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Extraction and Purification of Bromelain from Pineapple Fruit Pulp and Peel and Comparative Study of Enzymatic Activities

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Abstract—*Bromelain is a complex sulfhydryl proteolytic enzyme, which is prominently found in all parts of pineapple plant (Ananas comosus), i.e., fruit, stem and leaf. It has found immense applications in pharmaceutical industry as well as food, cosmetic, and leather industries. In the present study the extraction of bromelain was done from fruit pulp and peel and was purified through different purification steps. Purification was done by salt precipitation followed by membrane dialysis. The extract was further concentrated by using ultrafiltration and finally crystallized by lyophilization. Proteolytic activity of bromelain from both fruit pulp and peel were calculated in casein digestion unit (CDU) and compared at various stages of purification. It was found that bromelain obtained from fruit pulp showed higher proteolytic activity as compared with fruit peel. The proteolytic activity of purified bromelain was measured at different pH and temperature. The activity of purified bromelain was observed to be maximum at pH- 7.5, temperature- 50 to 60 ^{0}C. The 3-dimensional structure of fruit bromelain was further predicted using bioinformatics tool.*

Keywords*: Bromelain, proteolytic enzyme, extraction, lyophylization.*

1. INTRODUCTION

Bromelain is a cysteine protease enzyme which is obtained from pineapple plant (*Ananas comosus*). The term bromelain was originally used for the proteases extracted from the plants of bromeliacy family. Bromelain is found in almost all parts of pineapple plant i.e. stem, fruit, leaves and crown, out of which Stem and fruit contain higher concentrations of bromelain and have been extensively studied. Bromelain has been categorized as stem bromelain (EC. 3.4.22.32) and fruit bromelain (EC.3.4.22.33) on the basis of its source. The molecular weight of stem and fruit bromelain is 23.8 kDa and 33 kDa respectively [1, 2]. Bromelain hydrolyses proteins preferentially at glycyl, alanyl and leucyl peptide bonds [3]. Bromelain has a varied range of applications in food, cosmetics, leather and drug industries. Clinical applications of bromelain include inhibition of tumor growth, third degree burns and enhanced absorption of antibiotics. It is also used in reversible inhibition of platelet aggregation, treatment of bronchitis, sinusitis, surgical traumas etc. [3]. Food and drug administration (FDA) USA has approved bromelain as a food additive. It has been extensively used in food industry for meat tenderization, baking processes, protein hydrolysate production, beer clarification, as food supplement, and in prevention of browning of apple juice [4]. In leather industry bromelain is used for skin pre-tanning, softening and bating [5]. It is also used as active ingredient to provide gentle peeling effect in cosmetic industries [6]. Due to its high cost of purification, researchers have been following various strategies for extraction and purification in order to reduce the cost of the bromelain purification. Researchers have been trying to extract bromelain in highly pure form by using different combinations of extraction and purification techniques such as membrane filtration techniques-microfiltration, nanofiltration, ultrafiltration [7]; Reverse miceller system [8], precipitation techniques [9, 10]; aqueous two phase extraction [11], and chromatographic techniques [12]. In present work, bromelain was extracted from pineapple fruit pulp and peel by a series of purification steps which include ammonium sulfate precipitation followed by membrane dialysis, ultrafiltration and at last extracts were crystallized by lyophylization. Proteolytic activity of bromelain extracted from fruit pulp and peel were assayed and compared at each stage of purification. Three dimensional structure of fruit bromelain was predicted by using bioinformatics tool.

2. MATERIALS AND METHODS.

2.1 Materials

Ammonium sulfate was purchased from Rankem. Ascorbic acid, Bovine serum albumin (BSA), Ethylene diamine tetra acetic acid (EDTA), dialysis tube (MWCO 12kDa) were

purchased from Himedia Lab Chemicals. Comassie brilliant blue G-250, Sodium carbonate, Trichloroacetic acid (TCA) were purchased from SRL. Polyvinylpolypyrrolidone (PVPP) was procured from Sigma-aldrich. Disodium hydrogen phosphate (anhydrous) and Monosodium dihydrogen phosphate were purchased from Qualichems and Merck respectively. Pineapple fruit was obtained from vendor in BHU campus. Fruit was washed properly and used for further experiments.

2.2. Methods

2.2.1. Preparation of crude extract

The fruit pulp and peel were weighed separately and cut into small pieces. 0.1 M Sodium phosphate buffer solution containing 10 mM cysteine, 3 mM EDTA, 3 mM ascorbic acid and 1% w/v PVPP was prepared. Fruit pulp and peel were mixed separately with above mentioned 0.1 M Sodium Phosphate buffer in 1:1 ratio. The solution was homogenized and filtered by using a fine cloth. Filtrate obtained was centrifuged at 10,000 rpm in a cooling centrifuge at 4^0C. The supernatants were collected and used as crude bromelain. Samples were taken for protein and enzyme assay.

2.2.2. Ammonium Sulfate Precipitation

Fractional precipitation of pulp and peel crude extracts were carried out at 4^oC. $(NH_4)_2SO_4$ was gradually added to get 30% saturation (16.98 gram/100 ml) of fruit pulp and fruit peel crude extract separately, continuously stirring by magnetic stirrer for 30 minutes. The precipitated solutions were centrifuged at 10,000 rpm at 4^oC temperature for 20 min. Pellets were re-dissolved in minimal volume of 0.01 M sodium phosphate buffer. Volume of supernatant was measured and amount of $(NH_4)_2SO_4$ needed to get 70 % saturation was calculated. The precipitated protein solutions were further precipitated at 100 % ammonium sulfate saturation and centrifuged. At each step of fractional precipitation samples were taken for proteolytic activity determination and protein quantification after dialysis.

2.2.3. Dialysis

Fractionate obtained after precipitation were dialysed against 0.01 M sodium phosphate buffer by using dialysis membrane of 12KDa molecular weight cut off (MWCO).

2.2.4 Ultrafiltration

The fractionate obtained after 70% ammonium sulfate precipitation was subjected to ultrafiltration by using Amicon ultrafiltration unit at 25 psi pressure. Samples from permeate and retentate were collected for protein assay and enzyme assay.

2.2.5. Protein quantification

Protein content were determined by using Bradford protein assay, in which 0.2 mg/ml BSA stock solution was used and standard graph was plotted [13]. By using this standard graph protein content of samples were calculated.

2.2.6. Enzyme assay

Enzyme assay was performed according to Sigma Aldrich universal protease assay in which casein was used as substrate (0.65% casein along with 8 mM cysteine and 1 mM EDTA) and L-tyrosine (0.2 mg/ml) was used as standard. Absorbance was taken at 660 nm using spectrophotometer. From the standard curve the activity of protease in the samples was determined in terms of Units, which is the amount of tyrosine (micromoles) equivalents released from casein per minute per milliliter.

2.2.7. Temperature profile

The temperature dependence of proteolytic activity of purified bromelain obtained from fruit pulp was determined at constant pH (7.5) and substrate concentration (0.65%).

2.2.8. pH profile

The proteolytic activity of purified bromelain obtained from fruit pulp was determined at constant temperature of 37^oC and varying pH.

2.2.9. Structural analysis of fruit bromelain using bioinformatics tools

Amino acid sequence was retrieved from UNIPROT in FASTA format. Amino acid sequence in FASTA format was send to I-Tasser Server. Three dimensional structure was given by the I-Tasser server after 24 hour.

3. RESULTS AND DISCUSSION

3.1. Ammonium sulfate precipitation

Ammonium sulfate precipitation was primarily used for purification of crude bromelain followed by dialysis and ultrafiltration. After each step of fractional precipitation specific activity of fruit pulp and peel bromelain was calculated. Crude bromelain extract of fruit pulp and peel showed specific activity of 264.4 U/mg and 185 U/mg respectively (Fig. 1). At 30 % ammonium sulfate precipitation specific activity of fruit pulp and peel bromelain decrease to 203.3 U/mg and 71.9 U/mg respectively. At 70 % ammonium sulfate saturation the specific activity of fruit pulp and peel was found to be 162 U/mg and 138 U/mg respectively.

3.2. Purification using ultrafiltration

The extract obtained from 70% ammonium sulfate precipitation was further purified by using cross flow ultrafiltration unit at 25psi pressure. After ultrafiltration was completed protein samples were analyzed for their proteolytic activities. The specific activity was found to be 212 and 138 for fruit pulp and peel respectively.

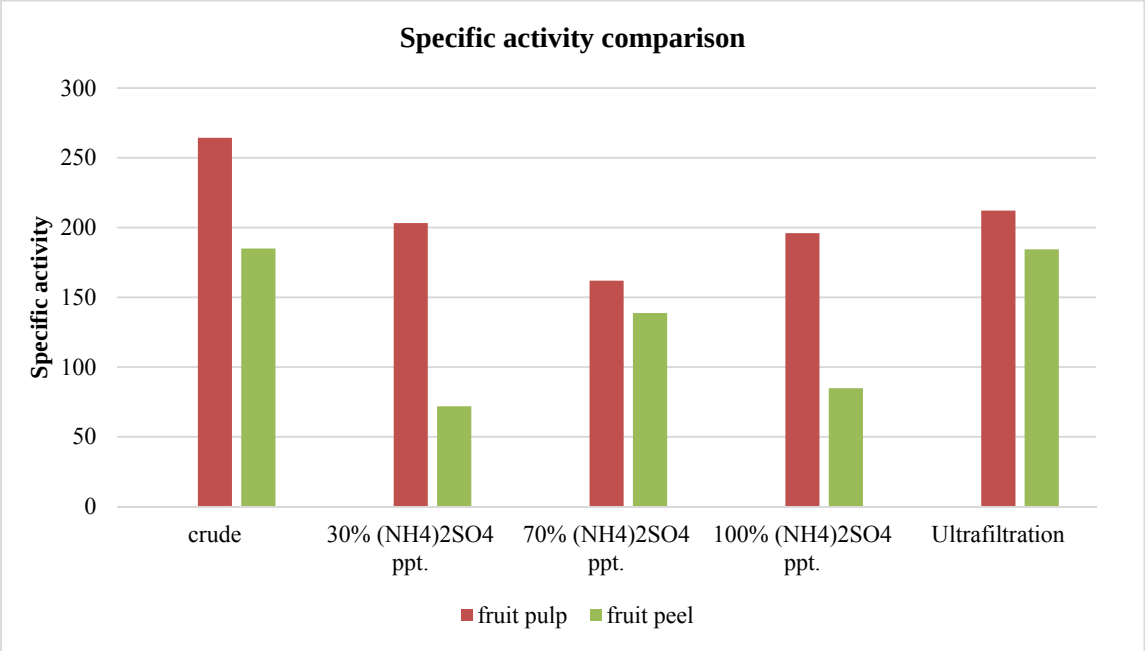

Fig. 1: Comparison of specific activity of fruit pulp and fruit peel bromelain at each purification steps

Table 1: Ammonium sulfate coupled ultrafiltration for extraction and purification of bromelain

		Activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Fold Purity	Total activity/100ml (U)	Yield (% recovery)
Crude	fruit pulp	4.23	0.016	264.4	-	423	100
	fruit peel	3.33	0.018	185	-	333	100
Ammonium sulfate ppt. (70 % sat.)	fruit pulp	6.48	0.04	162	0.61	648	153.19
	fruit peel	5.69	0.041	138.8	0.74	569	170.87
Ultrafiltration	fruit pulp	8.94	0.042	212.16	1.30	894	211.35
	fruit peel	7.74	0.042	184.4	1.32	774	232.43

3.3. pH profile

Fruit pulp bromelain showed proteolytic activity at a wide range of pH values (substrate concentration and temperature were kept constant). The optimal pH for the proteolytic activity was found to be 7.5. The activity decreases slightly when pH changes to acidic or basic.

3.4. Temperature profile

Proteolytic activity of purified bromelain was measured at a temperature range of 20-80^{o}C keeping pH constant at 7.5. The optimal temperature for proteolytic activity was found to be at 50-60^{o}C.

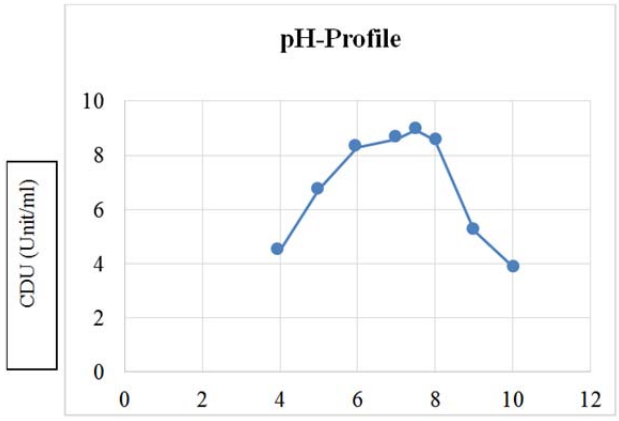

Fig. 2: Proteolytic activity-pH profile of fruit bromelain

3.5. Prediction of 3-D structure of fruit bromelain

Fruit bromelain amino acid sequence in FASTA format was retrieved from UNIPROT protein database and sent to I-TASSER server. The 3-D structure was given by the server after 24 hours. The fruit bromelain is composed of 352 amino acid residues. After cleavage of propeptides and signal peptides, only 230 amino acids are retained in three dimensional structure.

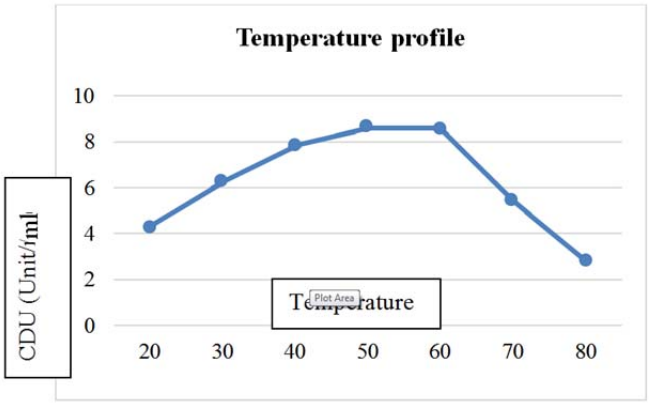

Fig. 3: Proteolytic activity-temperature profile of fruit bromelain

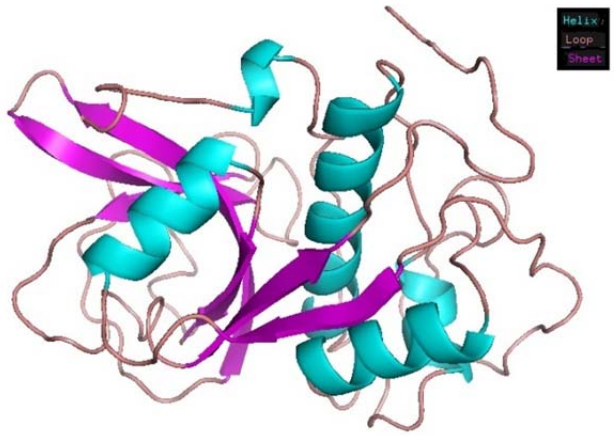

Fig. 4: Predicted 3-D structure of fruit bromelain

4. CONCLUSION

Fruit peel is a major waste material of pineapple juice industry. From this study it is concluded that fruit peel bromelain shows slightly lower proteolytic activity than fruit pulp at each level of purification. So that peel can be a major source for the bromelain production. Optimal pH and temperature of purified fruit bromelain for caseinolytic activity was found to be 7.5 and 50-60^0 C respectively.

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