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Full Length Research Paper

Isolation, partial purification and characterization of antifungal trypsin inhibitor protease from the seed of *Blighia sapida* K. D. Koenig (*Ackee*)

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Seed proteins have been evaluated for their nutritive value and biological activity. Proteases and proteinase inhibitors have been of immense benefit to both agriculture and therapeutic purposes. The seed proteins of *Blighia sapida* was evaluated for both structure and biological activity in this study. The matured dried seeds were pulverized and sequentially extracted using 10 mM Tris/HCl pH 7.4, 10 mM ammonium acetate and 10 mM sulphuric acid. Crude protein extracts were concentrated and the protein concentrations were estimated. Proteins were purified by 70% ammonium sulphate precipitation, Sephadex G50 reversed phase chromatography and finally by HPLC on a C18 column. Two bands were obtained from SDS-PAGE electrophoresis and they were identified by ESI/MS using in gel tryptic digestion. The seed protein from *B. Sapida* consists of two single polypeptide chains each with mass of about 24 to 27 KDa as established by a combination of SDS-PAGE and ESI/MS. Proteins exhibited protease activity, which was confirmed by zymography. Protease activity was characterized for effect of temperature, pH, divalent metal ions and chelating agents. The crude protein from the seed of *B. sapida* showed antimicrobial activity and the antifungal activity was comparable with the reference drug, Ticonazole.

Key words: *Blighia sapida,* chromatography, protease activity, in-gel trypsin digestion/mass spectrophotometry, antimicrobial activity.

INTRODUCTION

Ackee, the national fruit of Jamaica, is a food staple in many Jamaican diets (Sharma et al., 2009). The ackee tree is a tropical evergreen tree that can grow as tall as 40 feet. Its leaves are broad and pinnate; it's approximately 10 cm wide, 100 g of the fruit may be colored anywhere from straw to bright red. *Blighia sapida* is a woody perennial multipurpose fruit tree species native to the Guinean forests of West Africa. The fruit

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splits open while still on the tree to reveal 3glassy black seeds surrounded by a thick, oily, yellow aril. An association between ackee poisoning and Jamaican vomiting sickness was made in 1875 with a water-soluble toxic material in the seed and pods of the ackee fruit (Joskow et al., 2006; Moya, 2001; Gaillard et al., 2011). Hassal and Reyle (1954) were the first to isolate two toxic compounds in their crystalline form from the unripe fruit and the compounds were called hypoglycin A and B because of their hypoglycemic activity. The fruit should be allowed to open and ripen naturally on the tree as fresh arils of the ripened fruits are edible but the unripe fruit is not. *B. sapida* (Ackee) a known fish poison and the hypoglycin A and B have been identified as the toxicant in the seed (Bowen-Forbes and Minott, 2009; Moya, 2001; Natalini et al., 2000). The fruit is rich in essential fatty acids, vitamin A, zinc, and protein (Oladiji et al., 2009).

Commonly, seeds are evaluated for their suitability for nutritional and probable anti-feedant properties. Plants contain a variety of proteins which are resident in vacuoles and it includes storage proteins, hydrolases, proteases and α-amylase inhibitors as well as enzymes involved in plant defense (Hermann, 1994). Majority of proteinase inhibitors studied in plant kingdom originates from three main families namely leguminosae, solanaceae and gramineae (Richardson, 1991).

Leguminosae seeds contain high amounts of protein, a small portion of which consist the inhibitors that suppress proteolytic activity either *in vivo* or *in vitro*. Proteinase could be inhibited by various compounds including proteins by the formation of stable stoichiometric complexes, thus inhibiting their activity, and preventing proteolysis (Laskowsky and Kato, 1980). The presence of proteinase inhibitors in plants and seeds frequently accounts for the low nutritive value of uncooked vegetarian food (Liener, 1996). Studies have shown that human populations which are known to consume food with high concentration of proteinase inhibitors in their diet have lower rates of colon, breast, prostate and skin cancers. The protein proteinase inhibitors are divided into families according to the class of proteolytic enzymes inhibited, extensive sequential and structural homology among the members, the locations of disulfide bridges and the reactive site. The serine proteinases are the most widely studied (Macedo and Xavier-Filho, 1992; Macedo et al., 2002; Macedo et al., 2004). Various legume proteinase inhibitors have been classified as Kunitz-type, Bowman-Birk-type, potato I, potato II, squash, cereal super family, thaumatin-like and Ragi AI inhibitors (Richardson, 1991). Seed storage proteins often are not just protein stores for germination requirement and food for man alike but could provide a defense mechanism to protect the seeds from pathogen invasion. Many seeds such as *Adenanthera pavonina, Benincasa hispida, Areca catchu, Capparis spinosa* their seed proteins have been characterized as well as biological activity against common bean weevil and HIV-I reverse transcriptase activity (Macedo et al., 2004; Lam and Ng, 2008; Shih et al., 2001; Kusumoto et al., 2006). Preliminary study revealed potent antimicrobial and antifungal activity from the seeds and these considerations have led us to purify and characterize the seed proteins from *B. sapida*, which to the best of our knowledge has not been previously characterized.

MATERIALS AND METHODS

Chemicals

All chemicals used were either of analytical grade or the highest available purity. All solutions were prepared in MilliQ Water (Millipore, Bedford, MA, USA); EDTA (ethylenedinitrilo tetraacetic acid disodium salt) from Merck, Darmstadt, Germany; BIS (*N*, *N*-methylenebis-acrylamide), and acrylamide was obtained from Sigma (St. Louis, MO, USA); ammonium per sulfate (APS); bromophenol blue (3,3,5,5-tetrabromophenolsulfonephthalein); tetramethylethylene diamine (TEMED); 2-mercaptoethanol; glycerol, sodiumdodecyl sulphate (SDS), DL-dithiotreitol were purchased from Sigma as well. Crystallized and lyophilized trypsin, tris (hydroxymethyl) aminomethane hydrochloride (tris-HCl), ammonium bicarbonate, agar, divalent salts were from Sigma; all other reagents used were of the highest purity grade. Nutrient broth 'E' (Oxiod, England), Tryptone Soya Broth (Merck, Germany). Nutrient Agar, Sabouraud Dextose Agar and Sabouraud Dextrose Broth are all products of Becton Dickinson and company, U.S.A.

Microorganisms

Microorganisms used are *Staphylococcus aureus* (ATCC 13709), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (Clinical isolate), *Escherichia coli*, *Candida albicans* (ATCC 10231) and *Aspergilusniger.*

Seed collection and extraction

B. sapida seeds were harvested from the Botanical garden, University of Ibadan and were identified by Mr D Esimekhuai in the Herbarium of Botany Department, University of Ibadan, Ibadan, Nigeria. Seeds were air dried in Nutritional/Industrial Biochemistry laboratory in University of Ibadan and sorted to homogeneity. Air dried seeds were milled with coffee grinder and stored in air tight bags until required. Fifty grams of the matured dried seeds of *B sapida* were pulverized and was sequentially extracted by cold maceration for 24 h with 10 mM Tris /HCl pH 7.4, 10 mM ammonium acetate and 10mM sulphuric acid.

Protein concentration determination

The protein concentration was measured spectrophotometrically at 280 nm for the 3 fractions obtained from chromatographic separations or total protein concentration in the eluted fractions as well as the purified protein was determined using the method of Bradford 1976 with Bovine serum albumin (BSA) as standard.

Purification and fractionation of crude proteins

Crude proteins from the extractions were concentrated on Bucchi

Rotavap 114 at 30°C to reduce volumes to about 30ml. This was followed by ammonium sulphate precipitation (70%) and precipitates were separated by centrifugation at 10,000 rpm for 20 min at 4°C. Residues were dissolved using a few mills of 10mM ammonium acetate buffer containing 0.001% sodium azide and stored at -20°C. Crude fractions from ammonium sulphate precipitation was separated on Sephadex G50 on a Flex column 2.5 by 100 cm, the column using 10mM ammonium acetate buffer containing 0.001% sodium azide as running buffer. Flow rate was maintained at 1 ml/8min using a fraction collector and the eluent was monitored at 280 nm spectrophotometrically on GBC UV/V is 920 instruments to obtain chromatogram. Chromatogram from size exclusion sephadex G50 separation was used to obtain the different fractions; peaks 1, 2 and 3 from the three different extractions from *B. sapida* seeds and these were pooled together and concentrated using rotary evaporator.

Final purification of the seed protein was achieved by subjecting the protease active fraction from sephadex G50 separation to HPLC using C18 (5 µm, 46 × 250 mm; Vydac, the Separation Group, Inc., USA) Zymographic determination reversed phase column. The elution was performed under the following conditions: eluent A, 0.1% TFA in water (v/v), eluent B 100% acetonitrile and 0.05% TFA. Gradient program was 5% B for5 min, 0 to 60% B in 20 min followed by 100% B for 25 min on Agilent technologies 1200 series instrument. The flow rate was maintained at 1 ml/min and absorbance was monitored at 230 nm. Aliquots were taken from each purification stage for both Bradford protein concentration estimation and SDS/PAGE electrophoresis.

SDS polyacrylamide gel electrophoresis

The purity and molecular mass of the different fractions of partially purified enzyme and crude seed protein was established by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). The separating gel (10%) was prepared by mixing 2.0 mL deionized water, 1.25 mL Tris-HCl (buffer 1.5 mol/L, pH 8.8), 50 *µ*L SDS (10% (w/v)), 1.66 mL acrylamide (30% (w/v))/bismethylenacrylamide (0.8% (w/v)), 25 *µ*L APS (10% (w/v)), and 15 *µ*L TEMED. This gel was polymerized for 45-60 min at room temperature. Next the stacking gel (4%) was prepared by mixing 1.195 mL water, 0.5 mL Tris-HCl (0.5 mol/L, pH 6.8), 20 *µ*L SDS (10% (w/v)), 0.26 mL acrylamide (30%) and bis-methylenacrylamide (0.8%), 15 *µ*L APS (10% (w/v)), and 10 *µ*L TEMED. These gels were polymerized within 35-45 min. After 5 *µ*L of pure protein samples and 15 µL of impure protein extracts were heated at 100°C for 5 min and applied electrophoresis was run at 150 V over the gel. Finally the gel was stained by a solution of Coomassie brilliant blue (0.25% (w/v)) and destained in (10% acetic acid, 30% methanol and 60% deionized water (v/v) destaining solution overnight and gels in gel storage packs were scanned using 3 in 1 (2050 Hewlett-Packard printer).

Zymography

of the protease activity was done as above except for the incorporation of 1% gelatine in the gel as described by Lacks and Springhorn (1980) and samples were only dissolved in sample diluting buffer without heating. Briefly after electrophoresis, the gel was washed with 2.5% Triton-X 100 (3 × 20 min) with continual shaking to remove SDS followed by washing with water and incubation for 48 h at 37°C in activation buffer (25 mM Tris-HCl buffer added 5 mM $CaCl_2$ of pH 7.4). Finally, the gel was stained with coomassie gel staining dye and destained with 10% acetic acid and 30% methanol solution to visualize the clear bands of proteolysis against the dark background.

Protease agar plate /trypsin inhibitory assay

Method of Pfleinderer and Krauss (1965); Scumacher and Schill 1972 were used. Briefly, 0.5g of casein was dissolved in 25 mL of 50 mM Tris-HCL buffer pH 8.0 to which 1 mM calcium chloride and 0.01% sodium azide has been added with gentle stirring for 1 h, solution was made up to 50 ml with the pH adjusted to 8.0. 1 g of agar was heated in 25 ml of casein buffer in the microwave for about 50 s, it was cooled to about 50-60°C and mixed with casein solution (1:1) and poured into petri dishes and degassed to solidify in a Laminar Flow hood. Wells were formed using sterile cork borer.

Protein extracts at different concentrations were added to the wells and incubated at 37°C for 24 h for agar plate protease assay while trypsin and plant extracts was added to the wells for trypsin inhibitory assay. At the end of incubation agar plates are exposed to saturated ammonium sulphate (1M) and zone of white precipitate indicates catalytic activity. In the trypsin inhibitory assay difference in diameter between well of trypsin alone and that with plant extract was used to calculate inhibition from Trypsin activity unit (au) of 1645 in 1ml/mg.

Enzyme activity assay

Spectrophotometric protease activity of crude protein extract and partially purified protein fraction was done by slight modification of Kunitz and McDonald (1946). Briefly, 20 µg of crude protein, 100 µL 1% (w/v) Casein was dissolved in 0.1 M tris –HCl pH 8.0 buffer. The reaction mixture was incubated at 37°C for 30 min in Gefran 500, temp control unit and the reaction was stopped by adding 200 µL of 40% Trichloroacetic acid (TCA). Blank was prepared for each sample in a similar manner except that the 20 µg of crude protein was added after reactionhas been quenched with 40% TCA.The samples were centrifuged at 14,000 rpm in Eppendorf mini spin plus for 10 min and absorbance of TCA soluble peptides was measured at 280 nm. Activity unit was calculated using the difference in absorbance between sample and blank, and all measurement were done in triplicate and average values were used.

Effect of temperature and pH on protease activity

The effect of temperature on protease activity was determined by incubating the enzyme reaction mixture at different temperatures ranging from 37 to 90°C. Reaction mixture was 20 µg of respective crude protein, 100 µL 1% (w/v) Casein 0.1 M tris –HCl pH 8.0 buffer and was at 37, 45, 50, 60, 70, 80 and 90°C for 30 min in Gefran 500, temp control unit. The reaction was stopped by addition of 200 µL of 40% Trichloroacetic acid (TCA). Blanks were similarly prepared for each sample, incubated at the different temperatures, except that the 20 µg of crude protein was added after reaction has been quenched with 40% TCA. Samples were centrifuged at 14,000 rpm in Eppendorf mini spin plus for 10 min and absorbance of TCA soluble peptides was measured at 280 nm. Protease activity unit at the various temperatures were calculated using the difference in absorbance between sample and blank. Each measurement was done in triplicate and average values were used to obtain protease activity at the different temperatures.

The effect of pH on protease activity was determined by testing for protease enzyme activity using standard laboratory method earlier described except that 100 µL of 1% (w/v) Casein was prepared in 0.1 M buffers of different pH values.The buffers used were 50 mM Glycine-HCl buffer (pH 3.0), sodium acetate buffer (pH 4.0 to 5.0), sodium phosphate buffer (pH 6.0 to7.0), Tris-HCl buffer (pH 8.0 to 9.0) glycine –sodium hydroxide buffer (pH 10.0 to 11.0). Triplicates of reaction mixtures and the corresponding blanks (without) crude proteins were incubated for 30 min at 37°C and the assay was

continued as earlier described for protease activity determination.

Effect of metal ions and chemical agents

The protease activity was then measured as earlier described and percentage inhibition was calculated by comparison with optimum conditions. Divalent metal ions such as Sn, Mg, Pb, Fe, Mn, Cu and Co were used as the chloride salt to determine effect of metal ions. Metal chelator such as EDTA, sulphydryl agent ditthiothreitol and detergents such as triton-x, sodium dodecyl sulphate and urea were used for effect chemical agents. The effect of metal ions and chemical agents on protease activity was determined by incubating the enzyme with 1 mM of the respective chelating agent or metal ion for 30 min at 50°C. Briefly, assay mixture was prepared using 20 µg of respective crude protein, 100 µl 1% (w/v) Casein 0.1 M tris –HCl pH 8.0 buffer, 1 mM of the metal ion or chemical agent and incubating at 37°C for 30 min in Gefran 500, temp control unit. Reaction blank was similarly prepared except that the enzyme was added after reaction had been stopped using TCA and reactions were continued as earlier described for spectrophotometric protease activity measurement.

HPLC purification

Final purification of the seed protein was achieved by subjecting the protease active fraction on C18 (5 µm, 46 × 250 mm; Vydac, the Separation Group, Inc., USA) reversed phase column on Agilent technologies 1200 series instrument. The elution was performed under the following conditions:

Eluent A, 0.1% TFA in water (v/v), eluent B 100% acetonitrile and 0.05% TFA. Gradient program was 10% B for5 min, 0 to 100% B for 15 min.

The flow rate was maintained at 1 ml/min and absorbance was monitored at 254 nm. Representative HPLC chromatogram for BS Tris first peak is shown in Figure 3a, all *B sapida* extracts from Tris, Amac and Sulp had similar chromatographic profile except the observed decrease in the peak at 8 to 10 min possibly due to decreased concentration from subsequent extraction from the same starting see powder

In gel trypsin digestion / mass spectrometry

All gel pieces were cut into smaller cubes, the gel pieces were digested with 20 µL of a 10 ng/µL trypsin solution at 37°C overnight. The resulting peptides were extracted twice with 70% acetonitrile in 0.1% formic acid for 30 min, and then dried and stored at -20°C. Dried peptides were dissolved in 5% acetonitrile in 0.1% formic acid and 10 µL injections were made for nano-LC chromatography.

All experiments were performed on a Thermo Scientific EASY-nLC II connected to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nano-electropsray source. For liquid chromatography, separation was performed on an EASY-Column (2 cm, ID 100 µM, 5 µM, C18) pre-column followed by an EASY-column (10 cm, ID 75 µM, 3 µM, C18) column with a flow rate of 300 ml/min. The gradient used was from 5 to 40% B in 20 min, 40 to 80% B in 5 min and kept at 80% B for 10 min. Solvent A was 100% water in 0.1% formic acid, and solvent B was 100% acetonitrile in 0.1% formic acid. The mass spectrometer was operated in data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Data were acquired using the Xcalibur software package. The precursor ion scan MS spectra (*m/z* 400 – 2000) were acquired in the Orbitrap with resolution R = 60000 with the number of accumulated ions being 1 × 106. Data analysis was done using Thermo Proteome Discoverer 1.3 (Thermo Scientific, Bremen, Germany) were used to identify proteins via automated database searching (Mascot, Matrix Science, London, UK) of all tandem mass spectra against the Swissprot 57.15 databaseof more than $p<0.05$ and peptides of high and medium confidence as determined by Proteome Discoverer.

Antimicrobial assays

The agar diffusion method was used (Reeves et al., 1978). 0.1ml of 1 in 100 dilution of the overnight broth culture of each organism was used to seed sterile molten nutrient agar maintained at 45°C. The plates were allowed to solidify and six wells (8 mm diameters) were made in the seeded plates. Different dilutions of the crude *B. sapida* seed protein ethanol extract (400, 200, 100, 50 mg/ml) were added into the appropriate wells. The plates were incubated at 37°C for 24 h; Ampicillin (10µg/ml) was used as positive control and dimethylsulphoxide (DMSO) was used as negative control. Antifungal tests were performed in a similar manner seeded with fungi hyphae in sabouraud dextrose agar petri- dishes. A suspension of the organism (fungi) was prepared by inoculating a small amount into Typtone Soya Broth (TSB). Tioconazole ('Trosyd' 5mg/ml) was used as reference antifungal drug. All plates were subsequently incubated at room temperature for 48 h (Reeves et al., 1978; Cowan, 1974). The diameter of zone of inhibition was measured as an indication of activity.

RESULTS

Fifty gram *B. sapida* on extraction with 500 ml each of buffer, concentration and ammonium sulphate precipitation yielded the following:

10 mM Tris buffer PH 7.4 total volume 32 ml (3.027 mg/ml); 10 mM Ammonium acetate total volume 10 ms^{-1} (0.998 mg/ml) and 10 mM Sulphuric acid gave total volume 10 ml (2.046 mg/ml).

Impure protein from the three extracts from 50 g BS seed was 127.304 mg (0.2546%) percentage yield. Obtained chromatograms from size exclusion chromatography are shown as (Figures Ia, b and c) while the protein concentrations of the concentrated pulled chromatographic fractions are shown on Table 1. Separation with the sephadex G50 size exclusion yielded 60.181mg (47.27%) and about 2 fold purification. Similar chromatogram was obtained from OD_{280} measurements for BS Tris, BS amac and BS sulpsize exclusion chromatography using sephadex G50 as shown (Figures Ia,b and c) from which we obtained 3 peaks each. Preliminary screening using the agar plate assay for protease activity showed test tube 32 had highest protease activity, followed by 30 and 25, whiletest tube 51 showed minimal activity andtest-tubes 95, 92, 81 and 61 showed no activity.

SDS-PAGE electrophoresis bands from the crude *B. sapida* extract was rather broad but better bands were obtained after size exclusion chromatography and desalting (Figure 2a). All three peaks of *B. sapida* from

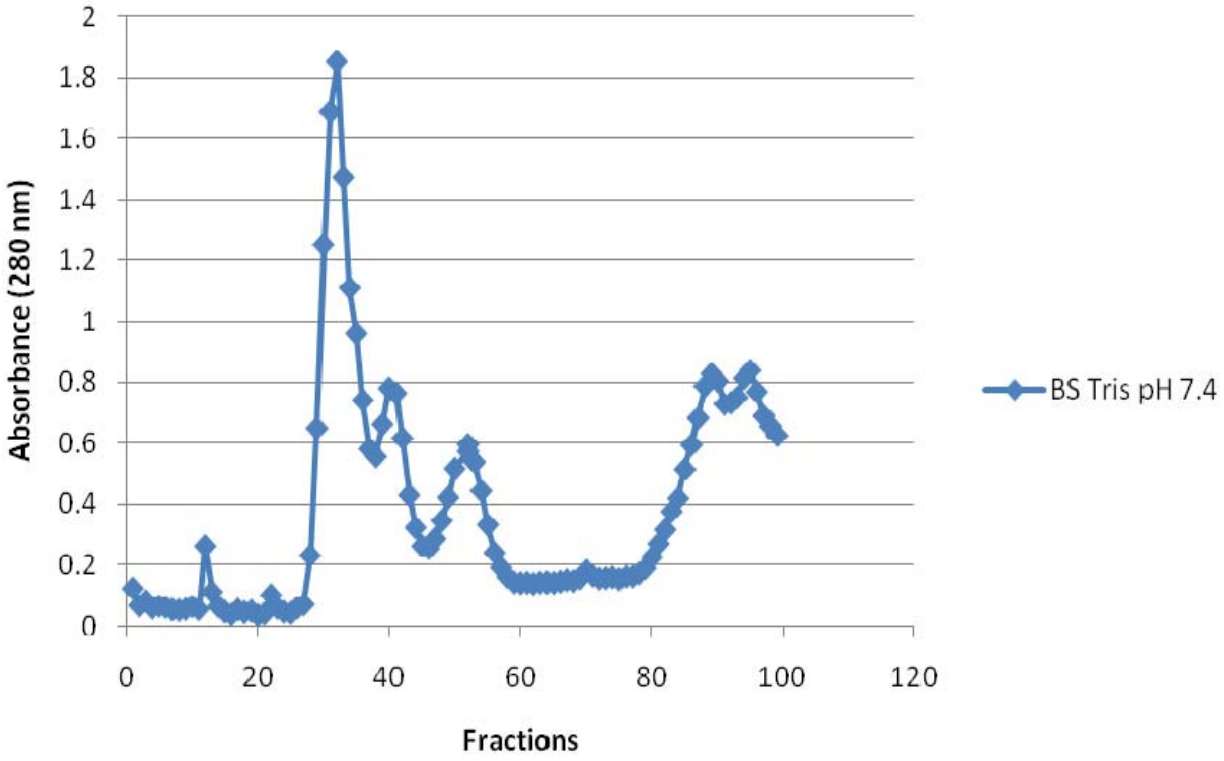

Figure Ia. Chromatogram for the sephadex G50 separation of *B. sapida* 10 mMTris /HCl pH 7.4 extract on Flex column 2.5 by 100 cm and flow rate of 1 mL/8min monitored at 280nm.

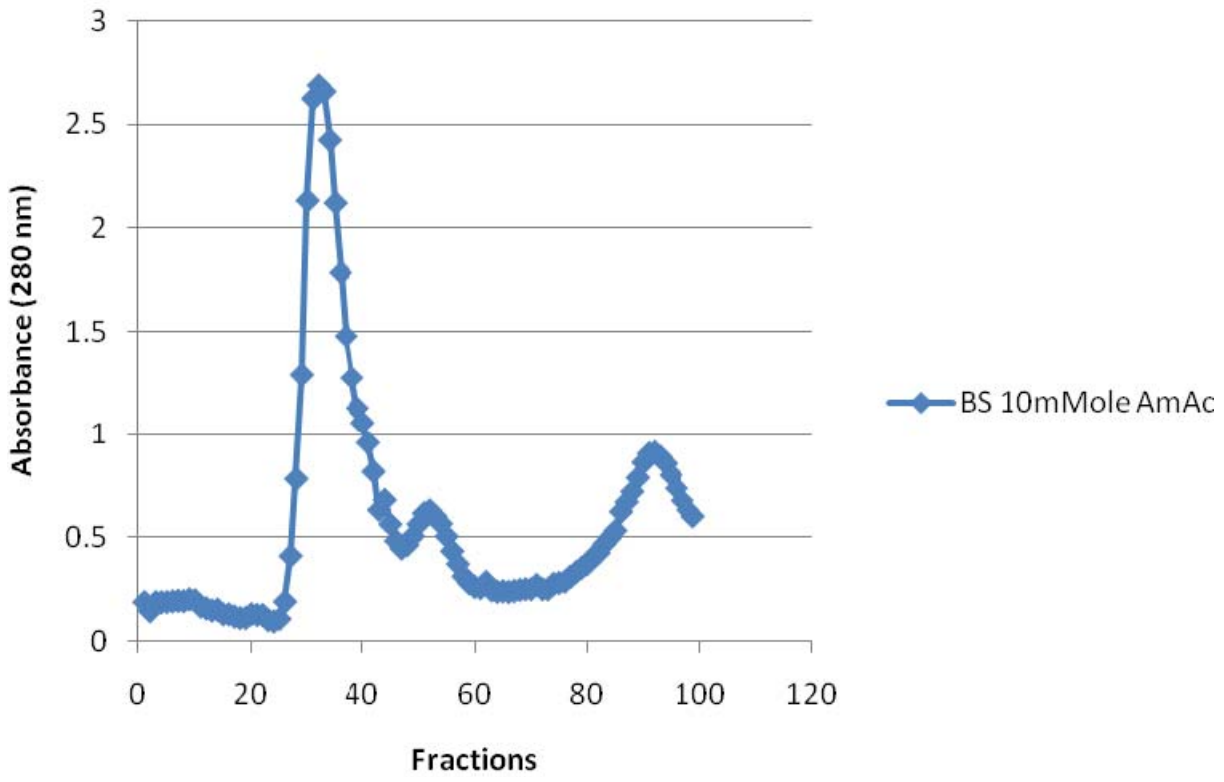

Figure Ib. Chromatogram for the sephadex G50 separation of *B. sapida*10 mM ammonium acetate extract on Flex column 2.5 by 100 cm and flow rate of 1 mL/8min monitored at 280 nm.

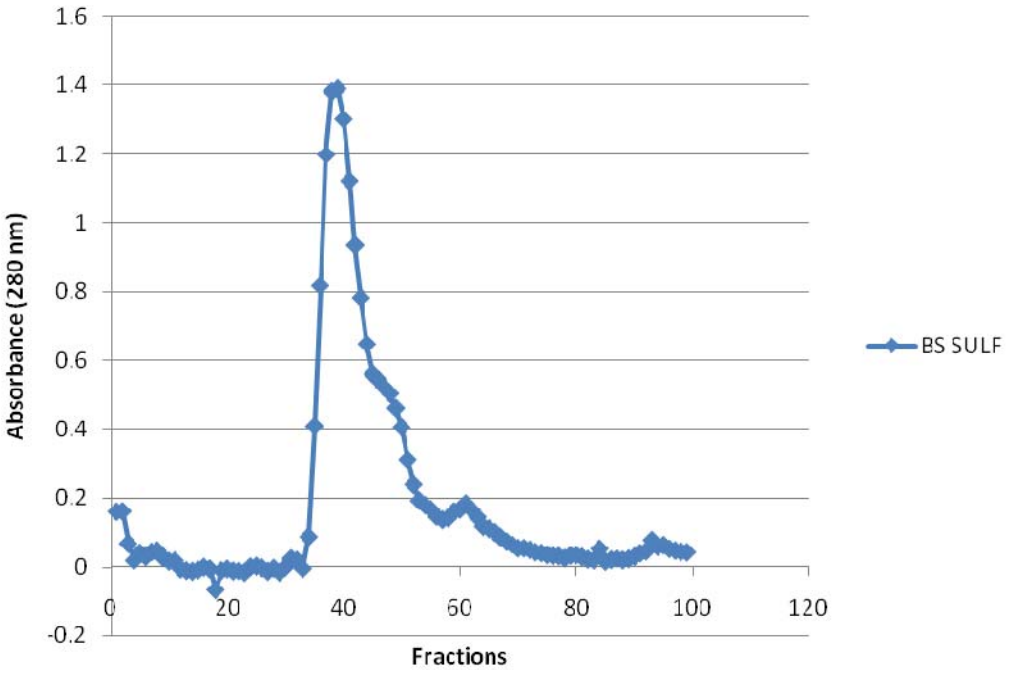

Figure Ic. Chromatogram for the sephadex G50 separation of *B. sapida* 10mM sulphuric acid extract on Flex column 2.5 by 100 cm and flow rate of 1 mL/8min monitored at 280 nm.

Table 1. Protein concentration estimations of size exclusion chromatography fractions using sephadex G50.

Peak	Amount of protein (mg)		
	10 mM Tris	10 mM Amac	10 mM sulp
1st	20.406	2.40	5.49
2nd	15.625	1.60	3.75
3rd	7.48	1.75	1.68
Total protein	43.511	5.75	10.92

sephadex G50 on SDS/PAGE gave two similar bands (Figure 2b) of about 20 to 30 KDA using marker proteins. The purity and molecular mass of the different fractions as well as purified enzyme was established by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Likewise, zymographic determination of the protease activity was established by light patch in the dark background of the 1% gelatingel as shown in Figure 2c. The nine chromatographic fractions (ie peaks 1, 2 and 3) from Tris, Amac and Sulp extracts from sephadex G50 (Figures Ia-c) where further purified using HPLC and we observed similarprofile for the fractions except decrease in the peak height of eluent at 9-10 and 14.7-15.3 min, which may possibly be due to decreased concentration from subsequent extraction from the same starting seed powder and purification processes. Representative HPLC chromatogram for BSTris first peak and BSsulp 3rd peak are shown in Figures 3a and b. In-gel mass spectrophotometry of the two bands from SDS/PAGE electrophoresis is shown on Figures 4a and b respectively.

Trypsin protease inhibitory agar plate assayed shows that the partially purified protease inhibited trypsin activity in a concentration dependent manner, and protease activity of extract only decreased from BS Tris> BS Amac> BS sulpas shown on Table 2 from diameter measurement of zone of inhibition. BS Tris extract was virtually inhibited by all divalent metals used in this study except Cu^{2+} with on 6.67% activity while BS Amac and BS Sulp had varying activity except with Co ions where protease activity was totally absent (Table 3a). Metal chelator, sulphydryl reagents and detergents had varying effect on protease activity (Table 3b). Optimum temperature and pH for protease activity were found to be 50°C and pH 5.0 respectively for BS Tris extract (Tables 3c and d). *B. sapida* crude extract showed

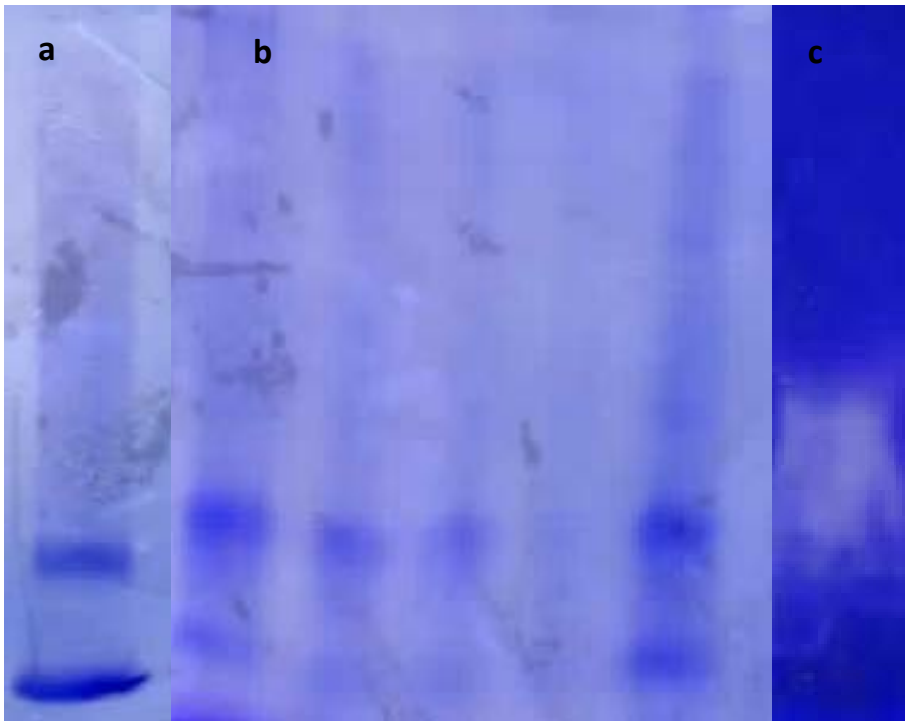

Figure 2. a) Examination of crude protein from the Sephadex G-50 run; b) SDS –PAGE electrophoresis showing Tris 1st, 2nd, 3rd peaks and Amac 1st and 2nd peaks from Sehpadex G50 separation; c) SDS-PAGE zymography of 1st peak of Tris extract with light patch showing zone of protease activity.

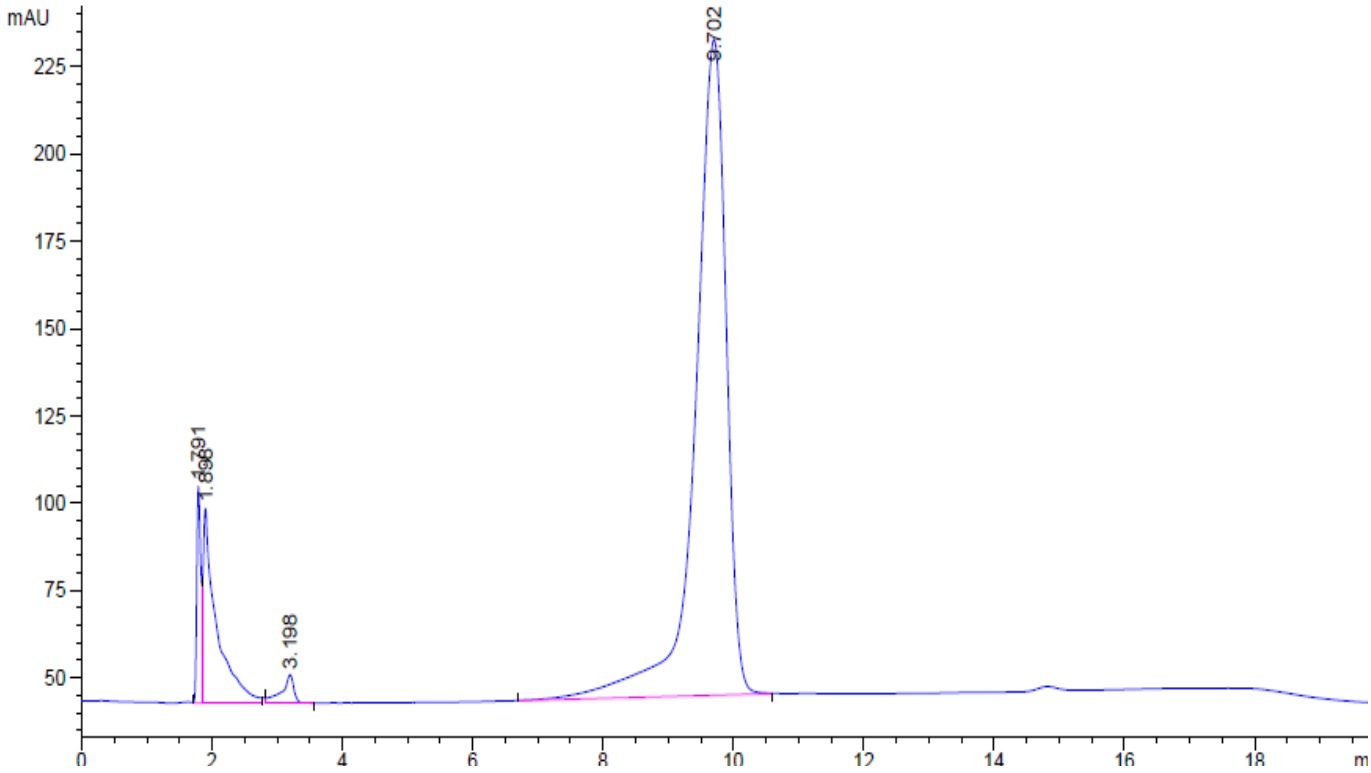

Figure 3a. HPLC chromatogram for the separation of *B.Sapida* 10 mMTris/HClIst peak fraction obtained from sephadex G50 size exclusion chromatography.

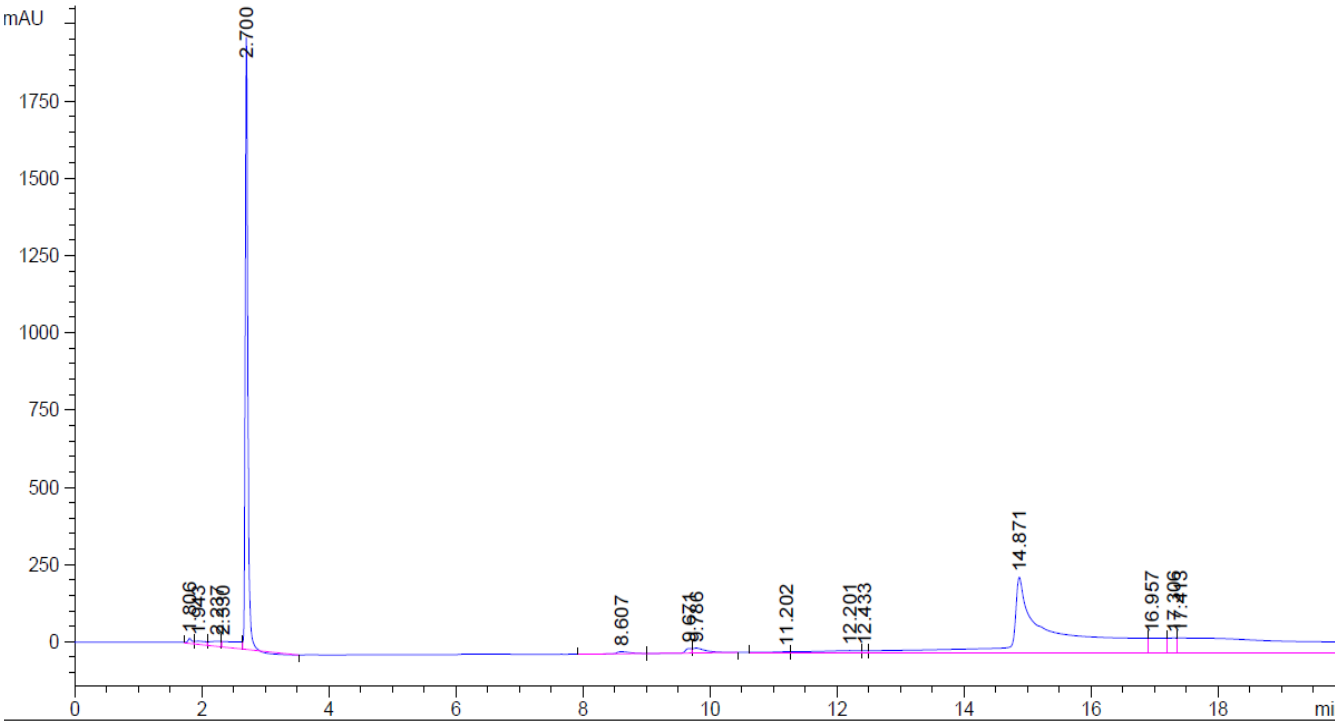

Figure 3b. HPLC chromatogram for the separation of B. *Sapida* 10 mMsulphuric acid 3rd peak fraction obtained from sephadex G50 size exclusion chromatography.

Table 2. Trypsin agar plate inhibitory assay of the crude protein extract of Blighia sapida.

Well content	Diameter zone of inhibition (cm)	Activity unit(au) calculated from % inhibition	
		10µg	20µg
Trypsin only	0.8 0	64.63	78.57
BsTris + Tryps	0.55	54.05	65.71
BsAmac + Trysin	0.46	37.60	45.71
BsSulp + trypsin	0.32		
BsTris (20µg)	0.60		
BsAmac (20µg)	0.48		
BsSulp (20µg)	0.35		

Diameters of zone of inhibition was measured from edge of well to periphery of white casein precipitated zone on the addition of saturated ammonium sulphate solution.

Table 3a. Effect of different divalent metal ions onspectrophotometric protease reactivity at 37°C.

Metal ion	Relative percentage protease activity (%)		
	BsSulp	BsTris	BsAmac
Sn	103.3	ND	82
Pb	80.83	ND	25
Mg	ND	ND	76.6
Fe	ND	ND	100
Cu	116.67	6.67	100.56
Co	ND	ND	ND
Mn	ND	ND	15.5

Table 3b. Effect of chemicals agents and chelators on protease activity (%) of *B. sapida* extracts from sephadex G50 separation.

Agent	T^1	T^2	T^3	A^1	A^2	A^3	S^1	S^2	S^3
EDTA	0	24.67	66.67	63.3	55.6	70.3	68.3	0	0
Urea	0	0	0	0	0	0	66.6	51.6	0
Dithioerythrol	83.3	0	0	0	82.2	0	0	0	0
Triton – X	0	0	0	0	4.84	0	4.8	0	30
SDS	0	0	34.6	76.7	75.5	51.1	61.6	0	50

Table 3c. Effect of temperature on*B.sapida* 10mM Tris/HCl sephadex G50 separated seed protease activity

Temperature in °C	Relative activity
37	0.018
45	0.02
50	0.026
60	0.015
70	0.014
80	0.005
90	Nd

Table 3d. Effect of pH on *B. sapida* 10 mM Tris /HCl sephadex G50 separated extract seed protease activity

pH	Protease activity
3	0.005
4	0.0165
5	0.0275
6	0.017
7	0.015
8	0.018
9	0.014
10	0.008
11	0.005

minimal antibacterial activity in the agar plate dilution assay which was concentration dependent (Table 4a) but the antifungal activity of the seed protein was comparable with the reference drug (Table 4b).

DISCUSSION

B. sapida seed proteins exhibited proteolytic activity in both agar plate assay, protease activity assay (spectrophotometric method) as well as exhibiting concentration dependent inhibition of trypsin. Protease activity for all nine fractions decreased slightly from 37 to 45°C before increasing from 50 to 80°C, above this temperature there was loss of activity (Table 3c). *B. sapida* exhibited protease activity over pH range 4.0 to 10.0 and above this pH there was loss of activity possibly due to denaturation (Table 3d). There was variation from decreased protease activity to non-detectable protease activity in the spectrophotometric assay using divalent metal ions such as Sn, Pb, Mg, Fe, Cu, Co and Mn by extracts of *B sapida* from Tris, Amac and Sulphuric acid fractions (Table 3a). However there was a slight increase in protease activity in the presence Cu ions BS amac (100.56%), BSsulp (116.67%) and Sn ions BSsulp (103.3%). Metal chelators such EDTA, sulphydryl reagent dithioerytrol and detergents such as Triton-X, SDS and Urea had varying effects on protease activity of BS fractions from Sephadex G50 separation (Table 3b) from inhibition to non-detectable activity.

All serine inhibitor families from plants are competitive inhibitors and all of them inhibit proteinases with a similar standard mechanism (Laskowki and Kato, 1980). *B sapida* proteases show a pH maximum at 5 to 7 and were very stable having thermophilic temperature range of protease activity between 50 to 70°C as shown on Tables 3c and 3d. The optimum activity of cysteine proteinases is usually in the pH range of 5 -7, which is the pH range of the insect gut (Murdoc et al., 1987).Serine proteinases have been described inmany plant species, and are universal throughout the plantkingdom, with trypsin inhibitors being the most common type(Lawrence and Koundal, 2002) possibly due to thefact that (mammalian) trypsin is readily available and is theeasiest of all the proteinases to assay using syntheticsubstrates, and hence it is used in screening procedures. On Table 2, the crude protein isolated exhibited but protease activity and trypsin inhibitory activity too. Such studies have provided a basic understanding of the mechanism of action (Huber and Carrel, 1989) that applies to most serine proteinase inhibitor families and probably to the cysteine and aspartyl proteinase inhibitor families as well.

Two analytical techniques are primarily employed in current proteomic research: two-dimensional (2D) gel electrophoresisforthe separation and visualization of proteins in crude extracts, and mass spectrometry (O'Farrel, 1975; Fenn et al., 1989) for the identification and characterizationof the separated proteins. SDS-PAGE electrophoresis, the proteins are separatedaccording to their molecular weights and a large numbers of proteinscan be separated and characterized by automated matrix-assisted laser desorption/ionization time-of-flight mass spectrometric

Table 4a. Antibacterial activity of the crude seed protein of *Blighia sapida.*

Microorganism	*B. sapida* (mg/ml)				Reference compound (mg/ml)- Gentamicin
	A	B	C	D	
Staphylococcus aureus	10	12	12	14	34
Bacillus subtilis	10	12	14	16	34
Pseudomonas aeruginosa	-	-	-	10	34
Escherichia coli	10	10	12	14	36

Gentamicin (25 µg/ml), A = 0.1250 mg/ml, B = 0.25 mg/ml, C = 0.5 mg/ml, D = 1 mg/ml corresponding to 12.5%, 25%, 50% and 100% dilutions respectively.Diameter of zone of inhibition (mm) 10 – 15 = +; 16 – 20 = + +; >20 = + + + ; 0 – 8 = no inhibition .Diameter of cup = 8mm

Table 4b. Antifungal activity of the crude protein extracts of *Blighia sapida.*

Microorganisms	*B. sapida* (mg/ml)				Tioconazole (Reference drug)
	A	B	C	D	
Aspergillus niger	-	10	12	14	20
Candida albicans	-	12	14	16	20

Tioconazole (5 mg/ml) A =0.125 mg/ml, B = 0.250 mg/ml, C = 0.5 mg/ml, D =1 mg/ml.
Diameter of zone of inhibition (mm): 10 – 15 = +; 16 – 20 = + +; >20 = + + +; 0 – 8 = no inhibition, Diameter of cup = 8mm.

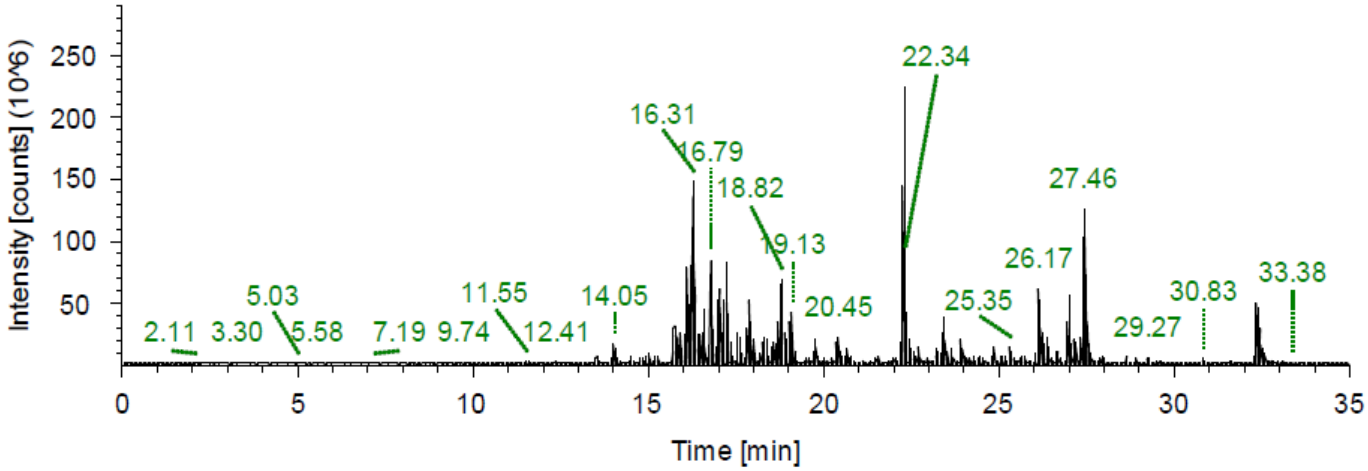

Figure 4a. In-gel trypsin digestion of the lower band of *B. sapida* was identified as Thaumatin like protein I peptide with 225 amino acid residues, 24.2KDA and pI of7.9

(MALDI TOF-MS) peptide mapping followed by extensive database searches (Henzel et al., 1993). In cases, wheremore structural information is required from the separated proteins, nano-liquid chromatography (LC)-electrospray ionization (ESI)-MS/MS is often employed (O'Farell 1975). Thedominant approach to proteomics includes theseparationof native proteins by 2-DE, their analysis bymass spectrometry (MS), and their identification basedon sequence information in databases.). Protein arrays provide a suitable sourceof a large number of recombinant proteins andthe storedMS information may be used to identify native proteinsfrom gels. The two bands from SDS/PAGE of *B. sapid*a crude seed extract were identified as Acidic endochitinase with 254 amino acid residues, mass of 27.6KDa, calculated isoelectric point of 6.02 and Thaumatin-like protein with 225 amino acid residues, mass of 24.2 KDa and a calculated isoelectric point (pI) of 7.9 (Figures 4a and b). This is in agreement with our data on effect of pH on protease activity especially as we only carried out partial

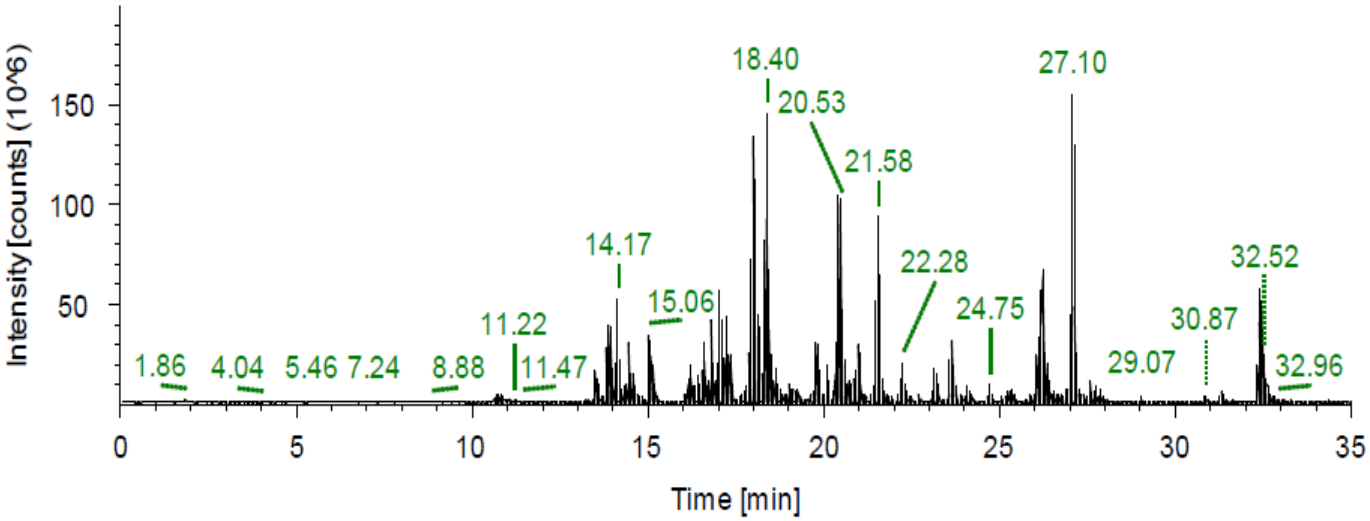

Figure 4b. In-gel trypsin digestion of the upper band of *B sapida* were identified as Acidic endochitinase with single peptide chain of 254 amino acid residues, KDA of 27.6, isoelectric point (pI) of 6.02.

separation. It might be necessary in future to collect the fractions from HPLC separations and identify the seed proteins individually. We observed antimicrobial activity in the crude seed protein, while the antifungal activity was approximately that of the reference drug Table 4b. This is not surprising as most seed proteins are not just present as seed storage proteins but protect the seed from pest and pathogens invasion.

In conclusion, we report the presence of two seed serine proteinase acidic endochitinase and Thaumatin-like proteins with mass of 27.2 and 24.2 KDa respectively in the seeds of toxic plant with promising antifungal activity. The seed is largely left to waste probably due to poisoning by hypoglycin A and B isolated from it but the protease and antifungal activity of the seed proteins might make for appealing industrial/technological application, especial as it retains proteolytic activity at high temperatures and over a wide pH range

Conflict of Interests

The author(s) have not declared any conflict of interest.

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