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AQUEOUS TWO PHASE EXTRACTION OF BROMELAIN FROM PINEAPPLE FRUIT

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ABSTRACT

Bromelain is a complex mixture of glycoproteases extracted from the stem, pulp, core and peel of pineapple fruit that has a wide range of industrial and medical applications. The Present work deals with extraction of Bromelain from pineapple fruit (pulp) by aqueous two phase systems (ATPs). Extraction of Bromelain with polyethylene glycol (PEG) and Ammonium Sulphate showed best partition in the system with 18% PEG 6000 and 15% Ammonium Sulphate with increased purity fold and recovery of 62%. Further Purification with Gel Filtration Chromatography showed 5 fold increases in purity. Molecular weight determination by SDS PAGE showed that fruit Bromelain has a molecular weight of about 24KDa. Studies on pH stability showed that Bromelain has an optimum pH of 7. This shows that ATPs can be used to purify Bromelain with acceptable purity.

INTRODUCTION

Bromelain is a group of proteolytic Enzymes found in the tissues of pineapple plant family Bromeliaceae. Although Bromelain have a broad range of specificity, they preferentially cleave glycyl, alanyl and leucyl bonds[2]. Bromelain is proved to be having antiedematous, anti inflammatory, antithrombotic, fibrinolytic and Immuno modulating properties. Apart from this, Bromelain is found to increase the absorption of antibiotics when they are administered together [2, 3]. These potent properties made them useful for medicinal and therapeutic purposes. Fruit Bromelain can find uses in various industrial applications including brewing, meat tenderization and functional protein predigestion[3]. The Present study uses Fruit Bromelain (3.4.22.33) which is also called Juice Bromelain, Ananase, Bromelase, Extranase, Pinase, Traumanace, etc[3].Many of the commercially available Bromelain in the market are extracted from the Pineapple Stem,though the waste from fruit is increasing day by day[1].

In the crude extract, Bromelain is contaminated with other components such as Phosphatases,Peroxidases,glycosidases,Carbohydrates and some protease inhibitors[3].Hence it necessities the need to go for purification. Commercial preparation of Bromelain involves Centrifugation, Ultra filtration and Lyophilization[2] .Although many techniques such as Precipitation with Acetone, Ethanol/Ammonium Sulphate,Reverse micellar Extraction,Ion exchange chromatography,etc were reported[4,5].Only a few papers deal with the extraction of Bromelain using aqueous two phase extraction.

MATERIALS & METHODS

SAMPLE PREPARATION

Mature pineapples were collected from local market,Tanjore.The pineapples were initially washed with sterile water and the skin was peeled off.After removing the crown and stem, the fruit pulp was made into slices and crushed in a juice blender. The crude juice obtained was filtered with cheese cloth,centrifuged at 8000rpm for 10min and the supernatant was collected, stored at 4^0C.

DETERMINATION OF PROTEIN CONTENT

The total protein content of the samples was determined by Lowry's method and the readings were measured at 580nm with BSA as standard.

$$Kp = \frac{\text{Protein Concentration in top phase}}{\text{Protein Concentration in top phase}}$$

Where Kp-Partition coefficient for protein

DETERMINATION OF ENZYME ACTIVITY

Enzyme activity of Bromelain was determined by the method of A.Ali et.al. The Reaction mixture contained 1ml of 2% casein and 1ml of crude Enzyme extract. The Reaction mixture was incubated for 30minutes at 37^0C.Then the Reaction was stopped by adding 2ml of 15% TCA and Cooled. The above mixture was centrifuged at 6000rpm for 10 minutes at 4^0C and to the 1ml of supernatant dilute folin Ciocalteau reagent (1:1 with water) was added and the total volume was made up to 10ml with distilled water. After 15minutes, the blue color developed was read at 625nm. One unit of Bromelain activity can be defined as amount of enzyme releasing product equivalent to lμmol of tyrosine/min.ml under assay conditions.

$$K_E = \frac{\text{Enzyme activity in top phase}}{\text{Enzyme activity in bottom phase}}$$

Where K_E- Partition coefficient for Enzyme

AQUEOUS TWO PHASE EXTRACTIONS

Aqueous two phase extractions were performed by adding PEG of different molecular weights at different concentrations in 10ml of crude extracts by keeping salt concentrations (Ammonium Sulphate)at 15% (W/V) in centrifuge tubes.The mixtures were vortexed thoroughly for 3minutes. Then the phases were separated by centrifuging at 7000rpm for 10 minutes at 4^0C[7].

Finally the protein content and enzyme activity were determined for each fraction.From the above data,the system with maximum partition coefficient and yield was choosed.The top phase of the above system was back extracted with 25% salt(W/V).

$$Y_t = \frac{K_E V_t}{1+KVo} \times 100$$

$$Y_b = \frac{1}{1+K_E V_b} \times 100$$

Where V_t - Volume of top phase, ml
V_b - Volume of bottom phase, ml
V_o - Total volume, ml
Y_t - Top phase yield
Y_b - Bottom phase yield

GEL FILTERATION CHROMATOGRAPHY

Gel filtrationchromatography was done to remove salts and other components. A sepharose column of length 5cm was washed with 70% ethanol and then with distilled water.Further the column was equilibrated with binding buffer (20mM Tris HCl at pH 7.6)and the sample was injected. Finally elution was done by using 0.5M NaCl. The fractions collected were determined for its protein content and Enzyme activity.

SDS-PAGE

The Molecular weight distribution was determined by using SDS PAGE according to laemmli method. The sample was mixed (GFC fraction) with sample buffer (0.5M Tris HCl, pH 6.8 containing 4%(W/V)SDS and 20%(V/V) glycerol). The proteins were loaded into gel and then subjected to electrophoresis at a constant voltage of 50mv.After separation; the gel was stained with 0.02% comassive Brilliant Blu-250 and destained with a mixture of acetic acid and methanol solution.

pH STABILITY

The pH profile was tested by assaying the protease activity of purified sample at different pH from 4-10. The pHstability was determined by incubating the isolated Bromelain at different pH buffers for 20 minutes at room temperature.

RESULTS AND DISCUSSION

AQUEOUS TWO PHASE EXTRACTION

EFFECT OF PEG ON BROMELAIN PARTITION

Partition of Bromelain in ATPS of different molecular weights (2000, 4000 and 6000) and concentrations (10-18%) of PEG were studied. It was found that the partition of Bromelain strongly depend upon the concentration and molecular weight of polymer (PEG). Higher values of K_P indicates that most of the proteins were shifted to top phase Similarly higher K_E values indicates that only target enzymes were partitioning to top phase[7]. Among all the ATPS studied, the system with 18% of PEG 6000 and 15% of Ammonium sulfate showed good results with a partition coefficient of 25.5 and recovery of 62%. This may be due to the increase in binding sites available for enzyme in polymer.

During Backward extraction with fresh 25%W/V Ammonium sulphate solution, all the proteins in the top phase were extracted into salt phase. This may be due to salting out effect of proteins.

TABLE: 1 PEG 2000 – 15 % Ammonium Sulphate

Percentage of PEG added W/V	Partition Coefficient for Protein K_p	Partition coefficient for Enzyme K_E	Top Phase Yield (Y_t) %	Bottom Phase Yield (Y_b) %
12	4.12	3.5	37.8	3.3
14	13.7	3.25	43.1	3.9
16	11.6	3.7	50.1	3.7
18	9.16	4.06	53.4	3.4
20	9.2	0.58	53.5	2.1

TABLE: 2 PEG4000 – 15 % Ammonium Sulphate

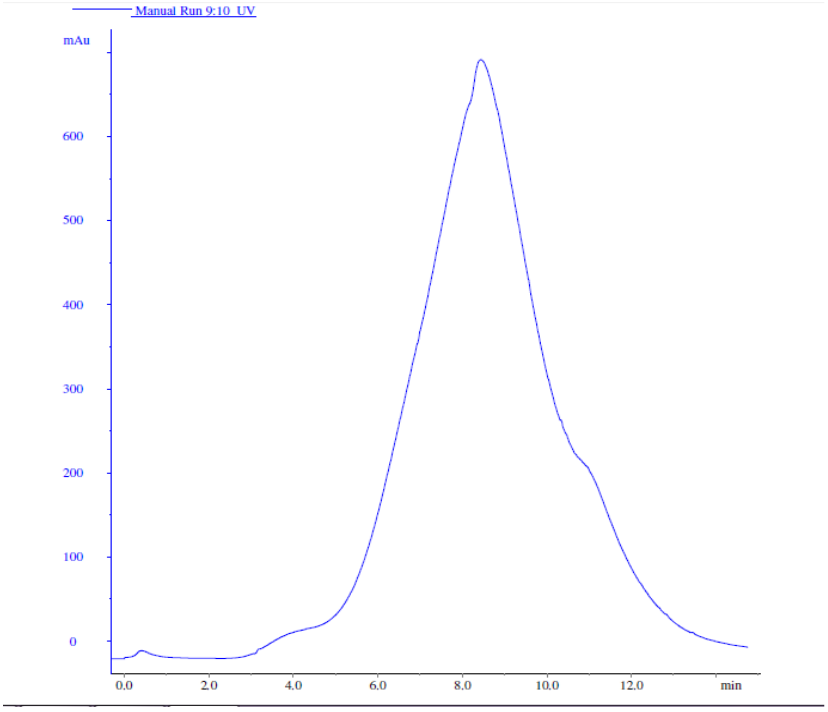
Percentage of PEG added W/V	Partition Coefficient for Protein K_p	Partition coefficient for Enzyme K_E	Top Phase Yield (Y_t) %	Bottom Phase Yield (Y_b) %
12	35	3.2	39.6	3.6
14	17.8	2.21	49.5	5.8
16	39.1	1.22	52	10.4
18	61.8	2.1	59.9	6.6
20	66	1.76	58.6	8.2

TABLE:3 PEG6000 – 15 % Ammonium Sulphate

Percentage of PEG added W/V	Partition Coefficient for Protein K_p	Partition coefficient for Enzyme K_E	Top Phase Yield (Y_t) %	Bottom Phase Yield (Y_b) %
12	6.66	12.25	38.6	1.01
14	14.7	16.6	45.6	0.7
16	29	14.28	53.9	1.01
18	110	25.5	62.6	0.6
20	7.42	11.8	58.02	0.15

GEL FILTERATION CHROMATOGRAPHY

The back extracted salt phase rich in enzyme was subjected to gel filtration chromatography. During elution a single, broad and long peak was observed which was found to have proteolytic activity. As the chromatogram showed single peak, the elute was directly analysed far pH stability and molecular weight in SDS PAGE. This step increased the purity fold to 5.

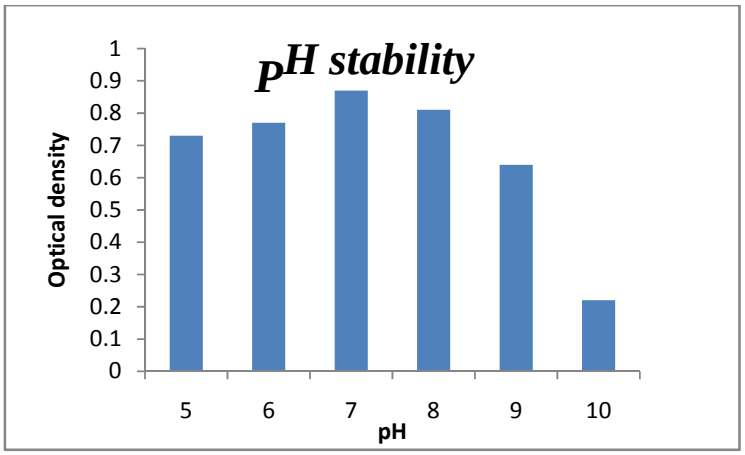


SDS PAGE ANALYSIS

SDS PAGE Analysis revealed the efficiency of the protocol to purity fruit Bromelain by Aqueous two phase extraction. A single Band of 24 KDa was shown which correspond with the result of Juliana et.al, indicating reasonable purification of enzyme.

pH STABILITY

The studies on pH stability of Bromelain showed that the optimum pH was found to be 7. But according to Sunantha et al., optimum pH was 8. The difference in these results may be due to the difference in pineapple species or the source as they used Bromelain from peel.



CONCLUSION

In conclusion, an aqueous two phase system can be effectively used for the partial purification of Bromelain with good purification fold. Although only few works were reported in Bromelain extraction using ATPS, it offers mild non deleterious environment for proteins, fast separation and simple operation.

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