IJPSR (2015), Vol. 6, Issue 12 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 16 June, 2015; received in revised form, 31 August, 2015; accepted, 03 October, 2015; published 01 December, 2015

EXTRACTION, PURIFICATION OF BROMELAIN FROM PINEAPPLE AND DETERMINATION OF ITS EFFECT ON BACTERIA CAUSING PERIODONTITIS

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Keywords:

Ananus Comosus, Extraction, Bromelain, Ion Exchange Chromatography, SDS-PAGE, Periodontitis

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ABSTRACT: The pineapple (*Ananas comosus*) is a multiple fruit belonging to the *Bromeliaceae* family, has a crude extract Bromelain accumulated in the stem, fruit, peel and leaves of the pineapple which shows proteolytic activity. The present study was carried out to assess the accumulation of bromelain in the different parts of the pineapple. The techniques homogenization with sodium acetate buffer, followed by filtration and centrifugation were used to extract bromelain from pineapple. The crude bromelain was then purified by ammonium sulphate salt precipitation, dialysis and finally subjected to ion exchange chromatography. Lowry's method and gelatine digestion unit (GDU) analytical method were performed to estimate the concentration and activity of the crude enzyme and purified bromelain from the different parts The molecular weight of the purified enzyme was determined by SDS-PAGE (sodium dodecyl sulphate - polyacrylamide gel electrophoresis) analysis and found to be 25 to 27 KDa. The final step was to find out the effect of bromelain from leaves and stem of pineapple on pathogens of periodontitis. The enzyme exhibited proteolytic activity against certain bacteria and its capacity to enhance the activity of antibiotics on pathogens causing periodontal diseases. The results of the study confirmed that the enzyme has a high commercial value and a wide range of applications in the field of medicine and biotechnology.

INTRODUCTION: The extracts of the plants have contributed to the work of the scientists for the discovery of new compounds in the different fields of biotechnology, such as food industry, textile industry and in the medical field. Pineapple (*Ananas comosus*) is a tropical plant cultivated in many parts of the world. Plant proteases have a very important place in the field of medicine and biotechnology.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.6(12).5284-94

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.6(12).5284-94

Proteolytic enzymes such as papain and bromelain are used in various forms of therapy [1]. Bromelain is a general name for a family of sulfhydryl protective enzymes obtained from various species of *Bromiliaceae* [2]. Bromelain is one of the best known plant protease that can be extracted from different parts of pineapple such as pulp, stem, peel and the leaves. The commercially available bromelain is produced from pineapple stem but the extract is removed from cooled pineapple juice by centrifugation, ultra filtration and lyophilisation [3]. In a recent study conducted on bromelain extraction it has been concluded that bromelain showed maximum activity at pH 7 at 50^0C at the simple extraction and most proteolytic activity at pH 8 to 60^0C when being precipitated by ethanol [4]. Bromelain assay for its activity was determined by

hydrolysis of gelatin and the activity was represented by using gelatin digestion unit [5]. The protease activity of bromelain was determined according to the casein digestion unit analytical method and tyrosine was used as the standard [6]. The bromelain in fruit of pineapple plant was 2.81fold pure at 40-60% saturation level using ammonium sulphate precipitation [7]. A recent study was conducted on the extraction of bromelain and used 12% SDS – PAGE to determine the molecular weight of purified protein [8]. The pathogens causing periodontal diseases are *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia* are together known as red complex. The major contributor of periodontitis is *P. gingivalis* and the disease leads to the development of atherosclerosis [9]. The reduction in collagen density is a common feature of periodontitis which is caused by the collagenase activity present often in bacteria *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis.*

The treatment plan should contain local antibiotic therapy along with scaling and root planning for the complete treatment of periodontitis caused by *A. Actinomycetemcomitans* [10]. Results of a dental study have showed that oral bromelain is an effective therapy to improve the quality of life after surgical removal of impacted lower third molars [11]. A project titled "In vitro Evaluation of Antibacterial Efficacy of Pineapple extract (bromelain) on Periodontal Pathogens" concluded that bromelain is an agent which has been widely used as anti inflammatory drug in the field of medicine and dentistry [12].

The objectives of the present study were to extract crude bromelain from different parts of the pineapple which was then purified so as to determine the concentration, activity and molecular weight of the enzyme. The purified enzyme was then evaluated for its inhibitory activity on the growth of the bacteria causing periodontitis. The final step was to determine the capacity of bromelain to enhance the activity of antibiotics.

MATERIALS AND METHODS:

Materials:

The pineapple was purchased from a local market in Bangalore and the present research was carried out in the laboratory of a renowned company. Aristogene Biosciences Pvt Ltd Bangalore in the year 2015. The reagents used were of research grade which included hydrogen peroxide (H_2O_2), sodium actetate (CH_3COONa) buffer, sodium azide, autoclaved demineralized water Folin Ciocalteau (FC), standard bovine serum albumin (BSA), 5% gelatin, 37% formaldehyde, ammonium sulphate, demineralized water(DM H_2O), distilled water (dH_2O), 2%sodium carbonate(Na_2CO_3) in 0.1N sodium hydroxide(NaOH), 1% copper sulphate solution, 2% sodium potassium tartarate, phosphate buffer (PB), Di-ethyl ammonium ethyl (DEAE) from GeNei India Ltd, Bangalore, regeneration buffer: sodium chloride (NaCl) buffer, equilibration or wash buffer: 20 mM phosphate buffer (PB),4 – band marker (containing BSA, ovalbumin, carbonic anhydrous and lysozyme of molecular weights 66 kDa, 45 kDa, 29 kDa and 14.3 kDa respectively), hydrochloric acid (HCL), sodium dodecyl sulphate (SDS), polyacrylamide gel, TEMED from Sigma Aldrich, potassium per sulphate (KPS), 30% acrylamide bisacrylamide solution, bromophenol blue, Coomasie blue dye, sample loading buffer (SLB), Tris – cl SDS, agarose, 1X – SDS (Tris-glycine) electrophoresis buffer and 70% ethanol.

All the chemicals were purchased from Hi Media Labs Ltd Mumbai. The bacterial strains of *Enterococcus faecalis, Streptococcus mutans, Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis* and *Serratia marcescens,* and the antibiotics ampicillin, chlorohexidine, amoxicillin and ciprofloxacin respectively were used for this study.BHI (brain heart infusion) with vancomycin agar/broth from DIFCO, MH (Mueller Hinton) agar/broth from HIMEDIA and BHI agar/broth from DIFCO were the growth media used.

Methods:

Extraction: The pineapple containing the leaves and stem was washed with 250 ml of 0.1% H_2O_2solution. The peel, pulp stem and leaves were cut into small pieces and weighed separately. The juice from the above mentioned parts of the pineapple were blended with CH_3COONa buffer, subjected to filtration and finally followed by centrifugation of the filtrates at 10,000 rpm. The

supernatants were refrigerated at 4^0C after adding 0.05% sodium azide.

Determination of the concentration of the enzyme in the crude extract by Lowry's method: After freezing 10 µl of the crude extracts were taken in four different test tubes and made upto 200 µl by adding distilled water. Standard BSA at range of 10 – 200µg were pipetted into test tubes and the volume was made upto 200 µl using distilled water. To each test tube 3ml of complex forming reagent was added, and incubated for 30 min at room temperature in the dark.0.3ml of FC reagent was added to all the test tubes and incubated for 10 min. The absorbance was measured using a UV-spectrophotometer at 660 nm, the readings were tabulated and a graph of protein (µg) on X-axis and absorbance on Y-axis was plotted. From this graph the unknown concentration of enzyme in the crude extracts were determined.

Determination of enzyme activity by GDU analytical assay: Four beakers each containing1 ml of the different extracts were labeled as test. Another four beakers each containing 25 ml of 5% gelatin were labeled as blank to which 100 µl of 3% H_2O_2 was added. The test samples were adjusted to pH 6 with 0.1N NaOH followed by adding 25 ml of the 5% gelatin substrate to all the four test samples. All the eight beakers were boiled in a water bath at 45^0 C for 20 minutes followed by adding 100 µl of 3% H_2O_2 to the test samples and boiling for another 5 min. 1ml of crude extracts were added to the four beakers labeled as blank and boiled on a water bath at 45^0 C for 5 min. The pH of all the beakers were adjusted to 6 by adding 0.1N NaOH followed by adding 10 ml of 37% formaldehyde to all the beakers. The pH was adjusted to 9 using 0.1N NaOH buffer. The enzyme activity for the crude extracts was determined by using the formula below

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

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)

14 is mg nitrogen per mM nitrogen

N is the normality of standardized NaOH

Purification:

The crude extracts (i.e. 48 ml of each) of the different parts of the pineapple were first purified by adding 20.61 g of ammonium sulfate salt with continuous stirring. The precipitated enzymes were centrifuged at 10,000 rpm and the pellets obtained were dissolved in 5 ml of 20 mM PB which was then subjected to dialysis.

These solutions obtained above were placed in four different dialysis bags and were then suspended in four different beakers each containing 20 mM PB. These beakers were refrigerated overnight at 4^0 C with continuous stirring. The next day the buffers in each of the four beakers were changed, refilled twice at an interval of 3 hours and finally the four dialysed extracts collected were frozen at -20^0 C overnight.

The next day these solutions were purified by ion exchange chromatography (IEC). DEAE: distilled water suspension in the ratio 1:1 was added to the column which was mounted vertically on a burette stand and clamped. 25 ml of 1 M regeneration buffer was added into the column and drained. The column was then equilibrated with 30ml of 20 mM PB. 5 ml of the dialysed peel sample was added to the column and allowed to stand by blocking the outlet. After 15 minutes the outlet was opened and 30ml of wash buffer was added to the column. The sample was collected and absorbance checked at 280nm using a UV-Spectrometer such that when the absorbance was less than or equal to 0.05, 40 ml of elution buffer was added into the column. 2 ml of the eluate solution was collected from the exit tubing in 14 accurately labeled test tubes. The absorbance readings of each of these eluates were determined at 280 nm and were tabulated. These readings give an indication of where protein is found in the eluate and were called as "fractions". 10 ml of regeneration buffer was added into the column. The column was refrigerated at 4^0C overnight and the purified samples were subjected to SDS-PAGE. The above procedure was repeated for the dialysed enzyme samples of the pulp, leaves and stem respectively.

Determination of the molecular weight of the crude and dialysed enzyme samples by SDS - PAGE: 2.97 mldH_2O, 3.6 ml of 30% acrylamide bis acrylamide, 2.25 ml 1.5 M Tris – cl SDS (pH 8.8), 9 µl TEMED and 0.18 ml KPS were mixed in a test tube so as to prepare 9 ml of the 12 % Separating gel solution. 2.075 ml dH_2O, 0.5 ml of 30% acrylamide bis acrylamide, 0.375 ml 1. M Tris – cl SDS (pH 6.8), 5 µl TEMED and 0.05 ml of KPS (potassium per sulphate) were mixed in a test tube so as to prepare 3 ml of the 5 % Stacking gel solution.

The separating gel solution and the stacking gel solution were loaded between the glass plate sandwich, followed by carefully inserting the comb into the stacking gel. The gels were allowed to polymerize completely before removing the comb. 10 µl of the crude enzyme extracts, dialysed samples and the 4 – band marker were mixed with 10 µl of the SLB and heated at100^{0}C for 5 min. The glass plates containing the solidified gel were removed from the casting apparatus and clipped to the electrophoresis apparatus. The comb was removed carefully from the gel followed by filling the top and bottom tank of the apparatus with 1X SDS electrophoresis buffer. The samples were loaded into the bottom of the wells using a flat – tipped pipette tips by carefully recording the contents of each well. This apparatus was connected to the power supply and was started at 100V. The power pack was switched off when the dye front came to 1 cm above the bottom of the gel. The gel plates were carefully removed and separated. The stacking gel was gently cut away and the separating gel was stained overnight in a plastic container which was filled with 20 ml of Coomasie blue stainer. The light coloured bands were observed the next day.

To assess the purity of the purified bromelain the above mentioned procedure was repeated. These purified bromelain samples so obtained were further subjected to quantitative estimation and enzyme assay so as to determine the concentration and proteolytic activity.

Determination of optimum temperature for enzyme activity: This was done to check the activity of the enzyme at different temperatures by varying the temperatures from 30^{0}C to 70^{0}C. Twenty test tubes were divided into 4 sets named as peel, pulp, leaves and stem respectively containing 5 test tubes each and labeled as 30^{0}C, 40^{0}C, 50^{0}C, 60^{0}C and 70^{0}C. 1 ml of the purified enzyme samples were added to the respective test tubes containing 1 ml of warmed gelatin substrate. These test tubes were incubated at the respective temperatures, were checked for solidification the next day and the results were tabulated.

Determination of optimum pH for enzyme activity: This was done to check the proteolytic activity of the purified bromelain enzyme at different pH with an acid (HCl) and a base (NaOH) from pH 5 to 8. About 1 ml of bromelain enzyme samples were taken in 16 test tubes of 4 sets each and were adjusted to different pH values ranging from 5 to 8 by adding about 1ml of the acid or base. 1ml of gelatin was added to the test tubes followed by sealing them with cotton plug to avoid any contamination. The tubes were incubated in refrigerator overnight and checked for solidification the next day.

Determination of the effects of the purified bromelain extracts on pathogens causing periodontitis: The plating of cultures was carried out in a laminar air flow chamber where 100 µl of the respective bacterial culture from the test tube were added onto the petri plate containing solidified agar media. The culture was uniformly distributed on the media by a sterile glass rod. The bottom of these petri plates were divided into four quadrants labeled as A for antibiotic, A + B for antibiotic with the respective bromelain extract, B for bromelain and W for distilled water (control).

Agar well diffusion assay was carried out for *Streptococcus mutans* by carefully pipetting 20µl of antibiotic, 20 µl of purified leaves bromelain extract along with 20µl of antibiotic, 20µl of purified stem bromelain and 20µl of distilled water (control) into a petri plate containing four separate 0.4 cm wells labeled as A, A + B, B and W respectively.

Disc diffusion assay was carried out to determine the antibacterial effect of the purified leaves and stem bromelain on the gram positive *Enterococcus*

faecalis and gram negative bacteria namely *Aggregati bacter actinomycetemcomitans, Porphyromonas gingivalis* and *Serratia marcescens* respectively. The two half inch diameter sterile filter paper discs (one impregnated with purified leaves bromelain and the other dipped in distilled water) and the two antibiotic discs (i.e. one plain antibiotic disc and the other antibiotic disc dipped in 20µl bromelain) were picked up with the sterile forceps and placed on the surface of the agar plate by slightly pressing down with a forceps so that the disks adhere to the surface of the medium.

This step was repeated for the 3 remaining petridishes containing the other 3 bacterial cultures. The above steps were repeated using purified stem bromelain. The agar plates were incubated in inverted position under anaerobic conditions at 37 ^{0} C overnight in an incubator and ZOI (zones of inhibition) in mm around the discs were observed the next day. The MIC (Minimum inhibitory concentration) was determined.

Determination of the MIC: Three different concentrations (100%, 75% and 50%) of the leaves bromelain extract were prepared by diluting with distilled water. Two sets of test tubes for the bacteria *A. Actinomycetemcomitans* and *P. gingivalis* were taken with each set containing 3 tubes according to the respective concentrations. 18 ml of BHI broth and 18 ml of BHI broth with vancomycin were prepared for *A. Actinomycetemcomitans* and *P. gingivalis* and poured into 2 sets of test tubes containing 3 each such that each tube contains 6 ml each.900 µl of the stem bromelain extract was added into three respective accurately labeled test tubes. This step was repeated for the bromelain extract obtained from leaves. After this step 100 µl of the respective bacterial suspension were inoculated into respective labeled test tube. After inoculation, these test tubes were kept in an incubator overnight at 37^0C under anaerobic conditions and results were observed in the form of turbidity. The next day the optical density (OD) was observed at 600 nm using U.V. Spectrophotometer and the results were tabulated.

RESULTS:

Quantitative estimation of enzyme in the crude samples by Lowry's method:

TABLE 1: ABSORBANCE OF STANDARD BSA TO DETERMINE THE CONCENTRATION OF THE CRUDE ENZYME EXTRACTS

Volume of std BSA (µl)	Volume of distilled water (µl)	OD at 660nm
0	200	0
20	180	0.087
40	160	0.29
60	140	0.302
80	120	0.32
100	100	0.39
120	80	0.476
140	60	0.55
160	40	0.627
180	20	0.71
200	0	0.83
Test Samples (µl)		
10 (Peel)	190	0.45
10 (Pulp)	190	0.351
10 (Leaves)	190	0.08
10 (Stem)	190	0.248

Determination of enzyme activity:

TABLE 2: ACTIVITY OF THE CRUDE BROMELAIN

Crude extract	Blank titre (Volume of 0.1N NaOH run down in ml)	Test titre (Volume of 0.1 N NaOH run down in ml)	Activity in units/mg
Peel	11.75	13.3	190.35
Pulp	12.6	13.76	178.89
Leaves	11.7	12	201.92
Stem	8.8	9.6	180.65

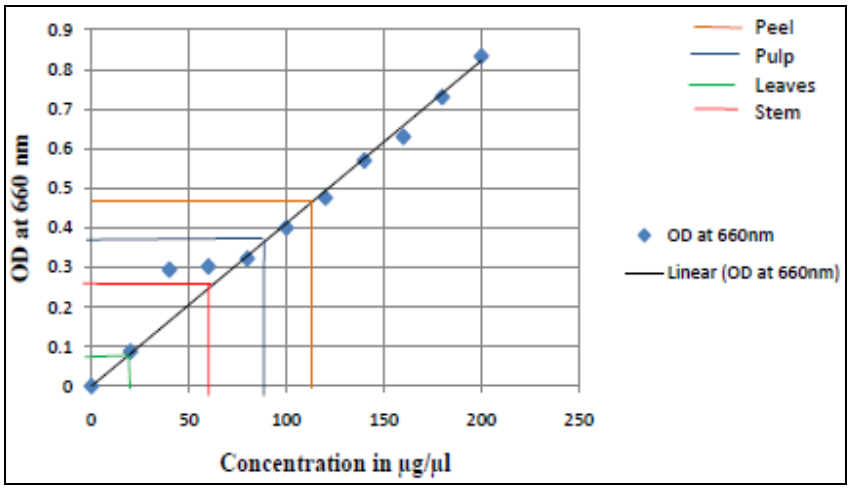

FIG. 1: STANDARD BSA GRAPH: QUANTITATIVE ESTIMATION OF CRUDE ENZYME

Determination of the molecular weight of the crude and dialysed enzyme samples by SDS – PAGE:

TABLE 3: SAMPLE LOADING PATTERN OF THE CRUDE AND DIALYSED SAMPLES ON THE GEL

Lane	1	2	3	4	5	6	7	8	9	10
Sample	4 – band marker	Gap	Crude Pulp	Crude Stem	Crude Peel	Crude Leaves	Dialysed Pulp	Dialysed Stem	Dialysed Peel	Dialysed Leaves

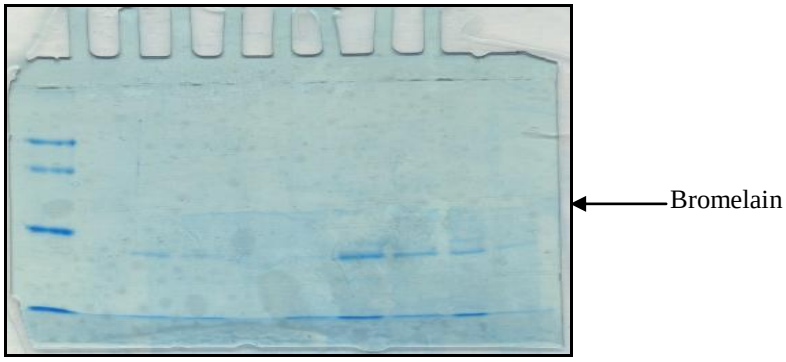

FIG.2: SDS - PAGE GEL OF THE CRUDE EXTRACTS AND DIALYSED SAMPLES

Purification of dialysed bromelain samples by IEC and determination of their purity by SDS – PAGE:

TABLE 4: ABSORBANCE AT 280 nm OF THE ELUATES FROM IEC COLUMN

Test tube	OD at 280 nm of Peel samples	OD at 280 nm of Pulp samples	OD at 280 nm of Leaves samples	OD at 280 nm of Stem samples
1	0.034	0.032	0.01	0.022
2	0.095	0.353	0.021	0.067
3	0.379	1.965	0.11	0.346
4	0.421	1.703	0.136	0.55
5	0.372	1.23	0.124	0.44
6	0.344	1.104	0.108	0.346
7	0.334	1.077	0.093	0.32
8	0.312	1.009	0.081	0.30
9	0.29	0.913	0.073	0.286
10	0.259	0.784	0.066	0.252
11	0.227	0.656	0.061	0.223
12	0.197	0.569	0.053	0.194
13	0.171	0.437	0.049	0.168
14	0.15	0.369	0.047	0.153

TABLE 5: SAMPLE LOADING PATTERNS OF THE MARKER AND ELUATES ON 4 DIFFERENT GELS

Lane	Peel Sample	Pulp Sample	Leaves Sample	Stem Sample
1	4 - band marker	4 - band marker	4 - band marker	4 - band marker
2	Test tube 4	Test tube 3	Test tube 2	Test tube 3
3	Test tube 6	Test tube 5	Test tube 4	Test tube 4
4	Test tube 8	Test tube 7	Test tube 5	Test tube 5
5	Test tube 10	Test tube 9	Test tube 6	Test tube 6
6	Test tube 12	Test tube 11	Test tube 7	Test tube 8
7	Test tube 14	Test tube 13	Test tube 8	Test tube 11

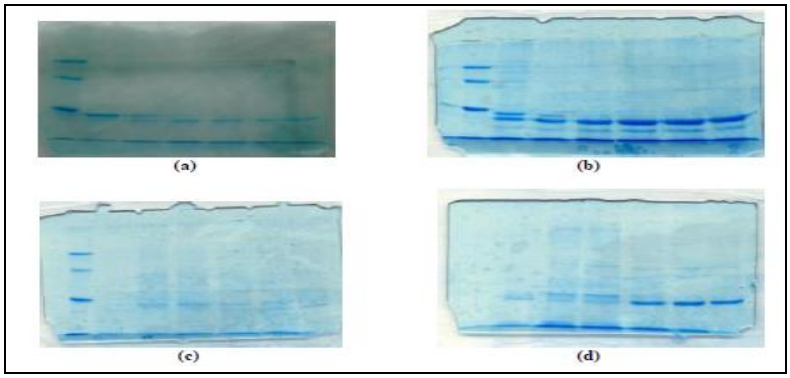

FIG.3 (a)SDS - PAGE GEL OF THE PURIFIED PEEL SAMPLE, (b)SDS - PAGE GEL OF THE PURIFIED PULP SAMPLE, (c)SDS - PAGE GEL OF THE PURIFIED LEAVES SAMPLE AND(d)SDS - PAGE GEL OF THE PURIFIED STEM SAMPLE

Quantitative estimation of bromelain in the purified samples by Lowry's method:

TABLE 6: ABSORBANCE OF STANDARD BSA TO DETERMINE THE CONCENTRATION OF THE PURIFIED BROMELAIN

Sl No.	Volume of std BSA (µl)	Volume of distilled water (µl)	OD at 660nm
1.	0	200	0
2.	20	180	0.053
3.	40	160	0.184
4.	60	140	0.17
5.	80	120	0.238
6.	100	100	0.281
7.	120	80	0.431
8.	140	60	0.44
9.	160	40	0.463
10.	180	20	0.531
11.	200	0	0.562
	Test Samples (µl)		
Peel	100	100	0.058
Pulp	100	100	0.188
Leaves	100	100	0.031
Stem	100	100	0.07

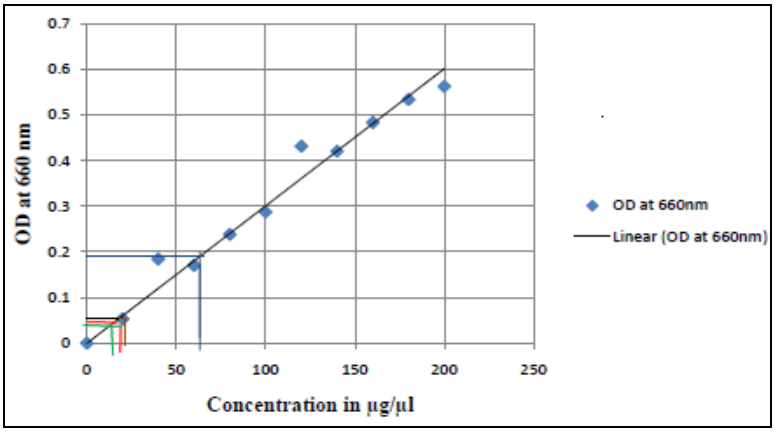

FIG.4 STANDARD BSA GRAPH: DETERMINATION OF CONCENTRATION OF PURIFIED BROMELAIN

Determination of optimum temperature for enzyme activity:

TABLE 7: DETERMINATION OF OPTIMUM TEMPERATURE

Temperature (^{0}C)	Enzyme activity based on state of gelatin
30	Solidified
40	Not solidified
50	Not solidified
60	Partially solidified
70	Solidified

Determination of optimum pH for enzyme activity:

TABLE 8: DETERMINATION OF OPTIMUM pH

pH	Enzyme activity based on state of gelatin
5	Solidified
6	Not solidified
7	Partially solidified
8	Solidified

Determination of bromelain activity by GDU analytical assay:

TABLE 9: ACTIVITY OF THE PURIFIED BROMELAIN

Purified extract	Blank titre (Volume of 0.1N NaOH run down in ml)	Test titre (Volume of 0.1 N NaOH run down in ml)	Activity in units/mg
Peel	9.2	9.3	636.36
Pulp	9.8	10	437.5
Leaves	12.55	12.7	1750
Stem	12.7	13	1750

Determination of the effects of the purified leaves and stembromelain on pathogens causing periodontitis

Gram positive bacteria:

1. *Enterococcus faecalis:*

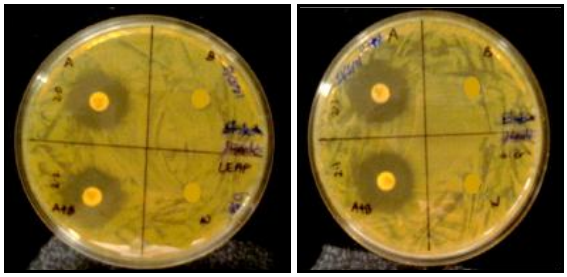

FIG. 5: EFFECT OF PURIFIED LEAVES AND STEM BROMELAIN EXTRACTS ON *E. FAECALIS*

2. *Streptococcus mutans:*

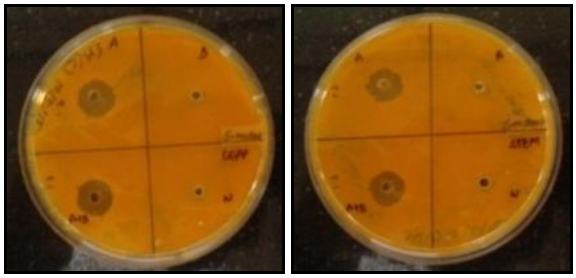

FIG.6: EFFECT OF PURIFIED LEAVES AND STEM BROMELAIN EXTRACTS ON *S. MUTANS*

Gram negative bacteria:

1. Aggregatibacter actinomycetemcomitans:

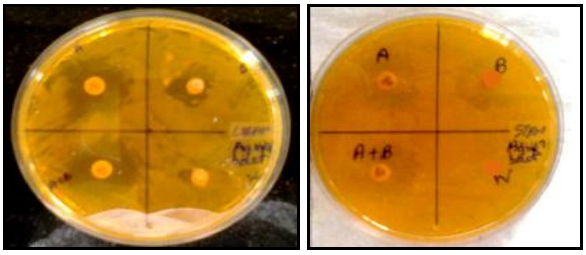

FIG. 7: EFFECT OF PURIFIED LEAVES AND STEM BROMELAIN EXTRACTS ON *A. ACTINOMYCETEMCOMITANS*

2. Porphyromonas gingivalis:

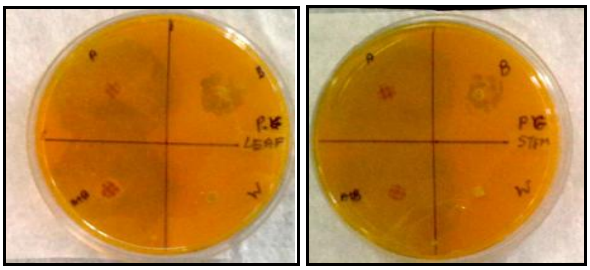

FIG. 8: EFFECT OF PURIFIED LEAVES AND STEM BROMELAIN EXTRACTS ON *P. GINGIVALIS*

3. *Serratia marcescens:*

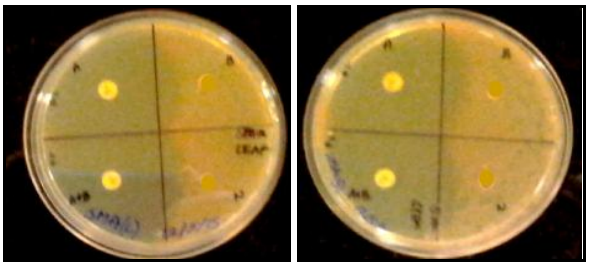

FIG. 9: EFFECT OF PURIFIED LEAVES AND STEM BROMELAIN EXTRACTS ON *S. MARCESCENS*

TABLE 10: ANTIBACTERIAL EFFECTS OF LEAVES AND STEM BROMELAIN ON GRAM POSITIVE AND GRAM NEGATIVE BACTERIA

Bacteria	Bromelain extract	Antibiotic (A) ZOI in mm	Antibiotic + Bromelain (A+B) ZOI in mm	Bromelain (B) ZOI in mm	Distilled water (W) ZOI in mm
E. faecalis	Leaves	20 ± 1	24 ± 1	0.0	0.0
	Stem	24 ± 1	25 ± 1	0.0	0.0
S. mutans	Leaves	25 ± 1	27 ± 1	0.0	0.0
	Stem	25 ± 1	28 ± 1	0.0	0.0
A.actinomycetemcomitans	Leaves	37 ± 1	40 ± 1	12 ± 1	0.0
	Stem	42 ± 1	50 ± 1	10 ± 1	0.0
P. gingivalis	Leaves	40 ± 1	42 ± 1	15 ± 1	0.0
	Stem	38 ± 1	41 ± 1	10 ± 1	0.0
S. marcescens	Leaves	45± 1	47± 1	0.0	0.0
	Stem	45± 1	48± 1	0.0	0.0

TABLE 11: DETERMINATION OF THE MIC OF BROMELAIN EXTRACTS

Bromelain Extract	Concentration of bromelain extracts	OD at 600 nm	
		A. actinomycetemcomitans	*P. gingivalis*
Leaves	100%	1.191	1.002
	75%	1.362	1.501
	50%	1.870	1.920
Stem	100%	1.102	1.203
	75%	1.501	1.426
	50%	1.983	2.010

DISCUSSIONS: The pineapple extracts from the different parts were subjected to quantitative estimation by Lowry's method and the concentration of the enzyme in the crude extracts of the peel, pulp, leaves and stem was found to be 11.4 mg/ml, 9 mg/ml, 2.08 mg/ml and 6.2 mg/ml respectively. Hence the concentration of the enzyme in the crude extracts was the highest in the peel and lowest in the leaves. Then the enzyme assay was conducted to determine the activity of the crude extracts and it was found to be the highest in the leaves followed by the peel, stem and was lowest in the pulp. The crude extracts were purified by ammonium sulphate precipitation and dialysis. The molecular weight of the crude and dialysed extracts were determined by SDS – PAGE and found to be ranging between 25 to 27 KDa (**Fig. 2**). The band representing the dialysed leaves extract was observed to be very light compared to those of pulp, peel and stem, which indicated that the enzyme concentration in the crude leaves sample was the least which was in accordance with the result obtained by Lowry's method.

The purified dialysed extracts were then subjected to IEC and the eluates from the respective test tubes were mixed with SLB, loaded on to the gel in the pattern as represented in **Table 5** to determine the purity of the peel, pulp, leaves and stem bromelain which are observed in **Fig.3 (a), (b), (c)** and **(d)** respectively. The lanes showing clear bands indicate that the bromelain fraction in these

lanes was pure. These purified fractions from the respective test tubes of the different parts of the pineapple were pooled together separately in order to determine the concentration, optimum temperature, optimum pH and activity of the purified bromelain.

The purified bromelain from the different parts were subjected to quantitative estimation by Lowry's method and the concentration of bromelain from the peel, pulp, leaves and stem was found to be 0.22 mg/ml, 0.64 mg/ml, 0.12 mg/ml and 0.24 mg/ml respectively. It can be observed that the concentration of the purified bromelain was the highest in the pulp followed by the stem, peel and lowest in the leaves. The concentration of the purified bromelain was less when compared to the crude enzyme concentration as the later contains the presence of other proteins. The gelatin had solidified in the test tubes incubated at temperatures 30^0C and 70^0C respectively, but the gelatin did not solidify in the test tubes which were incubated at 40 and 50^0C.

This is because the bromelain being a proteolytic enzyme breaks down the peptide bonds in gelatin. The raise in temperature above the optimum temperature might have led to the destruction of the peptide bonds which ultimately resulted in the inactivity of the enzyme. Hence the optimum temperature for bromelain activity ranged between 40and60^0C. It was also observed that the gelatin did not solidify at pH 6 after overnight incubation but it partially solidified at pH 7 and completely solidified in acidic solution at pH 5 and basic solution at pH 8. Hence the optimum pH of bromelain activity is at pH 6.When the purified bromelain samples were subjected to gelatin digestion unit (GDU) analytical assay the bromelain from the leaves and stem showed better proteolytic activity(**Table 9**) than the peel and pulp which is in accordance with the result of S S Gautam et al., (2010) that the stem bromelain possessed more enzymatic activity than the fruit bromelain. After purification, the purification factor of the pineapple peel, pulp, leaves and stem was found to be 3.34 fold, 2.45 fold, 8.67 fold and 9.68 fold respectively, which also contributed to the increase in proteolytic activity of purified bromelain.

In human beings due to repeated dosages of antibiotic drugs, the pathogens have developed resistance to these drugs. The indiscriminate use of antibiotic drugs may lead to serious side effects. To overcome this problem, alternative medicines in the form of food supplements, fresh fruit juices and plant extracts are emphasized, to fight or prevent common diseases. This study was designed to determine the antibacterial activity of bromelain and also its capacity to enhance the activity of the antibiotics that cure periodontitis. Periodontitis is a bacterial disease resulting in the destruction of periodontal tissues which can lead to tooth loss and other serious health problems.

Since the activity of the purified leaves and stem bromelain was the highest, the purified extracts from these two samples were used to determine their antimicrobial effect on the pathogens causing periodontitis. In the case of gram positive bacteria the pineapple leaves and stem bromelain did not show any antibacterial effect on *E. Faecalis* and *S. mutans,* but enhanced the activity of the antibiotics ampicillin against *E. faecalis* and chlorohexidine against *S. mutans* (**Table 10**).The purified leaves and stembromelain at 100 % concentration showed antibacterial activity against gram negative *A. Actinomycetemcomitans* and *P. gingivalis,* but did not have any effect on *S. marcescens* at any concentration.

Thus the result clearly indicates that the bromelain inhibits the growth of the pathogens *A. Actinomycetemcomitans* and *P. gingivalis* causing periodontal diseases. The minimum inhibitory concentration (MIC) of leaves and stem bromelain was found to be 0.024 mg/ml and 0.048 mg/ml respectively and can be conveniently used as an alternative medicine for antibiotics. The inference drawn from the present study clearly indicates that bromelain from the leaves and stem of *Ananas comosus* enhanced the activities of the antibiotics namely amoxicillin against gram negative *A. Actinomycetemcomitans* and *P. Gingivalis* and ciprofloxacin against *S. marcescens*, hence it could be given to the patients suffering from periodontitis along with antibiotics to enhance its activity in the case of those resistant to antibiotics.

CONCLUSIONS: The present study revealed that purified bromelain of the leaves and stem showed more proteolytic activity than the pulp and peel. The proteolytic activity of the pure enzyme increased as the concentration of enzyme decreased, due to the removal of other endopeptidase enzymes such as ananain and comosain initially present in the crude extract. The study also showed that bromelain had less effect on the gram positive bacteria than the gram negative bacteria. According to the present study purified bromelain can be conveniently used in the medical field as an alternative medicine especially in the field of dentistry. To validate the result obtained in this study more clinical trials will have to be conducted.

ACKNOWLEDGEMENT: We wish to thank Aristogene Biosciences Pvt Ltd Bangalore for providing the required equipments and assistance during this study.

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How to cite this article:
Aravind KV and Gokulakrishnan M: Extraction, Purification of Bromelain from Pineapple and Determination of Its Effect on Bacteria Causing Periodontitis. Int J Pharm Sci Res 2015; 6(12): 5284-94.doi: 10.13040/IJPSR.0975-8232.6(12).5284-94.