Research Article

In-vitro Hemolytic and Clot Buster Activity of the Extracts of Ananas Comosus (Pineapple)

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ABSTRACT

Bromelain refers to a family of proteolytic enzymes containing sulfhydryl group obtained from the Pineapple plant (*Ananas comosus*). The objective of this study was to determine the optimum pH and temperature for the hemolytic activity of the extracts from various parts of the Pineapple fruit and to test the clot buster activity. The crude extracts were partially purified by ammonium sulphate precipitation followed by dialysis and protease assay was performed. The partially purified extracts were tested for hemolytic activity for a range of temperature (4°C-95°C) and pH (pH 4-9). The optimum activity was observed at 9 pH and 45°C. The extracts with maximum hemolytic activity were assessed for clot buster activity. Extract SP-50 from stem pellet showed the highest clot buster activity of 68 %. The results indicate a potential use of the pineapple extracts for clot dissolution in the pharmaceutical, tannery and food industry in the near future.

Keywords: *Ananas comosus,* Ammonium sulphate precipitation, Dialysis, Hemolytic activity, Clot buster activity.

INTRODUCTION

Bromelain is a proteolytic enzyme that is typically extracted from fruit, stem and leaves of Pineapple (*Ananus comosus)* plant. Pineapple being an epiphyte utilizes these proteolytic enzymes (Bromelain) to break down organic compounds and obtain its supply of nitrogen and phosphorus[1].

Bromelain is essentially characterized under the family of cysteine proteinase hydrolytic enzymes owing to its ability to break peptide bonds. It is commonly referred to as sulfhydryl protease, since the free sulfhydryl group of cysteine residues is essential for its function[2,3]. Bromelain is an unusual complex mixture of different thiol endopeptidases with other components such as phosphatases, peroxidase, cellulases and glycoproteins[3]. Stem bromelain and fruit bromelain of Pineapple are the two types of enzymes. The stem bromelain, the most abundant cysteine endopeptidase, exhibits a broad specificity for protein cleavage. However it demonstrates a strong preference for Z-Arg-Arg-I-NHMec amongst the small molecule substrates. In contrast, fruit bromelain has a strong preference for Bz-Phe-Val-Arg-1-NHMec but has no action on Z-Arg-Arg-I-NHMec[3].

The stem of the pineapple reported for eight proteolytic active components and fraction F9 Ananin (23,464 Dalton), which comprises of about 2% of the total proteins is considered to be the most active fraction[4]. Bromelain is most stable at pH 4.0-7.5 and its activity remains no longer susceptible to the effect of the pH once combined with its substrate. Its effective temperature range was found to lie between 40°C-65°C with an optimum of 50°C-60°C.

Bromelain is usually activated by calcium chloride, cysteine, bisulphate salts, NaCN and benzoate while Hg++, Ag+, Cu++, antitrypsin and iodoacetate inhibits its activity significantly[5]. The enzyme displays catalytic activity and preferentially cleaves glycyl, alanyl, leucyl bond resulting in its absorption through the gastro-intestinal tract. It is hence used extensively for various therapeutic applications, for the prevention of platelet aggregation, modulation of cell adhesion and inhibition of tumor growth[3]. Bromelain has also been approved to treat post-surgery swelling and inflammation and is also useful in effectively reducing inflammation associated with infection, sinusitis, osteoarthritis and cancer[5].

In USA and Europe, Bromelain is used as a food supplement and is available in food stores and pharmacies. It finds extensive use in the food industry for various applications such as the clarification of beers, cheese manufacturing, softening of meat, as well as in preparation of infant and dietetic foods[2]. One of most important aspects of bromelain is its effect on blood coagulation and fibrinolysis. Blood coagulation by bromelain is by inhibiting the synthesis of fibrin by increasing the serum fibrinolytic ability. *In vivo* and *In vitro* studies have suggested that bromelain is an effective fibrinolytic agent. It stimulates plasminogen to plasmin conversion, thereby resulting in an increased fibrinolysis by degrading the fibrin[6]. Hemolytic activity is generally used to evaluate the toxicity of compounds on human red blood cells[7].

Bromelain prevents the aggregation of human blood platelets both *in vitro* and *in vivo* and it also minimizes the health effect caused due to diseases like transient ischemic and angina pectoris[6]. The objective of our present investigation, is to test the *in vitro* hemolytic activity of the partially purified extracts of Bromelain at different pH and temperatures and also to test clot buster activity.

MATERIALS AND METHODS

Collection of Materials

A pineapple with crown leaves was purchased from a local shop in Vellore district. All the chemicals and reagents used were purchased from Himedia laboratories, Mumbai.

Preparation of RBC Solution

Human B^+ whole blood was collected and centrifuged at 5000 rpm for 15 minutes. The supernatant was then aspirated and discarded while the pellet containing RBCs was retained. It was washed thrice with 0.9 % NaCl solution and further diluted with sodium citrate buffer to obtain a 10 % RBC solution[9]. Thus prepared RBC solution was used for hemolytic activity.

Preparation of Crude Extract

The leaves, body (fruit) and stem were cut using a sterile knife for further processing. The leaves were washed thrice in distilled water; cut into small pieces and were air dried. They were homogenized with sodium citrate buffer (2:3) in a blender. The homogenate was filtered using Whatman no.1 filter paper to remove the fibers and was centrifuged at 5000 rpm for 20 minutes. The supernatant obtained contained the enzyme of our interest, which was collected and stored at 4°C for future use. The same procedure was followed for extracting both stem and body extracts[3].

Partial purification by Salt Precipitation

20 ml of each of the crude extracts were subjected for partial purification by ammonium sulphate precipitation. The various saturated concentration of salts used were 10%, 30%, 50%, 70% and 90%.

The precipitation was done at 4°C with continuous stirring until the desired saturation was achieved. The saturated solution was centrifuged at 10,000 rpm for 15 minutes to obtain the supernatant and the pellet for further use[3].

The extracts were then labeled as follows: Crude body – CB; Crude leaf – CL; Crude stem – CS; BS and BP represents extracts prepared from the supernatant and pellet obtained after ammonium sulphate precipitation of body part. LS and LP represents extracts prepared from the supernatant and pellet obtained after ammonium sulphate precipitation of leaf part. SS and SP represents extracts prepared from the supernatant and pellet obtained after ammonium sulphate precipitation of stem part.

For all the extracts, numbers 10, 30,50,70,90 indicates the concentration of saturated ammonium sulphate used for precipitation.

Dialysis

The dialysis membrane was filled with the extracts obtained from salt fractionation and the experiments were carried out in a beaker containing sodium citrate buffer which was pre-cooled. The buffer was continuously stirred using a magnetic stirrer and replaced every 1 hour[3].

Protein and Protease Assay

The dialyzed samples were analyzed for protein content by Bradford`s method[8]. The protease assay for the partially purified extracts was performed by Folin`s method using Casein as the substrate. Absorbance was measured using a UV spectrophotometer at 660nm. Enzyme activity was subsequently determined[8].

Effect of pH on Hemolytic Activity

For testing the effect of pH on the hemolytic activity of the partially purified extracts, various pH buffers such as 0.2M Sodium citrate buffer (pH 4 - 6), 0.2 M Phosphate Buffer (pH 7-8) and 0.2M Glycine-NaOH (pH 9) were used. 250µL of the extract was added to 200µL of 10% RBC solution and the final volume was made up to 1mL using the respective buffers. The mixtures were incubated for 1 hour, centrifuged and finally absorbance was measured at 541 nm[10]. RBC lysis buffer (NH4Cl-8.02 g/L; NaHCO3-0.84 g/L; EDTA-0.4 g/L) was used as a positive control.

Effect of Temperature on Hemolytic Activity

The effect of temperature on the various extracts was tested. The extracts were incubated at various temperatures like 4°C, 37°C, 45°C, 60°C, 80°C, 90°C for 1 hour. The extracts were examined for hemolytic activity at respective temperature[10,11].

Calculation of Hemolysis

The percentage of hemolysis was calculated using the formula given below:

$$\%\ Hemolysis = \frac{Absorbance\ of\ sample - Absorbance\ of\ blank}{Absorbance\ of\ positive\ control} \times 100$$

Clot Buster Activity

The thrombolytic activity of the partially purified selected extracts was tested by *in-vitro* blood clot lysis assay as prescribed by Prasad and water was used as the Negative control[12,18]. The crude extract was obtained from the stem, leaf and body of pineapple (*Ananus comosus*) respectively, by homogenizing with sodium citrate buffer. The Bromelain present in the extract was subsequently subjected to partial purification by precipitating it with varying concentrations of ammonium sulphate. By centrifuging the solution, supernatant and pellet were obtained which were then further purified through dialysis to remove the excess salts. The crude extracts of stem, leaf and the body (fruit) and also the partially purified extracts were tested for their protein content (supernatant and pellet) by Bradford's method using Bovine Serum Albumin as standard (Table 1). All the extracts (crude and purified) were tested for their protease activity by Folin's method (Figure 1) in order to confirm the presence of Bromelin. The purified solution was then stored at 4°C for further use.

RESULTS AND DISCUSSION

Table 1: Analysis of Protein Content of the Partially Purified Extracts by Bradford's Assay.

Samples	Protein Conc. (mg/ml)	Samples	Protein Conc. (mg/ml)	Samples	Protein Conc. (mg/ml)
CB	1.5923	CS	2.4296	CL	2.0605
BS 10	0.7771	SS 10	1.1074	LS 10	0.6115
BS 30	0.6407	SS 30	0.5653	LS 30	0.6504
BS 50	0.5838	SS 50	0.5168	LS 50	0.6763
BS 70	0.3696	SS 70	0.2817	LS 70	0.834
BS 90	0.5181	SS 90	0.4462	LS 90	1.2293
BP 10	0.0846	SP 10	1.5529	L P10	2.4898
BP 30	0.0367	SP 30	3.062	L P30	4.0607
BP 50	0.0269	SP 50	2.1799	L P50	3.3686
BP 70	0.0256	SP 70	2.7325	L P70	2.6179
BP 90	0.0135	SP 90	3.2524	LP 90	0.8078

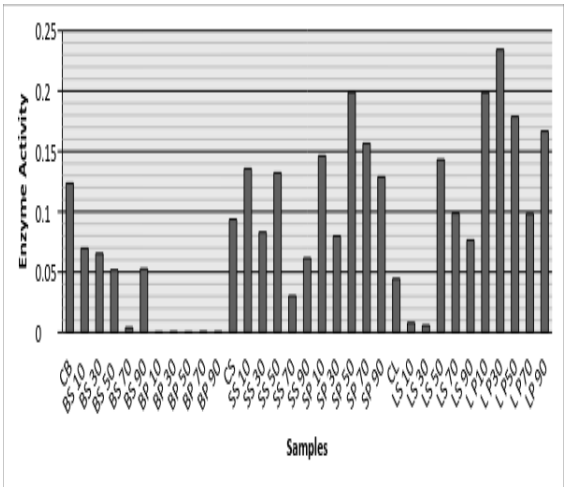

Figure 1: The graph illustrates the protease activity of partially purified extracts.

Effect of pH and Temperature

Hemolysis is the disruption of red blood cells due to the lysis of membrane lipid bilayer. It relates to the concentration and potency of the extract, which in turn is related to their chemical composition. An advantage of using erythrocytes as model is that RBCs are easy to isolate from the blood; and its membrane has similarities with other cell membrane.[16,17] According to Vinjamuri S chemical substances obtained from plants either exhibit hemolytic or anti-hemolytic activity on human red blood cells. The membranes of red blood cells are reported to have varying stability to different plant extracts.[13-15] A positive result was obtained and subsequent experiments were done to determine the optimum pH and temperature at which maximum hemolysis occurred.

The effect of pH on the Hemolytic activity of the extracts are shown in Figure 2(A), 2(B) and 2(C). Based on the results obtained, optimum or effective pH for the Hemolytic activity was observed to be at pH 9 for most of the extracts.

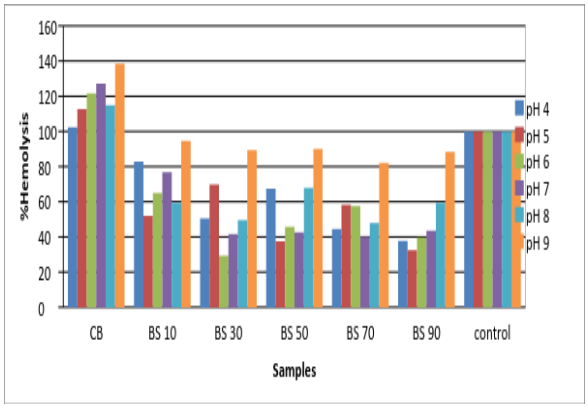

Figure 2(A): The graph illustrates the effect of different pH on the hemolytic activity of Crude and Fruit extracts.

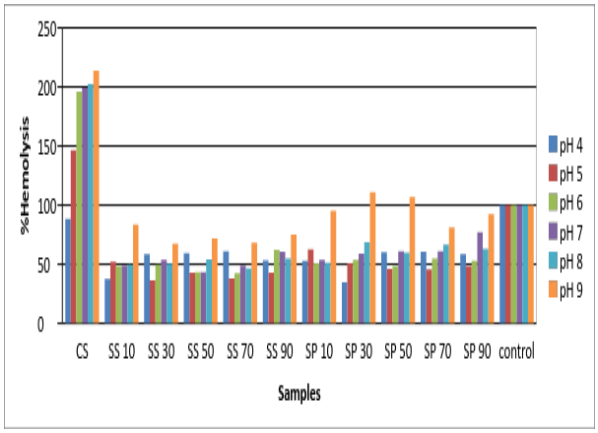

Figure 2(B): The graph illustrates the effect of different pH on the hemolytic activity of partially purified stem extracts.

Bromelain's proteolytic activity reported in literature showed maximum stability in the pH range of 4 to 7.5.[5] The crude extracts of the stem, body and leaves showed

relatively higher activity as compared to the other extracts. The extract from body pellet didn't show significant activity. In particular the crude extract of the leaf showed a significantly higher activity at pH 9 in both the crude as well as the purified extracts compared to that of the fruit and stem crude and partially purified extracts.

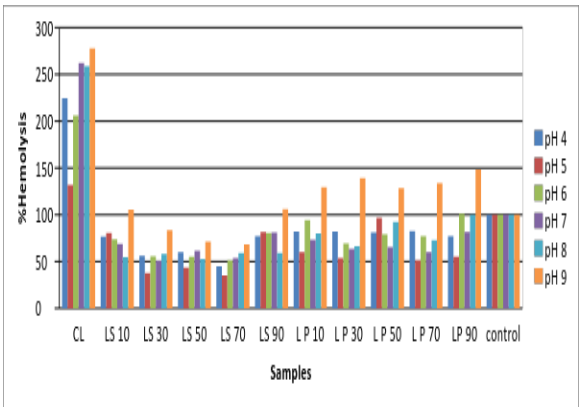

Figure 2(C): The graph illustrates the effect of different pH on the hemolytic activity of Leaf extracts.

The effect of temperature on the Hemolytic activity of the extracts are shown in Figure 3(A), 3(B) and 3(C). Based on the results obtained, the optimum or effective temperature for the Hemolytic activity was observed to be 45˚C for most of the extracts. The effect of temperature on the different extracts was tested by subjecting them to various temperatures such as 4°C, 37°C, 45°C, 60°C, 80°C and 90°C. The optimum temperature range for Bromelain was found to be reported between 40°C-65°C.[5] In the present investigation, the maximum hemolytic activity of the extracts was found to be at 45°C. It was observed that the extract, CB exhibited higher activity than CS10, CS30, CS50, CS70 and CS90, however precipitant's extracts did not show significant hemolytic activity. The stem and leaf part, pellet/ precipitant extracts after salt precipitation showed higher activity. In the stem part higher hemolytic activity was observed in extracts, CS, SP50, SP70 and SP90. LP10. LP30, LP50 and LP 70 of leaf part and CL exhibited higher activity. Thus effect of temperature on different parts and extracts exhibited different results, however maximum activity was observed at 45°C.

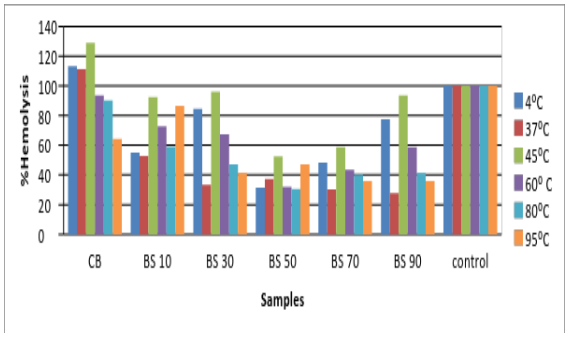

Figure 3(A): The graph illustrates the effect of different temperature on the hemolytic activity of partially purified fruit extracts.

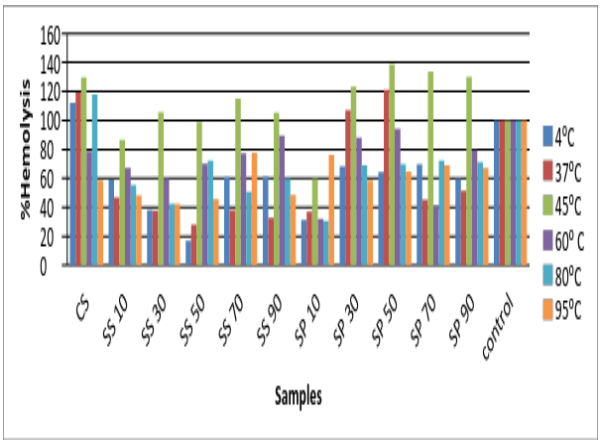

Figure 3(B): The graph illustrates the effect of different temperature on the hemolytic activity of partially purified stem extracts.

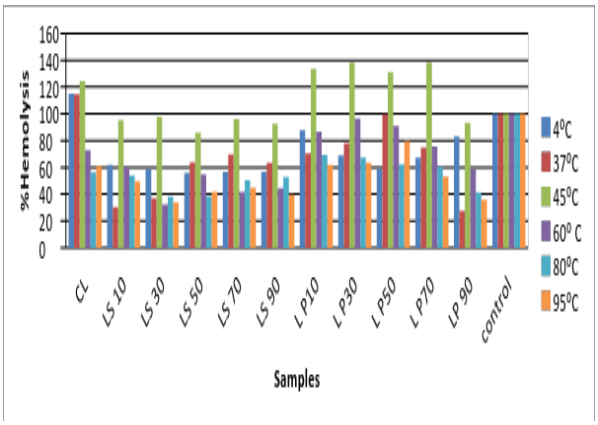

Figure 3(C): The graph illustrates the effect of different temperature on the hemolytic activity of partially purified leaf extracts.

Clot Buster Activity

The Clot Buster activity of the selected extracts is shown in Figure 4.

The extracts which showed the best hemolytic activity (CS, CB, CL, SP 50, LP 10, LP 30, and LP70) were further tested to assess their Clot buster activity. From the various extracts, the highest clot lysis activity was observed in SP50 (68%).

There are two main types of clot dissolving medications, fibrinolytic (fibrin specific) and thrombolytic (nonfibrin specific).

Bromelain falls under the category of thrombolytics, owing to its ability to lyse (systemic lysis) RBCs and hence destabilize and dissolve clots.[19]

The extract SP50 showed a better clot lysis activity (68 %) as compared to that of 100µL/mL of Staphylokinase (40%) as reported by Mohanasrinivasan.[18] *In vivo*, activation of plasminogen to form plasmin, by Staphylokinase, digests the fibrin clots leading to disruption of the fibrin meshwork.

The findings of our study are hence of importance in the current pharmaceutical and health care industry. Further

purification of the extracts and determination of the rate of thrombolysis would substantiate our results further.

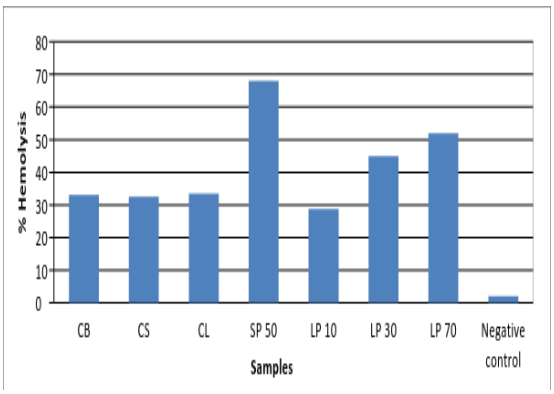

Figure 4: The graph illustrates the Clot Buster activity of the selected partially purified extracts.

CONCLUSION

The partially purified extracts of the pineapple showed hemolytic and clot buster activity which could be explored further for pharmaceutical and medical applications. The extracts exhibited clot buster activity could also have applications in the tannery and meat industry for the proper disposal of wastes containing blood.

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