

## Extraction, Purification and Characterization of Bromelain from Pineapple and *In Silico* Annotation of the Protein

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### Abstract

Bromelain which is one of the commercially important enzyme is an extract of pine apple (*Ananas comosus*) which is a member of bromeliaceae family. This particular study was performed to estimate the agglomeration of the enzyme bromelain from different parts of pineapple. The proteolytic enzyme was extracted using methanol, ethanol and water. The crude enzyme was then subjected to downstream processing for purification. Quantitative and Qualitative analysis were carried out to estimate the concentration and activity of the enzyme. *In silico* annotation was done to understand the protein bromelain and its function in various treatments. The results discussed show that the concentration of enzyme was found to be high in crown of the pineapple and protein sequence retrieval was done for further applications.

### Keywords

Bromelain, Proteolytic, *Ananas comosus*, *in silico* Annotation

### Introduction

#### Pineapple

Plants are classified into two classes such as Liliopsida and Magnoliopsida. Pineapple (*Ananas comosus*) belongs to the liliopsida class. This class can be further sub-divided into order and the Pineapple order is Poales. Pineapples are placed under *Ananas* genus. From all the natural sources available it is evident that magnesium, vitamin C, gallic acid are found in high concentrations in pineapple. This fruit has bromelain a proteolytic enzyme which is a great benefactor for medicinal applications. Antioxidant behaviour is one of the main property of pineapple. In the market pineapple is mainly consumed as fruit, juice or processed food, and the waste of pineapple is converted to animal feed in cattle industry.

#### Bromelain

Bromelain is an enzyme extracted from pineapple which is distributed throughout different parts of the fruit with varied concentrations. The evidences of separation of the enzyme from the fruit was recorded back in 1891 by Venezuelan chemist Vicente Marciano with as simple as fermentation of the fruit. Bromelain can be considered as a protease enzyme or a combination of other enzymes produced by the

extract from the fruit. Along with the varied benefits of the enzyme there are also few disadvantages associated with this protease enzyme such as the sore feeling in the mouth associated with the reaction between the enzyme and the proteins present on the tongue tissue. The main purpose of the study is to extract crude enzyme from pineapple which was then purified to estimate the concentration and activity of the enzyme and to view the structure of bromelain.

### Materials and Methods

#### Collection of the fruit

A Pineapple (*Ananas comosus*) weighing 850gm was collected from a local market in LB Nagar, Hyderabad.

#### Sample preparation

The pineapple was cleaned using distilled water and its peel was removed. The crown and fruit part of the pineapple were separated and cut into small sections and weighed individually according to the experiments performed.

#### Extraction of bromelain

Extraction and separation of Bromelain enzyme from the pineapple fruit material is essential. The pineapple fruit and crown were processed separately for the extraction of enzyme.

#### Procedure

1) 2gms of peeled fruit and crown were weighed separately and was ground in a mortar with pestle. The fine slurry was extracted using 10 ml of ethanol, distilled water and methanol separately. A 30 ml of extract of 10ml each was collected in 3 different chemicals (methanol, ethanol, deionised water) using 6 gm each of crown and fruit samples. The extract was transferred to labeled test tubes stored at 55°C and were left in the water bath overnight. Later, the samples were centrifuged at 10,000 rpm for 15 minutes. The supernatant was filtered using Whatman filter paper (150 mm) and the filtrate was collected in labeled falcon tubes. The samples were stored at 4°C until further use.

#### Purification

##### Salt precipitation:

##### Procedure

The stored enzyme extracts were poured into separate labeled beakers and the corresponding

saturation amount (50-100 %) of ammonium sulfate was added to each beaker. The salt was added slowly by continuous stirring till the salt gets dissolved completely. The beakers were left undisturbed for 30 minutes on ice. The contents of the beaker were transferred to 15 ml centrifuge tubes and were centrifuged at 10000 rpm for 10 minutes. The supernatant was discarded and the pellet was dissolved in deionized water and was centrifuged again at 10,000 rpm for 10 minutes. The supernatant was decanted and the pellet was suspended in 2 ml of deionized water.

#### Dialysis

6 strips of dialysis membrane of 9 centimeters each were taken (one for each sample). They were boiled in distilled water at 80°C for 10 minutes followed by boiling in 2% sodium bicarbonate solution for 10 minutes. They were rinsed with distilled water allowing the pores to open. One end of the dialysis membrane was sealed to avoid leakage. Equal quantities (2 ml) of the crude enzyme extracts were pipetted into separate dialysis bags and the other end was also sealed. The dialysis bags were soaked in buffer solution and kept on a magnetic stirrer and left overnight.

#### Quantitative estimation of bromelain by Lowry's method

##### Procedure

5 different concentrations of BSA ranging from 0.2 mg/ml to 1 mg/ml with a final volume of 1 ml were prepared and subjected to standard Lowry's method by measuring its optical density at a wavelength of 660 nm. 0.1 ml of the enzyme samples were pipetted into separate labeled test tubes and the volume was adjusted to 1 ml using distilled water. An exact volume of 4ml of complex forming reagent (alkaline copper sulphate) was added, mixed and kept at room temperature for 10 minutes. 1 ml of Folin-Cioaltea reagent was added, mixed thoroughly and incubated in dark surroundings for 30 minutes. Optical density was read on spectrophotometer at a wavelength of 660 nm and the readings were recorded. Calibration curve was plotted for standard protein concentration against optical density reading. The concentration of unknown samples were calculated by using the intercept of the curve.

#### Determination of enzyme activity by GDU

7 conical flasks were taken of which 6 were labeled with respective samples and the remaining flask serves as a blank. A 25 ml of gelatin substrate was added to the flasks and kept in water bath at 45°C. A 1 ml of respective enzyme assay solution was added to the substrate and stirred following which the substrate was left undisturbed for 20 minutes at 45°C. 2 drops of 3% hydrogen peroxide was added to the substrate in all the conical flasks. They were incubated at 45°C for 20 minutes. The conical flasks were taken out from the water bath and PH was

adjusted to 6 using 2N NaOH. Later, 10 ml of 37% formaldehyde was added to the substrate and titrated to PH 7.8 against 0.05N NaOH. The activity is calculated using the following formula.

$$GDU/mg = \frac{(T - B) * 14 * N * 1000}{\text{Concentration of the enzyme}} (mg/ml)$$

Where,

T is the Test titer (Volume in ml of 0.1 N NaOH run down)

B is the Blank titer (Volume in ml of 0.1 N NaOH run down)

N is the normality of NaOH used to neutralize the acid

#### Immobilization

Crude enzyme was dissolved in 1 ml of Distilled water and mixed with 3 % sodium alginate solution. The solution was pipetted out drop wise into 2% Calcium chloride solution. The beads formed were transferred into a petriplate and was stored in a refrigerator for further use.

#### Sequence retrieval from NCBI

To understand the sequence of the protein for further analysis and the information pertaining to the protein an open source database known as NCBI was used. The database has a vast collection of journals.

#### ProtParam

Theoretical simulation helps in characterizing the physical and chemical properties of the protein. PROTPARAM simulation tool helps in understanding and calculating the protein molecular weight, stability, estimated half-life etc. It is available at <http://web.expasy.org/protparam/> //place in appendix

#### Phyre2

In cases where the protein structure could not be obtained or simulated bio-informatics comes to help. One such tool is Phyre-2, it is a web-based tool used for predicting and analyzing unknown protein structure and function. It performs local alignment of the available sequence against fold library. It is available as an open-source software for non-commercial use.

<http://www.sbg.bio.ic.ac.uk/~phyre/index.cgi>

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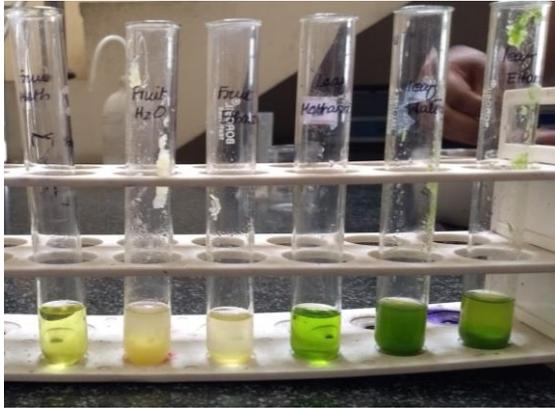
#### Protein data bank

The primary database for protein structure information is the **Protein Data Bank (PDB)** which is powered by the Protein Data Bank archive-information about the 3D shapes of proteins, nucleic acids, and complex assemblies that help in understanding the aspects of biomedicine and agriculture, from protein synthesis to health and disease.

**Results and discussion**

The enzyme bromelain was obtained from the crown and fruit of pineapple using methanol, ethanol and distilled water

**Fig 1: Crude enzyme extracts from crown and fruit of pineapple.**



The crude samples were purified by centrifugation, filtration and dialysis.

**Fig: 2 Crude enzyme samples packed into dialysis bags.**



**Quantitative Estimation of DNA**

**Fig 2: Showing the treated tubes with the standard samples at various concentrations.**

The quantitative estimation of the enzyme was done by Lowry's method that gave the following results.



**Table 1: Standard Graph Sampling is shown in the table below**

S.no	Vol. of BSA sample	Conc. of BSA (mg/ml)	Vol. of distilled water(ml)	Vol. of rea1(ml)	INCUBATE FOR 10 MINS	Vol. of Reagent 2 in ml	INCUBATE FOR 30 MINS	OD at 660 nm
1	0	0	1	4	INCUBATE FOR 10 MINS	1	INCUBATE FOR 30 MINS	0
2	0.2	0.2	0.8	4		1		0.207
3	0.4	0.4	0.6	4		1		0.221
4	0.6	0.6	0.4	4		1		0.245
5	0.8	0.8	0.2	4		1		0.251
6	1	1	0	4		1		0.302

The above table 1 shows that sampling procedure with the standard BSA which can be used as a reference for the calculation of concentration for Unknown samples.

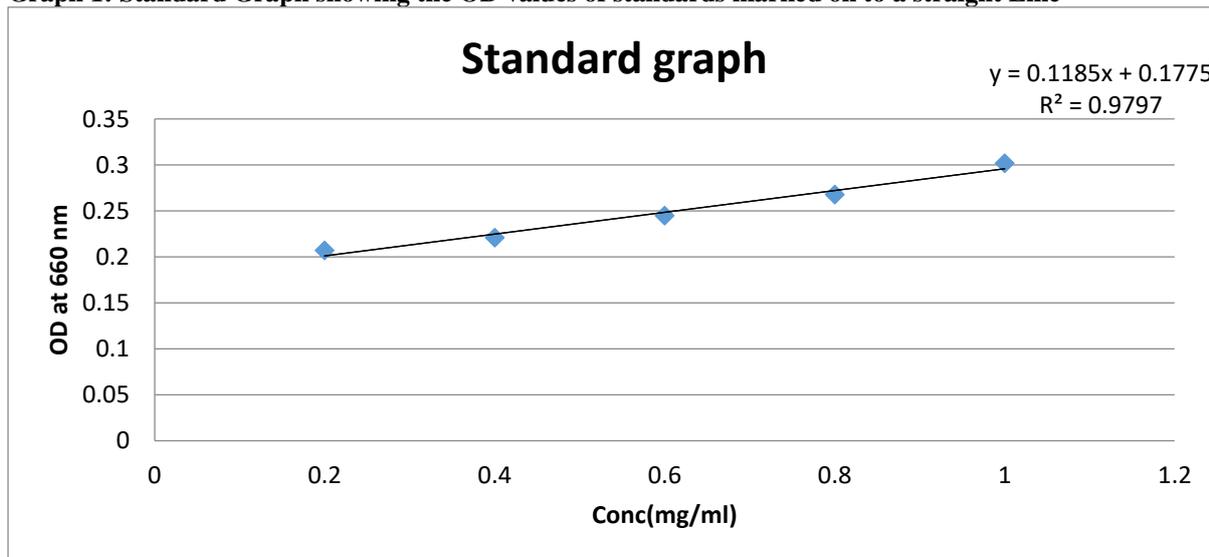
**Table 2: Unknown Samples protocol is shown in the table below**

S.no	Vol. of sample	Vol. of distilled water(ml)	Vol. of rea1(ml)	INCUBATE FOR 10 MINS	Vol of rea2(ml)	INCUBATE FOR 30 MINS	OD at 660 nm
FM	0.1	0.9	4	INCUBATE FOR 10 MINS	1	INCUBATE FOR 30 MINS	0.489
FE	0.1	0.9	4		1		0.271
FW	0.1	0.9	4		1		0.268
CE	0.1	0.9	4		1		0.237
CM	0.1	0.9	4		1		0.668
CW	0.1	0.9	4		1		0.237

The above table shows the OD values obtained for all the samples treated as the same as standard Protein

The OD values obtained in the above table1 and 2 are used to plot the graph which is a straight line plot in which the unknown samples are plotted and extrapolated to obtain their concentration.

**Graph 1: Standard Graph showing the OD values of standards marked on to a straight Line**



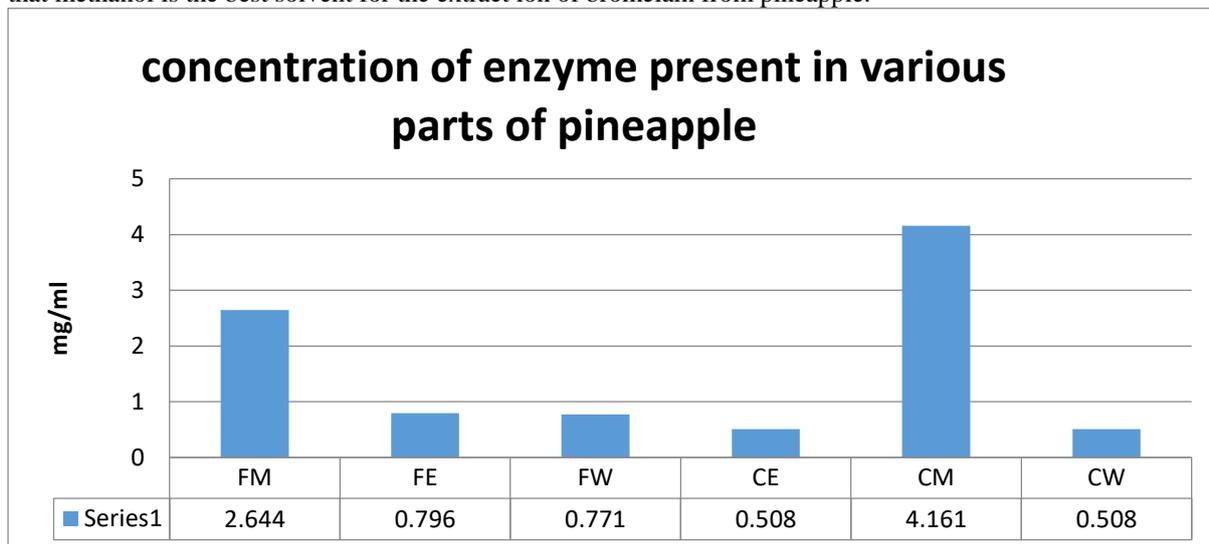
The concentration of all the unknown samples were determined using the equation  $y=0.1185x+0.1775$  which was obtained from the standard graph. The results are tabulated as below

**Table 3: Showing the Concentrations of proteins in each extract obtained from the Pine apple**

Sample	Conc. (mg/ml)
FM	2.644
FE	0.796
FW	0.771
CE	0.508
CM	4.161
CW	0.508

**Graph 2: Graph showing the Comparison of Proteins among Various extracts tested**

The highest concentration of enzyme was observed in methanolic extracts of both crown and fruit. This proves that methanol is the best solvent for the extract ion of bromelain from pineapple.



FM=Methanolic extract of fruit, FE=Ethanollic extract of fruit, FW=fruit extract in water, CE= Ethanollic extract of crown, CM= Methanolic extract of crown, CW= crown extract in water

Qualitative Assay of the Enzyme

Table 4: Gelatin Digestion Unit Assay for Crude Extracts of Bromelain

SAMPLE	VOLUME OF NaOH RUN DOWN(ml)
BLANK	9.2
FW	22
FM	26.8
FE	10.5
CW	10
CM	29.2
CE	20

Using the formula we calculate the activity of enzyme extracted from various parts of the pineapple.

$$\frac{GDU}{mg} = \frac{(T - B) * 14 * N * 1000}{Concentration\ of\ the\ enzyme} (mg/ml)$$

Table 5: Showing the calculation and final activity of the enzyme samples tested in all the extracts

SAMPLE	GDU/g
FW	11621.271
FM	<b>4659.606</b>
FE	1143.216
CW	1102.36
CM	3364.575
CE	14881.88

From the calculations it can be observed that the enzyme present in the **fruit shows more activity**. By considering the sample run down values and as per the calculation the fruit extract in methanol showed more activity compared to crown extract in methanol. Hence methanolic fruit extract forms an active enzyme.

Immobilization of Enzyme for Preservation

In order to utilize the enzyme activity in future or preserve the active enzyme it was trapped into beads using calcium chloride and sodium alginate which is termed as the immobilization of Enzyme. The final product obtained would be the enzyme entrapped in Calcium alginate which can be preserved in refrigeration.

Fig 3: Showing the Calcium Alginate beads containing the enzyme for preservation.



Insilico Analysis of the Sequence:

Bromelain sequence was retrieved from the NCBI protein data

>BAA21929.1 bromelain [Ananas comosus]  
 MAEYGRVYKDNDEKMRRFQIFKNNVNHITF  
 NNRNGNSYTLGINKFTDMTNNEFVAQYTGGI  
 SRPLNIEKEPVVSFDDVNISAVGQSIDWRDYG  
 AVTEVKDQNPCGSCWAFSAIATVEGIYKIVTG

YLVSLSEQEVLDCAVSNGCDGGFVDNAYDFII  
 SNNGVASEADYPYQAYQGDCANSWPNSAY  
 ITGYSYVRSNDESSMKYAVWNQPIAAIDAS  
 GDNFQYYNGGVFSGPCGTSLNHAITIIGYGQD  
 SSGTQYWIVKNSWGSSWGERGYIRMARGV  
 SSSGLCGIAMDPLYPTLQSGANVAVIKMVS  
 K  
 T

**Inference:** From the above sequence retrieved it can be inferred that it comprises of 312 amino acids and can be concluded that it is a linear peptide chain.

**Physiochemical Characterization of protein using Protparam**

The physical and chemical properties of the protein are clearly depicted by the protparam tool. The properties as shown below:

- Number of amino acids:** 312
- Molecular weight:** 34158.74
- Theoretical pI:** 4.64
- Total number of negatively charged residues (Asp + Glu):** 32
- Total number of positively charged residues (Arg + Lys):** 21
- Atomic Formula:** C<sub>150</sub>H<sub>2259</sub>N<sub>403</sub>O<sub>482</sub>S<sub>14</sub>
- Total number of atoms:** 4663

**Estimated half-life:**

The N-terminal of the sequence considered is M (Met).

The estimated half-life is: 30 hours (mammalian reticulocytes, in vitro).

- >20 hours (yeast, in vivo).
- >10 hours (Escherichia coli, in vivo).

**Instability index:**

The instability index (II) is computed to be 29.97 This classifies the protein as stable.

**Aliphatic index:** 69.07

**Grand average of hydropathicity (GRAVY):** -0.312

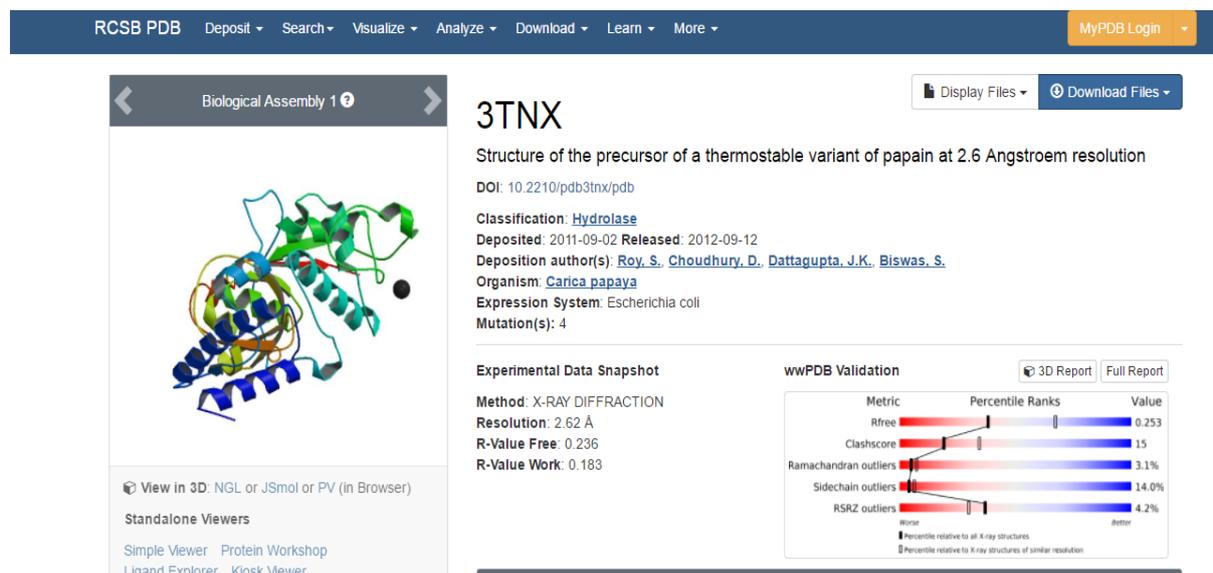
**Interpretation**

From the above information it can be inferred that the software computes and characterizes different parameters of the protein such as the number of amino acids, molecular weight, total composition total number of atoms, estimated half-life etc. Thus it plays a vital role in protein study and characterization.

**Phyre for Tertiary Structure Analysis**

This gives the scope code starting with c or d. Other results gives the 3D model of the structure, identification percentage and template information. The Protein data bank, PDBID obtained for the given protein sequence was 3TNX.

**The Fig 4: The below Picture from PDB shows that the structure coding for 3TNX is the structure of Papain**



**Inference:** From the above results it can be inferred that there is no proper structure of Bromelain stored in the PDB. The most similar structure for the sequence was found to be the Crystal structure of Papain. Thus further Structure prediction has to be performed for Bromelain protein. Further it can also be inferred that the sequence of papain and bromelain share a good degree of similarity because of their similar functions.

**Conclusion**

In view of the importance of Bromelain enzyme at the commercial scale either in the food industry or the medical industry, the work aimed to design an economic and easily feasible protocol for the extraction of enzyme. As mentioned in the above procedure the enzyme was successfully extracted. Several purification techniques like filtration, Centrifugation, Salt Precipitation, Dialysis etc., have

been employed in a sequence to obtain the fully pure enzyme. Characterization of the protein was performed both quantitatively by Lawry’s method and qualitatively by Gelatin digestion method. Both the techniques showed that the quantity and quality of the enzyme produced from the methanolic extract of fruit sample was higher than all the other variations. Further the Sequence was analyzed for its properties and Structural parameters using various

*insilico* tools. Structural analysis of the protein showed that the 3D structure of it is not available at PDB. Thus the structure of this protein has to be identified yet.

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