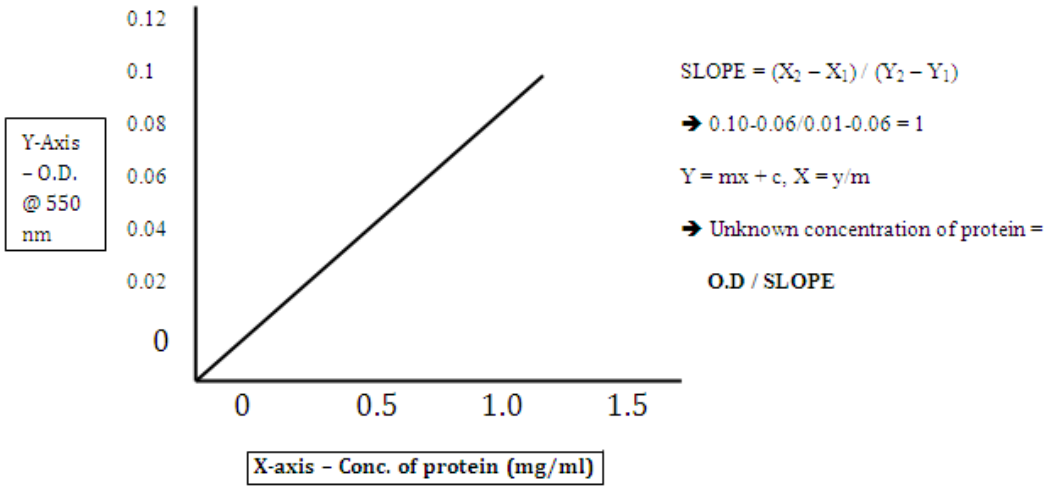

Figure 7
Lowry's Graph

SDS-PAGE of ENZYME SAMPLES

The crude samples obtained from precipitation with different acetone and ammonium sulphate concentration were then subjected to SDS-PAGE analysis. It was observed that a single band was observed between 24-45KDa range which might corresponds to major pineapple protease "BROMELAIN".

DISCUSSION

There are some research papers which have been published detailing the purification of bromelain from *Ananas comosus.* However there have been reports that another protease present in *Ananas comosus* similar in characteristics to actinidain, present in *Actinidia chinensis.* I am trying to purify proteases from the fruits and screen for that particular protease which has not been worked upon yet. Therefore all the work I have done and the results discussed are based on my original work. Protein concentration was quantified using Lowry's and Bradford method of protein quantification. The concentration is proportional to the absorbance. The different range of acetone, ammonium sulfate & jell-o were chosen for precipitation and protein quantification was performed for precipitated proteins obtained from this range. For acetone from 40% to 100% range was chosen as from established sources it was found that 40%-100% acetone range give maximum concentration of protein. For Ammonium Sulphate precipitation, 45% and 65% saturated ammonium sulphate was chosen as 45-65% range generally gives maximum protein concentration. Acetone is generally used for precipitation as it is volatile, and easily evaporates after precipitation. It was observed that for pineapple, 80% acetone and 45% ammonium sulphate gave maximum concentration of protein.

CONCLUSION

The protease from the pineapple fruit was purified using "Silica Gel Anion Exchange Chromatography". The fractions obtained, containing the proteases similar to Bromelain, the major protease of pineapple as when the fractions were subjected to SDS-PAGE analysis, the multimeric bands were observed in the range of 24-45 KDa. In case of pineapple, a single faint band was observed between 24-45 KDa also the range in which molecular weight of Bromelain lies. The presence of protease in these fractions was also confirmed using other Assay's, where activity of purified protease was found. The extra fraction obtained in the chromatogram of the *Ananas comosus*, sample fraction showed no activity when subjected to other assay's indicating the absence of protease in the corresponding fraction thus conforming the absence of any other major protease similar to that of Bromelain in pineapple.[2] In future, the purified fractions from both pineapple and kiwi is intended to go for crystallographic or sequencing analysis to conform structurally the presence of major protease actinidain in kiwi and bromelain in pineapple.[3]

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