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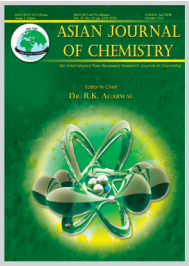

Extraction and Purification of Bromelain

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Bromelain is a crude protein extract obtained from pineapple fruit and stem, which comprises a variety of proteolytic enzymes. It is applied for reduction of muscle and tissue inflammation, alleviation of bronchitis and sinusitis, as an aid in digestion and for relieving symptoms of gout. The most important application of bromelain includes meat tenderization. Pineapple was peeled and crushed to extract the juice containing soluble bromelain enzyme. Further processing included, precipitation, dialysis and ion exchange chromatography of enzyme. Ion exchange elutes (pure enzyme) reveal the activity of bromelain by titrimetric enzyme assay method.

Key Words: Bromelain, Proteolytic enzyme, Dialysis, Ion exchange.

INTRODUCTION

Bromelain is a general name for a family of sulfhydryl containing, proteolytic enzymes obtained from Ananas comosus, the pineapple plant. It can function in the pH range 3 to 9 but once it is combined with substrate, the activity is no longer susceptible to the effect of the pH. The effective temperature range is 40-65 ºC with the optimum being 50 ºC. Bromelain can be activated by calcium chloride, cysteine, bisulfate salt, NaCN, H_2S, Na_2S and benzoate. Hg^{2+}, Ag^{+}, Cu^{2+}, α-1-antitrypsin, estatin A and B, idoacetate, inhibits bromelain. First introduced as a therapeutic compound[1] in 1957, bromelain's actions include: (1) inhibition of platelet aggregation[2-4]; (2) fibrinolytic activity[5]; (3) antiinflammatory action[6]; (4) antitumor action[7]; (5) modulation of cytokines and immunity[8]; (6) skin debridement properties[9]; (7) enhanced absorption of other drugs[10]; (8) mucolytic properties[11]; (9) digestive assistance[12]; (10) enhanced wound healing[13] and (11) cardiovascular and circulatory improvement[14,15]. Various steps that include this research work are, extraction of juice from pineapple, enzyme assay of the crude extract, purification of crude extracts by salt precipitation, dialysis, ion exchange chromatography, quantitative estimation of protein by Folin-Lowery's method. The Venezuelan chemist Vicente Marcano *et al.*[16] recorded the first isolation of bromelain in 1891 from the fruit of pineapple.

EXPERIMENTAL

Pineapple is purchased from local fruit market in Hyderabad (A.P., India). The major equipment used in the present research work includes UV spectrophotometer, ion exchange, chromatograph, gel filtration chromatograph, cooling centrifuge. Sodium acetate buffer was prepared by dissolving 25.5 gm of sodium acetate and 24.5 mL of acetic acid in water and the solution is made up to 200 mL to get a pH of 4.5. Gelatin solution (5%) was prepared by heating 5 gm of gelatin in 100 mL of water at 80 ºC for 20 min. 3 % Hydrogen per oxide was prepared by dissolving 3 mL of H_2O_2 in 100 mL water.

Monobasic and dibasic solution are prepared as follows (Table-1): For monobasic solution 3.12 g of sodium di hydrogen orthophosphate is taken and dissolved in 100 mL distilled water. For dibasic solution 3.55 g of disodium hydro orthophosphate is taken and dissolved in 100 mL distilled water. Then prepared the buffers according to the Table-1.

TABLE-1
PREPARATION OF THE BUFFER SOLUTIONS OF VARYING pH

pH	X (mono basic) (mL)	Y (dibasic) (mL)
2.0	20.00	0.05
2.5	17.00	0.10
3.0	15.00	0.15
3.5	13.00	0.20
4.0	12.25	0.25
4.5	12.125	0.25
5.0	11.875	0.875
5.5	11.60	0.75
6.0	10.96	1.56
6.5	8.56	3.93

Preparation of monobasic and dibasic solutions with different pH values: The other reagents used are carboxyl methyl cellulose (CMC) buffer, formaldehyde, 0.05 % NaOH, 0.1 N HCl, 3 % sodium alginate, 2 % calcium chloride, 1 % calcium chloride, 1 % mercuric chloride, stock arylamide solution (30 % acrylamide and 0.8 % *Bis*-acryl amide), separating gel buffer, stacking gel buffer, electrode buffer (0.025 M (w/v) glycine, 0.025 M (w/v) *Tris* HCl and 0.1 % (w/v) SDS, Staining solution (Coomassie brilliant blue 0.025 g, methanol 40 mL, acetic acid 10 mL, distilled water 100 mL) destaining solution (methanol 40 mL, acetic acid 10 mL and distilled water 100 mL) Bovine serum albumin (BSA) solution, alkaline copper sulfate (reagent A: 2 % Na_2CO_3 in 0.1 N NaOH and reagent B: 0.5 % $CuSO_4$ in 1 % sodium potassium tartarate) reagent A and B were added in the ratio 50:1 to get alkaline $CuSO_4$ solution and FC reagent in 1:1 dilution with distilled water.

Extraction of juice from pineapple: The main steps involved in extraction are as follows: About 160 g of pineapple was taken and it was cleaned well using water. It was crushed into pieces. Then adding sodium acetate buffer it was made into paste. The extract was separated by using filter paper. Later the extract was centrifuged at 6000 rpm for 10 min. The supernatant was collected and stored by adding 1.2 g of benzoic acid in it.

Crude extract enzyme assay: The following procedure was adopted for enzyme assay: Two test tubes were taken and marked as test and blank. 2.5 mL of gelatin was added in each test tube. The test tubes are incubated at 45 ºC for 10 min. 0.1 mL of crude enzyme extract was added in test a test tube and mixed well. Now the test tubes are incubated at 45 ºC for 20 min. After incubation 10 µL of hydrogen peroxide was added in both test and blank and mixed well. pH of both test and blank was adjusted to 6.9 by using acid (HCl) and base (NaOH). 1 mL of formaldehyde was added in both the test tubes. These are titrated against NaOH and adjusted to a pH of 7.8 and the readings are noted.

Salt precipitation: Salt precipitation was carried out in the following sequence: 4.4 g of ammonium sulphate was added to 100 mL crude extract at 4 ºC for 45 min to 1 h and then kept at 4 ºC for overnight incubation. Later, the extract was centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and 10 mL of tris HCl was added to this pellet, mixed and stored.

Dialysis

Activation of dialysis membrane: 100 mL of distilled water is boiled. Then 7 cm of dialysis membrane was added into boiling water. Water was boiled for further 10 min, then added 2 % sodium bicarbonate and boiling continued for another 10 min with 50 mL of distilled water was boiled and into this dialysis membrane was transferred and boiled for another 10 min.

Dialysis: Into 100 mL of acetate buffer taken in a beaker, enzyme sample (pellet obtained from precipitation) was transferred into the dialysis bag. Both ends of the dialysis bag were tagged. Then dialysis bag was dipped in beaker and left for over night incubation at 4 ºC.

Ion-exchange chromatography: Dialysis sample was added into the chromatography apparatus. The pH of resin was maintained at 7.8 by adding tris HCl and kept undisturbed for 15 min. Buffer of pH 4 is added into the column and eluted after 5 min. Buffers ranging from pH 4-7.5 were used for this process. All the elutes were stored in the refrigerator.

Enzyme assay of different elutes: The procedure followed for enzyme assay is as follows: Nine test tubes were marked. One (out of nine) is marked as blank and others as test. 2.5 mL of gelatin was added to each test tube. The test tubes are incubated at 45 ºC for 10 min. To this, 0.1 mL of elutes were added and mixed well. Again the test tubes were incubated at 45 ºC for 20 min. After incubation 0.1 mL of elute was added in the blank. Now 10 µL of hydrogen peroxide was added in both test and blank and mixed well. The pH of both test and blank was adjusted to 6.9 by using acid (HCl) and base (NaOH). Later 1 mL of formaldehyde was added in each test tube. These are titrated against NaOH and adjusted the pH to 7.8 and the readings are noted. Maximum enzyme activity in the different elutes was determined.

Gel filtration chromatography: The procedure followed was bead was washed using acetate buffer solution and the flow rate was adjusted. 2 mL of elute (from ion exchange chromatography) was added to the column and was left for 20 min with out disturbing the bead. Elutes were collected (4 mL each) into 20 tubes at equal intervals of time. Optical density of all 20 elutes was taken at 280 nm.

Enzyme assay of gel filtration elutes: Four test tubes were taken and marked as blank and others as Test. 2.5 mL of gelatin was added in each test tube. Then test tubes were incubated at 45 ºC for 10 min. Elutes (0.1 mL) were added in test and mixed well. The test tubes were incubated at 45 ºC for 20 min. After incubation added 0.1 mL of elute in the blank. 10 µL of hydrogen peroxide was added in both test and blank and mixed well. The pH of both test and blank were adjusted to 6.9 by using acid (HCl) and base (NaOH). 1 mL of formaldehyde was added in each test tube. Now these are titrated against NaOH and the pH is adjusted to 7.8 and the readings are noted. Maximum enzyme activity in the different elutes was determined.

Quantitative estimation of bromelain by Lowry's method: Standard BSA (0.5 mg/mL) was pipetted in 5 test tubes in increasing volumes and in test samples (0.1 mL) in remaining tubes. The volume was adjusted to 1 mL with distilled water. 5 mL of complex forming reagent (alkaline copper sulphate) was added, mixed and kept at room temperature for 10 min. 0.5 mL of Folin-Cioalteu reagent was added, mixed thoroughly and incubated in dark for 0.5 h. Optical density was read on spectrophotometer at 660 nm and recorded the readings. Calibration curve was plotted by taking optical density reading on Y axis against standard protein concentration (µg) on X axis. Value x was recorded from the graph corresponding to the optical density reading for the test sample. The protein concentration was calculated using the following formula:

Protein concentration in test sample = x/v (mg/mL).

where, x - value from graph in µg; v - volume of sample in µL.

RESULTS AND DISCUSSION

Table-2 gives the data on the results of the crude enzyme assay.

TABLE-2
ENZYME ASSAY OF CRUDE EXTRACTS

Test tube no.	Vol. of gelatin (mL)	Incubation for 10 min at 45 ºC	Vol. of enzyme (mL)	Incubation for 20 min at 45 ºC	Vol. of hydrogen peroxide (µL)	Adjust the pH to 6.9	Vol. of formaldehyde (mL)	Adjust the pH to 7.8	Vol. of NaOH (mL)
Blank	2.5		-		10		1		1.0
Test	2.5		0.1		10		1		1.1

TABLE-3
ENZYME ASSAY OF DIFFERENT ELUTES ION EXCHANGE CHROMATOGRAPHY

pH of the contents in the test tube	Vol. of gelatin (mL)	Incubation for 10 min at 45 ºC	Vol. of enzyme (mL)	Incubation for 20 min at 45 ºC	Vol. of hydrogen peroxide (µL)	Adjust the pH to 6.9	Vol. of formaldehyde (mL)	Adjust the pH to 7.8	Vol. of NaOH (mL)
Blank	2.5		-		10		1		1.0
4.0	2.5		0.1		10		1		1.1
4.5	2.5		0.1		10		1		0.4
5.0	2.5		0.1		10		1		1.3
5.5	2.5		0.1		10		1		0.4
6.0	2.5		0.1		10		1		0.8
6.5	2.5		0.1		10		1		1.0
7.0	2.5		0.1		10		1		1.1
7.5	2.5		0.1		10		1		1.0

TABLE-4
OD VALUES OF DIFFERENT ELUTES OBTAINED FROM GEL FILTRATION CHROMATOGRAPHY

Test tube no	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
OD at 280 nm	0.03	0.02	0.03	0.077	0.01	0.02	0.02	0.02	0.03	0.01	0.06	0.05	0.02	0.04	0.02	0.01	0.02	0.02	0.076	0.017

TABLE-5
ENZYME ASSAY OF DIFFERENT ELUTES FROM GEL FILTRATION CHROMATOGRAPHY

Vol. of the contents	Vol. of gelatin (mL)	Incubation for 10 min at 45 ºC	Vol. of enzyme (mL)	Incubation for 20 min at 45 ºC	Vol. of hydrogen peroxide (µL)	Adjust the pH to 6.9	Vol. of formaldehyde (mL)	Adjust the pH to 7.8	Vol. of NaOH (mL)
Blank	2.5		-		10		1		0.9
4	2.5		0.1		10		1		1.0
11	2.5		0.1		10		1		1.1
19	2.5		0.1		10		1		1.2

Bromealin being a proteolytic enzyme acts on gelatine and degrades it into amino acids and oligopeptides. Hydrogen peroxide is used to stop the reaction after 20 min. It helps in oxidation. When pH is adjusted to 6.9 there will not be any amino acids. Formaldehyde breaks the amino group of amino acids to release H^+ ions. Number of H^+ ions is proportional to amino acids. Sodium hydroxide is added to neutralize H^+ ions. Amount of NaOH needed for titration proportional to number of H^+ ions, this is proportional to number of amino acids, proportional to activity of enzyme. Volume of NaOH needed for test is greater than blank (containing no enzyme) indicates test tube containing enzyme shows activity.

Table-3 provides the information about enzyme assay of different elutes ion exchange chromatography. The enzyme elutes of different pH were tested for activity of gelatine. As the contents in the test tubes with pH values of 4, 5 and 7 shows greater activity (more amount of NaOH needed for titration).

The data on the optical density values of different elutes of gel filtration chromatography is presented in Table-4.

Gel filtration was carried out during ion exchange chromatography elute pH-4 and elutes were collected in 20 different test tubes. Using spectro photometer optical density was checked for all 20 tubes. Test tube 4, 11, 19 yielded highest optical density values inclined, these have maximum concentration of pure enzyme after gel filtration.

The results enzyme assay of different elutes from gel filtration chromatography are given in Table-5. The three elutes of gel filtration was tested for activity on gelatin. As test tube-19 required maximum amount of NaOH. It was considered to contain pure enzyme.

Protein concentration in enzyme sample was determined using standard volume of BSA and 0.1 mL of crude enzyme extract, dialysis sample, ion exchange chromatography elute and gel filtration chromatography elute. The optical density values of 100 BSA solution were plotted against its volume and extrapolation gave volume of protein present in enzyme sample. Highest was found in gel filtration chromatography sample (GF 11) (Table-6).

TABLE-6
QUANTITY ESTIMATION OF BROMELAIN BY LOWRY'S METHOD

Test tube no.	Volume of BSA (mL)	Volume of dist. water (mL)	Volume of alkaline $CuSO_4$ (mL)		Volume of Fe reagent (mL)		Optical density at 660 nm
Blank	0.2	1.0	5		0.5		0.0
1	0.4	0.8	5		0.5		0.31
2	0.6	0.6	5		0.5		0.52
3	0.8	0.4	5		0.5		0.64
4	1.0	0.2	5	Incubate at	0.5		0.85
5	0.1	0.0	5	room	0.5	Incubate in	0.87
6 (crude)	0.1	0.9	5	temperature for	0.5	dark for 0.5 h	0.48
7 (dialysis)	0.1	0.9	5	10 min	0.5		0.33
8 (IE 4)	0.1	0.9	5		0.5		0.178
9 (IE 5)	0.1	0.9	5		0.5		0.37
10 (IE 7)	0.1	0.9	5		0.5		0.25
11 (GF 4)	0.1	0.9	5		0.5		0.146
12 (GF 11)	0.1	0.9	5		0.5		0.208
13 (GF 19)	0.1	0.9	5		0.5		0.140

Conclusion

According to the procedure described, the enzyme bromelain is extracted and is purified by salt precipitation, dialysis, ion exchange and gel filtration chromatography. After the complete purification it is observed that the amount or volume of protein is highest in gel filtration elute, as compared to ion exchange elute and dialysis elute. The process of gel filtration achieved highest degree of purification.

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