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Isolation, purification and characterization of a protease from the seeds of *Artocarpus heterophyllus*

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Proteases are being widely used in various industries like detergent, leather, food and pharmaceuticals.Protease was purified to homogeneity from the seeds of *Artocarpus heterophyllus*. The enzyme was found to be a tetramer having molecular mass of 74 kDa. Gelatin zymography showed a clear band of proteolysis. The enzyme isolated and purified was a serine protease, as indicated by its inhibition with PMSF. The enzyme was stable at broad pH and temperature ranges with pH and temperature optima at 8.5 and 50°C, respectively. The presence of some divalent ions enhanced the activity. With the addition of calcium, change in absorption and emission spectra was observed in spectrofluorometric analysis. The Km and V_{max} for the enzyme was found to be 0.229 µM and 0.014 µM min^{-1}, respectively, using BAPNA as a substrate. The enzyme consisted 4.44% alpha helix and 44.17% beta sheets when measured by CD spectra. Dynamic light scattering of the protease for particle size distribution revealed the mono-dispersity of the sample. Easy purification and paramount stability of protease makes it a good candidate for industrial and pharmaceutical applications.

Keywords: *Artocarpus heterophyllus*, BAPNA, CD spectra, Dynamic light scattering, Proteases, Serine endopeptidase

Proteolytic enzymes are intricately involved in many biological processes of plant life cycle like they play a central role in plant growth and development[1]. These proteases help in plant defence by producing hypersensitive response upon attack of any pathogens[2], by involving in the processes of plant's innate immunity[3] they generate an immune response[4]. Plant proteolytic enzymes also play indispensable role in plant germination by helping in proteolysis of the proteins accumulated in seeds[5]. Some proteases that are present in chloroplast play a key role in the maintenance of photosystem eventually helping in photosynthesis[6]. These proteases have a major function in programmed cell death and senescence signalling cascades[7]. Proteases are the proteins encoded by plant genome which are highly stable and can work in wide pH ranges and temperatures[8]. Plant proteases can be obtained from various plant sources, for instance seeds, roots, flowers, leaves, latex, *etc*. It has been used from ancient times as a folk medicine as anthelmintic, antitumor, antimicrobial, analgesic, antioxidant, anti-inflammatory, to clear skin infections, to enhance wound healing[9]. Hence, plant proteases has a vital role in therapeutics and this make them potential target for research in unexplored medicinal values[10]. Artocarpus tree is a member of Moraceae family, also named as mulberry family. Many different species of this genus are known and found in India and other continents of Southeast Asia. These are: *Artocarpus heterophyllus*, commonly known as jackfruit; *A. integer*, also known as cempedak*; A. camans*, known as bread nut and *A. altilis*, also known as breadfruit[11]. Although, no evidences have been found about industrial applications of Artocarpus proteases yet. Some studies have concluded that some plant proteolytic enzymes are very specific to their target or substrate. Purification and characterization of a protease from *Artocarpus heterophyllus* and delineation of its specific substrates is hereby being reported.

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Abbreviations: BAPNA, Na-Benzoyl-D,L-arginine 4-nitroanilide hydrochloride; CD, Circular dichroism; DLS, Differential light scattering; DMSO, Dimethyl sulfoxide; EDTA, Ethylenediamine tetraacetic acid; EGTA, Ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; PMSF, Phenylmethylsulfonyl fluoride; SEC, Size exclusion chromatography

Materials and Methods

Materials

Artocarpus heterophyllus seeds were procured from the local market in Indore (India). Ammonium sulfate, sodium dodecyl sulphate (SDS), was purchased from SRL, N-α-Benzoyl-DL-arginine þ-nitroanilide

(BAPNA), N-succinyl-Phe-p-nitroanilide, N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide, casein, gelatin, Diethylaminoethyl (DEAE)-cellulose 52, and pre-stained markers were purchased from Sigma-Aldrich. N,N,N,N-Tetramethylethylenediamine (TEMED), coomassie brilliant blue (CBB), Trichloroacetic acid (TCA) sulfuric acid, β-mercaptoethanol (β-ME), bromophenol blue, acrylamide, N, N-methylene-bisacrylamide, Dimethyl sulfoxide (DMSO), methanol, isopropanol, butanol, Triton X-100, Tween-80, Labolene and glacial acetic acid used were of analytical grade.

Methods

Seed extract preparation

Seeds were washed properly with distilled water prior and their seed coats were removed using a scalpel. Further seeds were soaked in 0.05 M Tris-HCl buffer pH 8 for 24 h. Soaked seeds were then homogenized in the same buffer using a mixer blender and stirred for 1 h on a magnetic stirrer to ensure proper mixing of the homogenate. Then it was centrifuged at 10000 rpm for 30 min to remove all insoluble materials and the supernatant was collected.

Purification of protease

The supernatant was subjected to 30% ammonium sulfate fractionation and then centrifuged at 10000 rpm for 20 min. The precipitate was stored in -20°C and the remaining supernatant was further precipitated with 30%-50% and then 50%-80% ammonium sulfate. The supernatant obtained after 30-50% ammonium sulfate fractionation was found to have highest protease activity as measured using BAPNA as substrate. This fraction was dialyzed against the 0.05 M Tris-HCl buffer pH 8 and was loaded on DEAE Cellulose-52 (21 × 60 mm) column which was pre- equilibrated with 0.05 M Tris-HCl buffer pH 8. The bound protein was eluted with a linear gradient of 0–0.8 M NaCl at a flow rate of 0.5 mL min^{-1}. Fractions of 3 mL were collected and their absorbance at 280 nm recorded. Casienolytic activity using casein as a substrate and amidolytic activity using BAPNA as a substrate was measured to confirm the presence of protease. The partially purified fractions with high protease activity were pooled and concentrated to 18 mg mL^{-1} by centrifugation using Centricon device (Amicon; molecular mass cut-off 10 kDa) at 3500 rpm. 0.3 mL of concentrated protein was further subjected to Mono Q Sepharose chromatography using Mono Q^{TM} 5/50GL column (5 × 50 mm) on the AKTA-pure system (GE Healthcare) which was pre-equilibrated with 0.05 M Tris-HCl buffer pH 8. After washing the unbound protein, the bound protein was eluted with a linear gradient of 0-100% 1 M NaCl at a flow rate of 1 mL min^{-1}. The peak portion was collected and absorbance was monitored, after which activity with BAPNA was examined in each peak.

Oligomeric status estimation of the purified protease

The protein isolated from ion exchange chromatography was concentrated and further subjected to size exclusion chromatography (SEC) on Superdex-200 10/300 GL column (10 × 300 mm) on the AKTA-pure system (GE Healthcare), calibrated with molecular weight standards: β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa) and blue dextran (2000 kDa). The elution volume (V_e) was divided by the void volume of the column (V_o) of different molecular weight markers and plotted against the Log10 of their molecular weight for the standard plot to estimate the molecular weight of SEC purified protein. The oligomeric status of SEC eluted protein (0.5 mg mL^{-1}) was determined by a dynamic light scattering (DLS) experiment by using a DLS instrument "Zetasizer µV" (Malvern) at 25°C. Apart from that, DLS analysis was also carried out to see the aggregation of the purified protease on increasing the temperature. Zeta average size or mean hydrodynamic size of the sample was observed for analysing the aggregation pattern of the protein.

Estimation of Protein Concentration

At every stage of purification of the protease protein concentration was measured by the method of Lowry *et al*[12] using BSA as a standard and absorbance at 280 nm was also recorded.

Protease activity assay

Caseinolytic activity

Caseinolytic activity of a protease was monitored using 1% casein as a substrate with certain modifications in the method given by Singh *et al*[2]. The reaction mixture consisted of protease solution (50 µg) in 0.5 mL of 0.05 M Tris-HCl buffer pH 8 and 0.5 mL of 1% (*w/v*) casein to make the final reaction volume of 1 mL. Mixture was incubated at 37°C for 30 min. The reaction was stopped by using 0.5 mL of 10% TCA, incubated further for 10 min at room temperature and centrifuged at 10000 rpm for 10 min. Absorbance at 280 nm of soluble peptides

present in the supernatant was noted. For control, a reaction without any enzyme was done to use as a blank while measuring absorbance. One unit of enzyme activity was defined as the amount of enzyme that increases the absorbance at 280 nm by 1 unit per minute of reaction in the given assay condition. The number of units of enzyme activity per milligram of protein was described as specific activity of the enzyme.

Amidolytic activity

N-α-Benzoyl-DL-arginine þ-nitroanilide (Trypsin substrate), N-Succinyl-L-phenylalanine-p-nitroanilide (Chymotrypsin substrate), N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide (Elastase substrate) were synthetic substrates used to assay amidolytic activity of the purified protease using spectrophotometer. BAPNA stock (1 mM) was prepared by dissolving in minimum amount of DMSO and volume was made up finally using the 0.05 M Tris-HCl buffer pH 8. The reaction mixture was prepared according to Dos Santos *et al*[13] with slight modification, it consisted of protease solution (50 µg) in final volume of 0.5 mL of 0.05 M Tris-HCl buffer pH 8 and 0.5 mL of 1 mM BAPNA to make the final reaction volume of 1 mL. Mixture was incubated at 37°C for 60 min. The reaction was terminated by adding 0.2 mL of 30% acetic acid and finally absorbance was taken at 405 nm. Similarly other two substrates were also made and used. N-Succinyl-L-phenylalanine-p-nitroanilide and N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide stock had the concentration of 5 mM.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS– PAGE)

Homogeneity and molecular mass determination under denaturing conditions was done by SDS PAGE on 15% gel under reducing condition by the method described by Lamelli *et al*[14]. Protein bands were visualized using 0.25 % (*w/v*) Coomassie brilliant blue R-250 stain. Standard protein molecular weight markers were used.

Gelatin zymography

Detection of presence of active protease was achieved by zymography by the method of Toth *et al*[15] using gelatin as substrate. SDS-PAGE gel (7.5%) was co-polymerized with 0.4% gelatin. Samples were mixed with non-reducing SDS-loading buffer without heating to avoid loss of protease activity. Following electrophoresis, the gel was washed in washing buffer (2.5% Triton-X100 in 50 mM Tris (pH7.4), 5 mM $CaCl_2$, and 1 µM $ZnCl_2$) twice for 30 minutes to remove SDS from the gel. After that buffer was changed with incubation buffer (1% Triton-X100 in 50 mM Tris (pH7.4), 5 mM $CaCl_2$, and 1 µM $ZnCl_2$) and incubated for 24 h to provide necessary cofactors for proteinase activity to occur. Further gel was stained with Coomassie Brilliant Blue G-250 dye and was de-stained to visualize the clear band against the dark blue background.

Cleavage pattern of Casein

10 µg of purified protease was incubated with 20 µg of Casein at 37°C for 2 h and 4 h. The resulting fragments were separated on 12% SDS-PAGE and cleavage pattern were observed using the method of Li *et al*. with some modifications[16].

Effect of substrate concentration on the reaction velocity

Effect of increasing concentration of substrate on reaction velocity of the purified protease was determined using BAPNA as a substrate at pH 8 and 37°C for 1 h. BAPNA was used in the range of 0.02 µM–1 µM, while the protease was taken at the concentration of 0.3 mg. Assay was performed similarly as described in amidolytic activity protocol. Specific substrate concentration range without any enzyme was taken as a blank. Km was calculated using Lineweaver- Burk plot.

Protease activity and stability at varying pH range

For the assessment of the optimum pH for protease activity, the enzyme assay was performed with different buffers (pH 2.3–10.0) at a concentration of 100 mM for 60 min at 37°C. For the stability test, the enzyme was incubated for 60min at 37°C in different pH buffers. Finally, the pH was shifted back to optimum pH for activity *i.e*, pH 8 and to which substrate was added and again kept the reaction mixture at 37°C for activity for 60min. Reaction was stopped as described in the enzyme assay method. The highest detected activity was attributed as 100% activity and used to determine the relative activity at different pH.

Protease activity and stability at varying temperature range

The enzyme assay was performed at different temperatures (0-90°C) for assessment of the optimum temperature for activity. While for the thermal stability test, the enzyme was pre-incubated for 30 min at different temperatures (0–90°C). Subsequently, the reaction was continued as described for the enzyme assay. The highest detected activity

was attributed as 100% activity and used to determine the relative activity at different temperature.

Effect of protease inhibitors on the protease activity

The effects of protease inhibitors on protease activity were investigated. The protease inhibitors (PMSF, Idoacetamide, EDTA and EGTA) were used in final concentration of 1 mM. E-64 and Pepstatin-A were used in final concentration of 0.01 mM while Aprotinin was used at 5 µg mL^{-1} in the assay. The proteases were incubated for 30 min before the assay with the different protease inhibitors. The reaction was initiated by adding a substrate and incubated at 37°C for 60 min. The control activity was considered as 100% that was devoid of any inhibitors.

Effect of various solvents and surfactants on protease activity

The stability of purified Artocarpus protease was monitored in the presence different organic solvents (Methanol, Ethanol, Isopropanol, Butanol, DMSO,) and surfactants (Triton X-100, Tween-80, SDS, Labolene [Laboratory cleaning detergent]. The protease was incubated with different concentrations of the solvents or surfactants for 30min. The reaction was initiated by adding a substrate at 37°C and incubated for 60min for protease to act on substrate. The control activity was considered as 100% that is devoid of any solvents or surfactants. The activity was calculated in terms of percent residual activity.

Determination of carbohydrate content

The carbohydrate content of Artocarpus protease was determined by using the method of DuBois *et al*[17]. To 1 mL of protease, 1 mL of 5% phenol solution was added, followed by 4 mL of concentrated H_2SO_4. After the solution gets cooled the colour intensity was estimated at 490 nm spectrophotometrically. Standard curve was made using dextrose for calculating carbohydrate content of the protease.

Effect of metal ions on protease activity

The effects of various metal ions on protease activity were investigated by adding (K^+, Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+}, or Hg^{2+}) metal ions in the 5 mM final concentration to the reaction mixture. The enzymatic assay was performed as mentioned in the assay procedure. The proteolytic activity of the purified enzyme without any additive metal ion was considered as 100 % and was used to determine the relative activity.

Fluorescence Spectroscopy

Fluorometric analysis was done of 10 µg protease using spectrofluorometer Fluorolog®-3 (Horiba). The excitation wavelength was at first fixed at 280 nm and emission spectra was recorded in the range of 320-440 nm. Further the emission wavelength was fixed at 343 nm according to the emission maxima and then excitation spectra was recorded at the range of 250-320 nm. The effect of calcium addition on both excitation and emission spectra was also observed by doing three simultaneous additions of 10 µL of 10 mM calcium chloride and finally effect of chelating agent was seen by adding 10 µL of 10 mM EDTA.

Secondary structure determination by Circular Dichroism spectroscopy

Purified Artocarpus protease (0.5 mg mL^{-1} in 5 mM sodium phosphate pH 8.0 and 50 mM NaCl buffer) was subjected to circular dichroism (CD) spectroscopy with a Jasco J-815 spectropolarimeter. CD spectrum was recorded from 200 nm to 255 nm at 25°C and optical path length of 1 mM. Secondary structures of the protein were predicted using Delta Epsilon $\Delta\varepsilon$ in M^{-1} cm^{-1} values obtained experimentally using the K2D2 program[18].

Statistical analysis

Results are represented as mean ± standard deviation of three replicates. Statistical analysis was done using Origin Pro8 software (Origin Lab Corporation) by one-way analysis of variance (ANOVA). P-value was calculated using ANOVA which stands for probability and it measures the probability of the observed difference between groups to be due to chance. Tukey's test was done for comparison of more than two means where F-value was calculated which signifies ratio of between group and within group variations. The larger the F-value, larger would be the between group variation and smaller within group variation. It is a multiple comparison test.

Results

Isolation, purification and oligomerization

A new protease from the seeds of *A. heterophyllus* was extracted and purified to 93.6-fold successively by the step wise procedure described in the method section. The results of the purification procedure are summarized in the (Table 1). In the first step, the crude extract or homogenate was precipitated. The precipitate obtained at 30– 50 % ammonium sulphate showed the specific activity (3.9 U/mg). At this stage of purification, the enzyme was purified by 1.43-fold with yield of 26.6%. The precipitate was then subjected to anion exchanger DEAE-Cellulose52. This procedure

Table 1— Summary of the purification of the protease from *A. heterophyllus.*

Purification steps	Total protein(mg)	Yield (%)	Specific activity (Units/mg)	Fold purification
Crude extract	13068	100	0.0039	1
Ammonium sulfate precipitate 30-50%	3478	26.6	0.0056	1.43
Ion-exchange (DEAE-Cellulose52) chromatography	686	5.24	0.0407	10.4
Ion-exchange(Mono Q^{TM} 5/50GL)chromatography	206	1.57	0.1184	30.35
Size exclusion (Superdex 200) chromatography	10	0.07	0.3651	93.6

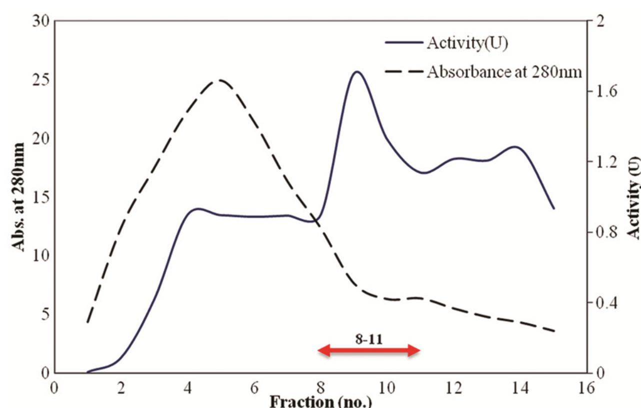

Fig. 1 — Chromatogram of anion exchange chromatography using DEAE Cellulose resins. Elution profile of protein from 30-50% ammonium sulfate fractionation step on anion exchanger DEAE-Cellulose column. The unbound proteins were washed out with the equilibration buffer (0.05M Tris-HCl buffer pH 8) and the column was eluted with 0.1 M NaCl. Fractions of 3 mL were collected. Each fraction was assayed for protein content and Proteolytic activity. Fractions (8-11) indicated with an arrow were pooled

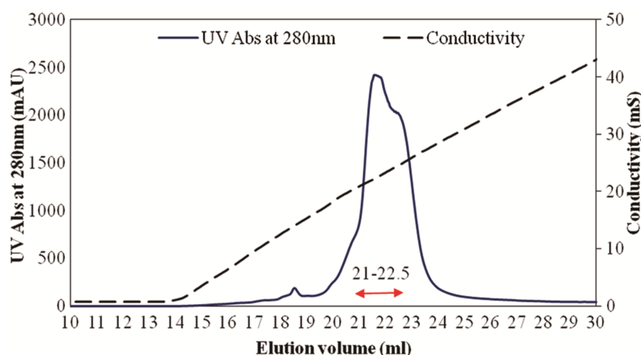

Fig. 2 — Chromatogram of Mono Q Sepharose chromatography using Mono Q^{TM} 5/50GL anion-exchanger. Active fractions from DEAE-Cellulose column were collected and applied to a Mono Q Sepharose column, equilibrated with (0.05M Tris-HCl buffer pH8). The enzyme was eluted with a linear gradient of NaCl (0–1 M) in the same buffer at a rate of 60 mL/h. Fractions (21-22.5) indicated with an arrow were pooled

yielded one broad peak of protease with 0.1 M NaCl and highest activity was present slightly after the peak of highest absorbance at 280 nm as shown in (Fig. 1). It resulted in 10.4-fold purification with 5.24% yield. Active fractions of this peak were pooled and then loaded on a Mono Q^{TM} 5/50G Lanion-exchange

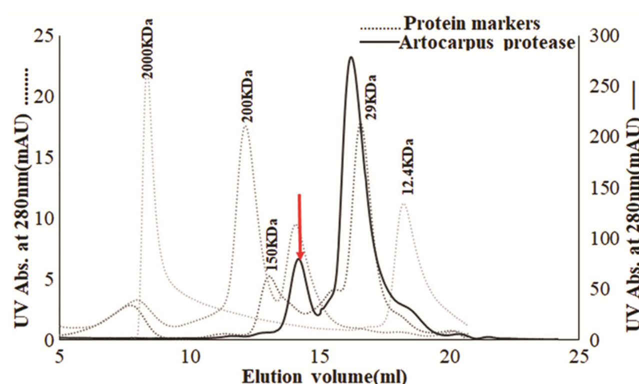

Fig. 3 — Size exclusion chromatography (SEC) profile of Artocarpus protease using a Superdex 200 10/300 column equilibrated with 0.02 M Tris–HCl pH 8.0, 0.2M NaCl buffer. The column was pre-calibrated with standard molecular weight markers (Sigma Aldrich; Blue dextran, 2000 kDa; β-amylase, 200 kDa; Alcohol dehydrogenase, 150 kDa; Carbonic anhydrase, 29 kDa; Cytochrome C, 12.4 kDa). Both peaks collected were assayed for protease activity. Among them only the peak eluted at 14.2 mL elution volume (indicated with an arrow) consisted of protease activity

chromatography. Bound proteins were eluted with a linear gradient of NaCl concentrations from 0 to 1.0 M. Protease activity appeared in a highest peak (Fig. 2) that resulted in 30.35-fold purification with 1.57% yield. At the final purification step, the protein was injected in Superdex 200 column pre-calibrated with standard molecular weight markers for SEC that showed two peaks out of which only one peak eluted at 14.2 mL elution volume had protease activity (Fig. 3) and the one eluted at 16.3 mL was devoid of protease activity. This procedure resulted in specific activity of 365.1 U/mg, 93.6-fold purification with 0.07%yield using BAPNA as a substrate. In respect to the standard protein markers, the purified protease showed the approximate molecular weight of 74 kDa.

DLS measurement on SEC eluted purified protease (Fig. 4) showed single peak on volume weighted particle size distribution with a hydrodynamic diameter of 7.05 ± 2.2 nm. The hydrodynamic diameter of purified protease corresponds to molecular weight nearly around 74 kDa (similar to SEC), that was estimated from the DLS instrument website[19].

Homogeneity and nature of the purified protease

The purified protease from SEC gave a single band on SDS-PAGE that confirms the homogenous nature of the protein. The purified enzyme has a molecular weight of approximately 18.45 kDa (Fig. 5A), estimated in reference to known protein standards. Microcarpain from latex of *Ficus macrocarpa* was found to have approximate similar molecular weight of 20 kDa[20], some other plant proteases with approximate molecular weight in the range or slightly bigger of Artocarpus protease are Procerain (28.8 kDa) and procerain B (25.7 kDa) isolated from the latex of *Calotropis procera*[21],[22]; Pergularain e I (23.356 kDa) extracted from the latex of *Pergularia extensa*[23]; Quercifoliain I (26 kDa) was isolated from the latex of *Vasconcellea quercifolia*[24]; asclepainc II which is a papain like protease (23.590 kDa) was isolated from latex of *Asclepias curassavica* L.[25]; Eumiliin (30 kDa)from the latex of *Euphorbia milii* var. hislopii; an alkaline protease (27 kDa) was purified from *Fusarium* sp. BLB[26]. A single band in the SDS PAGE gel infers that molecular weight of the purified protease is 18.45 kDa but the molecular weight estimated from SEC and DLS was 74 kDa. It is concluded that the purified protease in native conditions exists as a tetramer of 74 kDa. The homogeneity of the enzyme was also evaluated and justified using 7.5% gelatine zymograph activity staining. As shown in (Fig. 5B), the SDS-PAGE gel shows a fine resolved clear band of hydrolysis using gelatin as a substrate in non-reducing condition, indicating the purity of the purified Artocarpus protease. The endopeptidase nature of the enzyme was further investigated by casein digestion. As shown in (Fig. 5C), the purified protease cleaves casein and forms an additional fragment other than present in the lane of control casein. This indicates the cleavage at internal site that makes the protease an Endopeptidase.

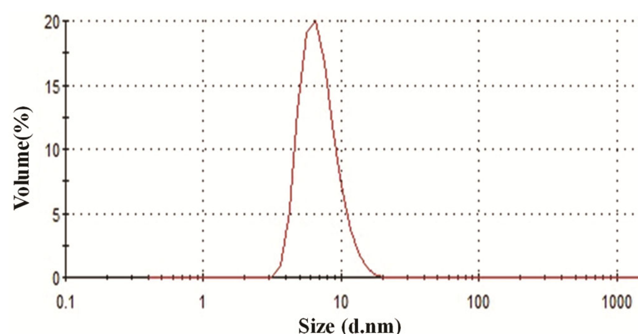

Fig. 4 — Dynamic light scattering (DLS) distribution of SEC eluted Artocarpus protease, displayed one major peak of population with a hydrodynamic diameter of around 7.05 ± 2.2 nm, that corresponds to molecular weight of ~74 kDa. It suggests that protein was present as a tetramer and monodisperse form in solution (d. nm: Diameter in nanometres)

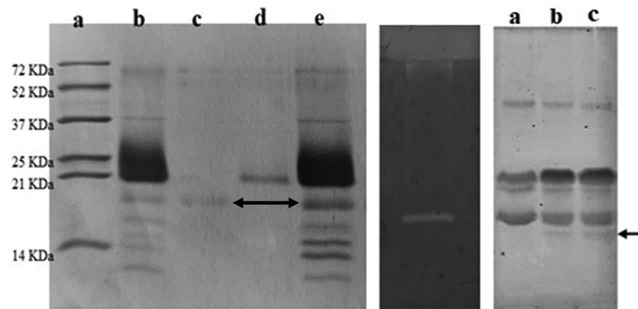

Fig. 5 — (A) SDS–PAGE gel (15%) of purified Artocarpus protease; Lane a: Standard molecular-mass markers (kDa); Lane b: Mono Q™ 5/50GL eluted active fraction; Lane c: SEC eluted active protease; Lane d: SEC eluted second peak (non-active); Lane e: DEAE-Cellulose column eluted active fraction; (B) 7.5% GelatinZymograph of proteolytic activity of the purified Artocarpus protease; and (C) SDS-PAGE gel (12%) showing cleavage pattern of casein. Lane a: control casein Lane b: casein incubated with protease for 2 h ; Lane c: casein incubated with protease for 4 h. Arrow indicates the cleaved fragment

Substrate specificity

Artocarpus protease hydrolysed natural substrates like casein and gelatin with remarkable activity. However, among the synthetic substrates used in the study, *e.g.*, N-α-Benzoyl-DL-arginine þ-nitroanilide (Trypsin substrate), N-Succinyl-L-phenylalanine-p-nitroanilide (Chymotrypsin substrate) and N-Succ-inyl-Ala-Ala-Ala-*p*-nitroanilide (Elastase substrate {F=64954.62, $P< 0.05$}). Artocarpus protease cleaved only N-α-Benzoyl-DL-arginine þ-nitroanilide (Trypsin substrate) and did not cleave the substrates of chymotrypsin and elastase as shown in (Fig. 6).

Kinetic parameter of purified protease

The effect of increasing substrate concentration on the velocity of the enzyme-catalyzed reaction was

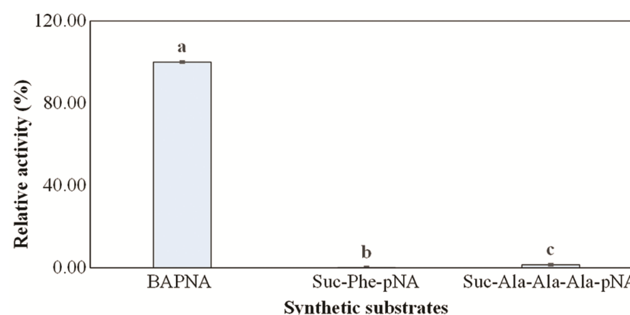

Fig. 6 — Protease activity against various synthetic substrates. A standardized amount of isolated protease (50µg) was used to measure relative activity using appropriate substrates (F=64954.62, $P< 0.05$). Columns with error bars above the column represent Mean±SD, respectively. Different letters above the bar are statistically different as determined by Tukey's test

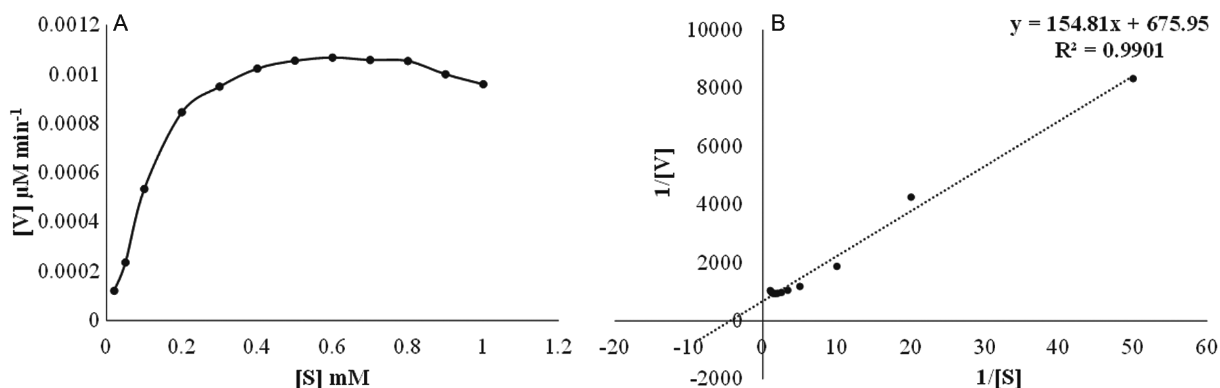

Fig. 7 — Kinetic parameter of purified protease Effect of substrate concentration on reaction velocity was studied on BAPNA (F=1911.03, $P< 0.05$). (A) Hyperbola curve; and (B) Lineweaver–Burk plot. Km values for the substrate was calculated from the Lineweaver–Burk plot

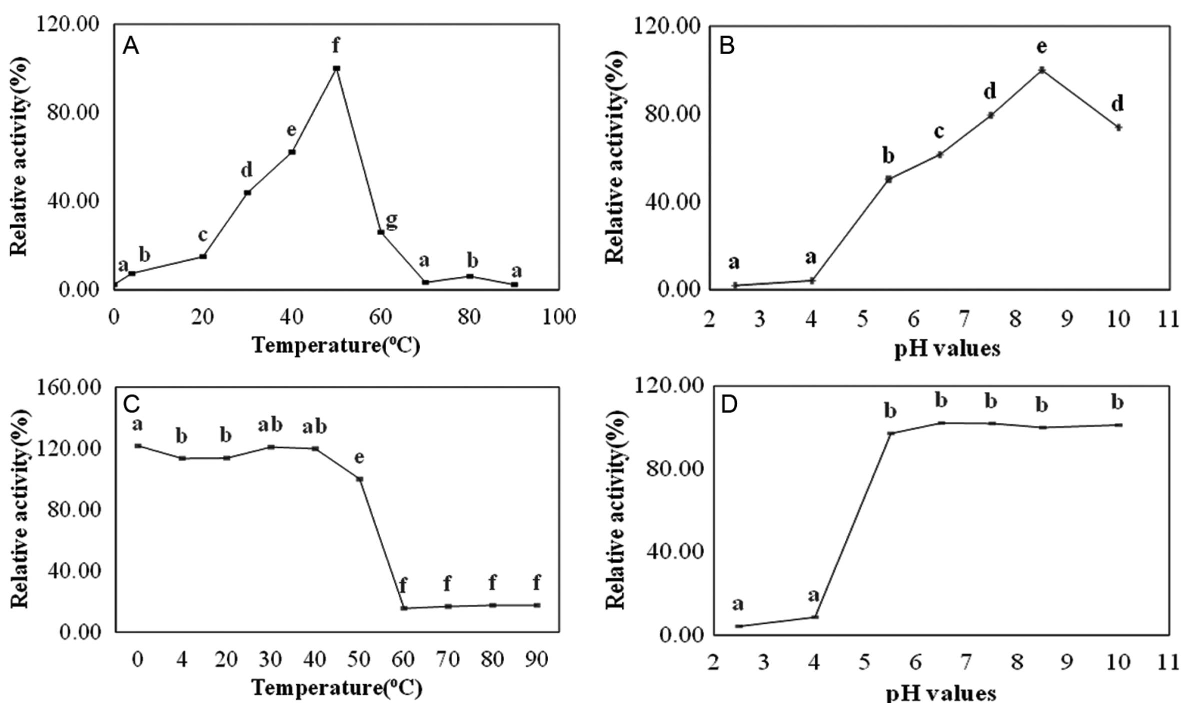

Fig. 8 — Effects of temperature and pH on activity of Artocarpus protease. (A) Activity at different temperature for 60 min {F=3919.91, $P< 0.05$}; (B) Activity at different pH for 60 min{F=392.22, $P< 0.05$}; (C) For temperature stability, enzyme was pre-incubated for 30 min at different temperatures and then activity was done at 37°C for 60 min{F=1031.82, $P< 0.05$}; and (D) For pH stability, enzyme was pre-incubated for 60min at different pH and then activity was done at 8.5 pH for 60 min {F=1366.38, $P< 0.05$}. Points with error bars above column represent Mean±SD, respectively. Different letters shown above bars are statistically different while same letters do not differ as determined by Tukey's test

studied using BAPNA as the substrate at pH 8 at 37°C. The effect of increasing substrate concentration on the velocity of the enzyme obeys the Michaelis–Menten equation {F=1911.03, $P< 0.05$}. The Lineweaver–Burk plot was plotted with 0.3 mg of protease and the substrate was in the range of 0.02-1 µM (Fig. 7). The value of K_m and V_{max} was 0.229 µM and 0.014 µM min^{-1}, respectively.

pH and temperature activity and stability

The proteolytic activity was monitored at different temperatures from 0–90°C and pH from 2.5-10. The temperature and pH optima for the activity were 50°C and pH 8.5, respectively. The activity got increased as the temperature increased from 0–50°C while the activity steadily decreased as the temperature rose above 55°C (Fig. 8A) {F=3919.91, $P< 0.05$}. It also

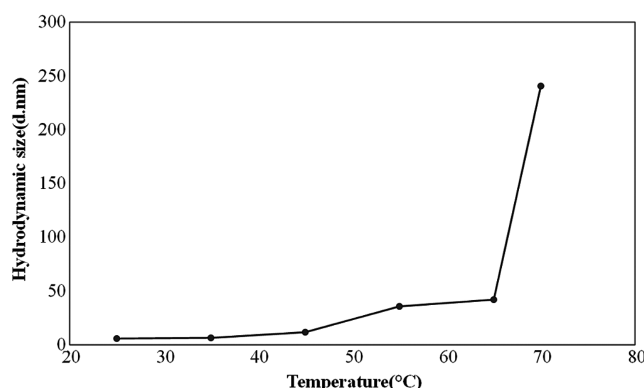

Fig. 9 — Temperature-dependent aggregation of purified protease. Hydrodynamic diameter (d.nm) of protein was recorded against raising temperature. Hydrodynamic size was uniform until 45°C but as temperature rose to 55°C, aggregation of the protein was seen

showed proteolytic activity at broad pH range of 5–10 (Fig. 8B) {F=392.22, $P< 0.05$}. The enzyme remained stable upto 50°C and gave 100% activity without any loss while at 60°C, the activity dropped to around 15% when the enzyme was pre-incubated for 30 min at different temperatures (0–90°C) {F=1031.82, $P< 0.05$} [Fig. 8C]. The enzyme remained stable with 100% activity above pH 5 but got permanently inactivated at acidic pH while remained stable at alkaline pH {F=1366.38, $P< 0.05$} (Fig. 8D).

Aggregation of the purified protease was also confirmed by DLS. The hydrodynamic diameter was recorded from 20°C-70°C temperature range (Fig. 9). The hydrodynamic diameter was uniform and stable up to 45°C. However, as the temperature rose above 45°C slight increase in the diameter was observed that suggested initiation of the aggregation of the protein and finally after 65°C, the hydrodynamic diameter increased tremendously which shows the presence of larger protein aggregates.

Effect of protease inhibitors on the protease activity

To determine the class of the isolated protease, different specific protease inhibitors were used as shown in (Table 2) {F=202.94, $P< 0.05$}.The enzyme activity was lost in the presence of PMSF and Aprotinin, while IAA, E-64, Pepstatin A, EDTA and EGTA had no effect on its activity. The enzyme got inhibited by PMSF and showed 77% inhibition at 2 mM concentration. Comparatively, Aprotinin was less effective and inhibited the enzyme up to 55% at 30 µg/ mL concentration. As the enzyme was inhibited exclusively by serine protease inhibitors, it is inferred as a serine protease. Other inhibitors failed to inhibit the purified proteases.

Table 2 — Effect of Protease inhibitors on purified protease from *A. heterophyllus*. Each value represents the mean of three replicates. Activity is written in terms of Mean±SD (SD: Standard deviation)

Protease inhibitor	Concentration	Residual activity (%)±SD
Aprotinin	5 µg/mL	91.29±0.005
	10 µg/mL	52.22±0.003
	30 µg/mL	45.74±0.007
PMSF	1 mM	87.40±0.001
	2 mM	23.33±0.001
E-64	10 µM	96.66±0.006
IAA	1 mM	84.63±0.011
Pepstatin A	10 µM	105.18±0.032
EDTA	1 mM	106.85±0.001
EGTA	1 mM	98.51±0.013
No Inhibitor	-	100±0.005

Effect of various solvents and surfactants on protease activity

Effect of different organic solvents and surfactants on protease activity is shown in (Table 3). The purified enzyme retained more than 80% proteolytic activity in the presence of Methanol. Beside this, the activity retained up to 60% in the presence of DMSO at 10% concentration while the enzyme got strongly inhibited in the presence of ethanol and butanol, which resulted in 60% loss in activity at 10% concentration. All the five solvents (Methanol, Ethanol, Butanol, Isopropanol and DMSO) at 10% concentration and control were compared using multiple comparison test {F=134.27, $P< 0.05$}, when compared at 30% concentration {F=181.94, $P< 0.05$} and at 50% concentration {F=306.62, $P< 0.05$} activity was significantly different.

The isolated protease was more than 80% activity in Triton-X 100 and Tween 80 at 1% and 0.5% concentrations, respectively, (Table 3). However, the enzymatic activity got increased on increasing the concentration of Triton-X 100. SDS and Labolene completely inhibited the protease activity. All the four surfactants (SDS, Triton-X100, Tween80 and Labolene) at 0.2% concentration and control were compared using multiple comparison test {F=97.38, $P< 0.05$}, when compared at 0.5% concentration {F=253.90, $P< 0.05$} and at 1% concentration {F=473.53, $P< 0.05$} activity were significantly different.

Table 3 — Effect of different solvents and surfactants on purified protease from *A. heterophyllus*. Each value represents the mean of three replicates. Activity is written in terms of Mean±SD (SD: Standard deviation)

Chemical	Concentration (%)	Residual activity (%)±SD
Control	0	100±0.007
Methanol	10	84.3±0.003
	30	33.3±0.006
	50	13.7±0.003
Ethanol	10	45±0.001
	30	5.6±0.001
	50	4.5±0.002
Butanol	10	39.8±0.005
	30	4.5±0.004
	50	3.3±0.003
Isopropanol	10	24.5±0.001
	30	2.1±0.001
	50	0
DMSO	10	62.7±0.002
	30	23.5±0.003
	50	12.7±0.001
SDS	0.2	14.46±0.003
	0.5	6.7±0.011
	1	5.91±0.008
Triton-X 100	0.2	71.78±0.029
	0.5	70.02±0.005
	1	88.89±0.005
Tween-80	0.2	88.71±0.014
	0.5	86.42±0.016
	1	68.43±0.009
Labolene	0.2	3.35±0.003
	0.5	1.23±0.003
	1	11.13±0.004

Carbohydrate content estimation

Carbohydrate content of the protease purified from the seeds of Artocarpus was estimated using phenol sulphuric acid method of DuBois {$F= 195.51$, $P< 0.05$}. Standard curve made using glucose was used to estimate the concentration of sugar present in the purified enzyme and it was found to be 58.4 µg per mg of protein. The result showcased the glycoprotein nature of the purified enzyme.

Effect of metal ions on protease activity

The effect of different metal ions (5 mM) on the purified Artocarpus protease activity was studied at pH 8.5 and 37°C by adding salts for divalent ions (Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+} or Hg^{2+}) to the reaction mixture {$F=992.52$, $P< 0.05$}. The activity without any metal ion was considered 100%. As shown in (Fig. 10), the protease activity was increased slightly

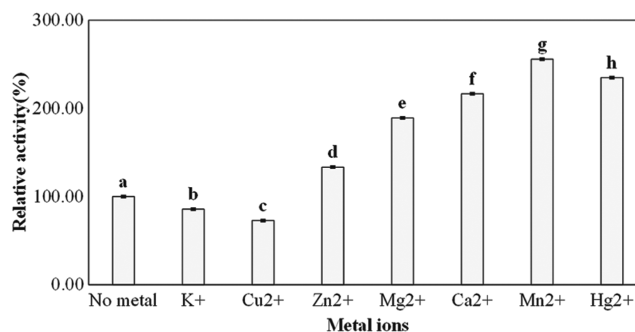

Fig. 10 — Effect of metal ions (5 mM) on protease activity {$F=992.52$, $P< 0.05$}. Columns with error bars above column represent Mean±SD, respectively. Different letters shown above bars are statistically different while same letters do not differ as determined by Tukey's test

up to 133% in the presence of Zn^{2+} while got slightly inhibited with Cu^{2+}.However, the addition of Ca^{2+}, Mg^{2+}, Mn^{2+}, and Hg^{2+} increased the proteolytic activity by more than 200%.

Fluorescence Spectroscopy

Fluorescence spectra of the purified protease was recorded to see the changes in the spectra in the presence of calcium as a ligand. The excitation and emission optima was found to be 282 nm and 343 nm, respectively. The excitation wavelength was fixed at 280 nm and emission spectra was recorded in the range of 320-440 nm while excitation spectra was recorded at the range of 250-320 nm after fixing emission spectra at 343 nm. On addition of calcium at 0.1 mM, 0.2 mM, and 0.3 mM final concentration, the emission spectra showed 70.68%, 67.97% and 62.56% quenching, respectively. While in case of excitation spectra, 96.80%, 84.82% and 82.61% quenching in fluorescence was observed on addition of 0.1 mM, 0.2 mM, and 0.3 mM of calcium, respectively. However, addition of 0.1 mM EDTA neither affect excitation spectra nor the emission spectra (Fig. 11).

3.11. Circular dichroism spectroscopy

The CD spectrum of Artocarpus protease eluted from SEC was recorded in the 255–200 nm wavelength range (Fig. 12). It showed pattern indicating the presence of both α-helix and β-sheet. CD spectrum was analysed using K2D2 program to predict the secondary structure of the protein. The tool used Delta Epsilon Δε values for the interpretation and indicated that the purified protease contains nearly 4.44% alpha helix and 44.17% beta strand.

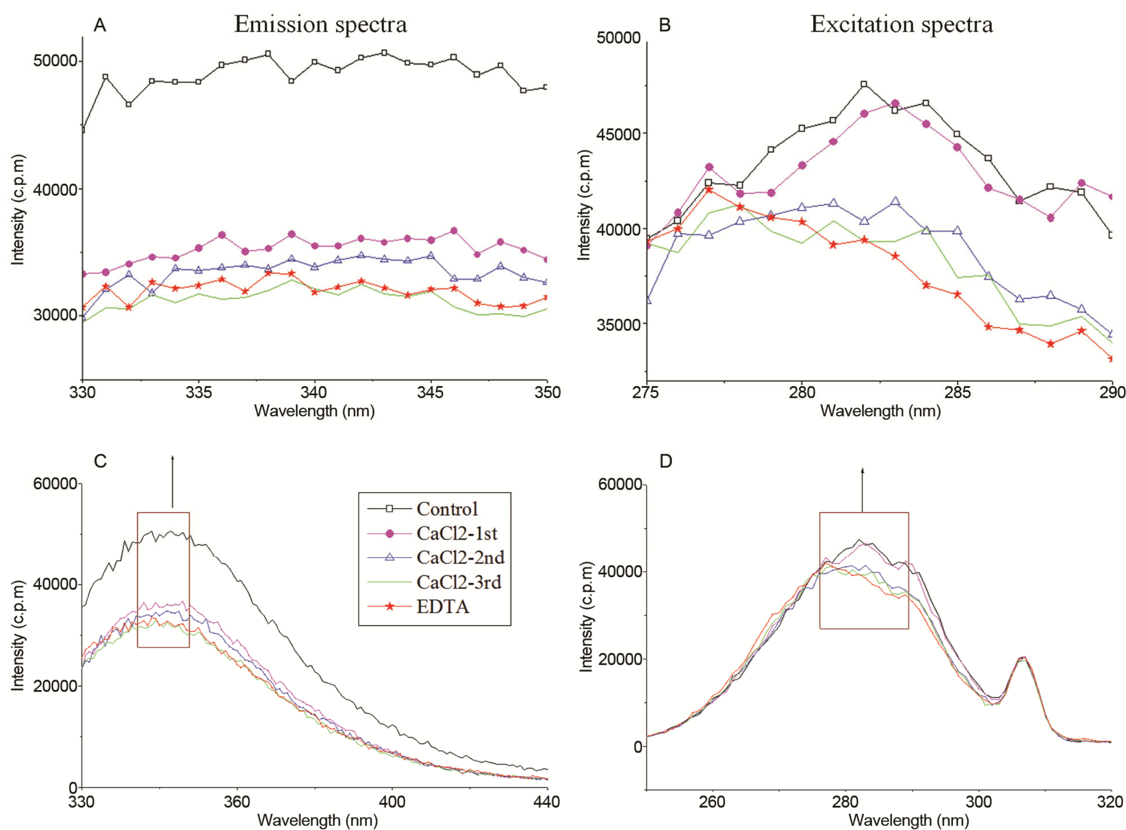

Fig. 11 — Fluorescence spectra of purified protease. A. Emission spectra with fixed absorption spectra at 280 nm. Three subsequent addition of 0.1 mM $CaCl_2$ to 7 µg/mL purified protease was done and one addition of 0.1 mM of EDTA; B. Excitation spectra with fixed emission spectra at 343 nm. $CaCl_2$ and EDTA were added in a similar manner as before. [c.p.s means counts per second]

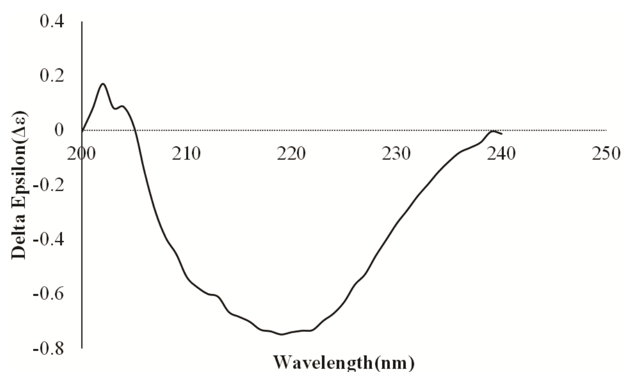

Fig. 12 — Circular dichroism spectra of purified protease using a J-815 spectropolarimeter with 5 mM sodium phosphate pH 8.0, 50 mM NaCl buffer. The spectra was collected between 200 and 255 nm wavelength range at 25°C and plotted against Delta Epsilon (Δε). Protein was showing signature features of α-helix (4.44%) and β-sheet (44.17%) determined by using K2D2 program

Discussion

The isolation, purification and characterization of a serine protease from the seeds of *Artocarpus heterophyllus* was done. Earlier a serine protease of molecular weight 79.5 kDa named Artocarpin[27] and a 48 kDa antimicrobial protease (AMP48) with antimicrobial property[28] from the latex of *A. heterophyllus* had been reported. Furthermore, AMP48 was reported to show fibrinolytic and fibrinogenolytic activity with optimal activity between 55 and 60°C temperature at pH 8[29]. Also, a protease has been partially purified from the seeds of *Artocarpus integrifolis* and is shown to have a native molecular weight of 22 kDa. The drawback in the study is that ammonium sulfate precipitated protein was directly loaded on gel filtration chromatography, the column being calibrated only with three broad range markers. Ion exchange chromatography and SDS-PAGE or Native PAGE was not performed. Thus it is concluded that the above study[30] is very preliminary in nature and could not be compared to. Hence, purification of the enzyme was done by sequential chromatographic methods. The molecular weight of the purified protease was estimated at 18.4 kDa using reducing SDS gel while 74 kDa using SEC and DLS. It appears that the purified protease may be present as a tetramer form in the nature and its monomer is of around 18 kDa. The similar result was

obtained in the tamarillin, a protease isolated from tamarillo fruit (*Cyphomandra betacea Cav*.) The molecular mass of the tamarillin was approximately 70 kDa under non reducing condition and approximately 23 kDa in reducing condition[31]. The molecular weight of the serine proteases usually lie between 19-110 kDa; among them most of them are between 60-80 kDa[32].Gelatin zymography gave a clear band even in the presence of 0.1% SDS in the gel and 1%SDS in the sample buffer. This confirms the stability of the enzyme in the presence of denaturant. Looking into the substrate specificity of the enzyme it was found that enzyme was able to cleave trypsin substrate BAPNA. This observation suggests that the Artocarpus protease has specificity of cleaving carboxyl end of arginine (Arg). Artocarpus protease failed to cleave at the C terminal of aromatic amino acid and aliphatic amino acid. As the chymotrypsin and elastase synthetic substrates do not have Arg or Lys, this may be the possible reason for the inertness of the purified Artocarpus protease toward them. The enzyme obeys the Michaelis–Menten equation and the Km 0.229 μM of the enzyme was found to be similar to the serine protease from *Helicoverpaarmigera*[33].

The purified enzyme displayed great pH and temperature stability that makes it a good candidate for industrial application. Similar pH optima was obtained in serine protease isolated from latex of *Crinum asiaticum*[34] while similar temperature optima was found of a protease isolated from wheat[35] and a serine protease from leave of *Lactuca sativa*[36]. The purified protease got strongly inhibited by PMSF and partially by Aprotinin in spite of both being serine protease inhibitors, this probably would be because of the reversible binding of the Aprotinin with the enzyme while PMSF binds irreversibly. This indicates that the enzyme is a serine protease. Surprisingly, Triton-X100 (detergent) that inhibited protease activity of major proteases, surprisingly increased the activity. Similar finding was obtained in the case of plasmin[36]. The purified protease was found to be of glycoprotein nature and according to one report this carbohydrate moiety might be of functional importance to the enzyme and responsible for the thermal stability of the protein[38].

Interestingly, the enzyme activity got increased in the presence of various divalent ions except Cu^{2+}. The Hg^{2+} which is generally a strong inhibitor for the proteases increased the activity. Similar findings were reported for protease from ginger rhizome[39]. Most of the proteases reported so far were generally found to get inhibited by Hg^{2+} ion as it act on sulfhydryl residues in proteins but in contrast presence of Hg^{2+} doubled the enzyme activity. The possible explanation could be as mercury tends to generate reactive oxygen species (ROS), both *in vivo* and *in vitro*, to scavenge the generated ROS due to mercury accumulation, the enzymatic activity got increased[40]. For validating the result obtained the antioxidant property of the purified protease need to be explored further. Presence of Mn^{2+} and Ca^{2+} lead to the maximum protease activity which was found similar with that of trypsin[41].

Fluorescence quenching observed on addition of calcium to the purified protease could happen because of binding of calcium to a tryptophan containing site thereby quenching its fluoresecence. Secondary structure of the protease consisted of 4.44% alpha helix and 44.17% beta strand, majorly comprised of β sheets which provides stability to the protein structure as the β-pleated sheet are made from extended β-strand polypeptide chains, linked by hydrogen bonds. Due to this, β-sheets resist stretching[42].

A simple purification procedure to purify the protease from *A. heterophyllus* to homogeneity is provided in this report. The enzyme could be used for large scale production due to its economic purification procedure and easy availability of the seeds. It could be used for the industrial applications after studying the 3D structure and related function of the enzyme. However, the high stability of the purified protease against various conditions and the striking properties makes it a potential candidate for pharmaceutical and biotechnological industries.

Conclusion

The present study emphasises on the isolation and purification of a protease from the seeds of *Artocarpus heterophyllus*. The enzyme was purified to homogeneity, it was achieved by ammonium sulphate precipitation (30–50 %), anion-exchange chromatography on both DEAE-Cellulose and Mono Q^{TM} 5/50GL column and size exclusion chromatography on superdex-200 column. After the final purification step, the enzyme was purified 93.6-fold with a specific activity of 365.1 U/mg and 0.07% recovery. The purified protease appeared as a single band on SDS-PAGE, and its molecular weight was estimated to be 18.4 kDa while in size exclusion its estimated molecular weight was found to be 74 kDa. This confirms the tetrameric structure of the protease

in natural environment. Apart from this the protease showed specificity for trypsin substrate. The kinetic parameters K_m and V_{max} were 0.229 μM and 0.014 μM min^{-1}, respectively, using BAPNA as a substrate. The optimum pH and temperature were 8.5 and 50°C, respectively. Divalent metal ions boosted the enzyme activity. Binding of calcium ions showed quenching of the fluorescence spectra. Secondary structure consisted 4.44% alpha helix and 44.17% beta strand. This Artocarpus serine protease could be used in pharmaceutical and biotechnology applications due to easy purification and availability.

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Conflict of interest

All authors declare no conflict of interest.

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