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Research Article

Purification and characterization of a new cysteine protease from *thevetia peruviana* (pers.) merr.

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Abstract

A new cysteine protease was purified from the latex of *Thevetia peruviana* (Pers.) Merr. to electrophoretic homogeneity by a procedure involving pre-treatment of the latex followed by DEAE-cellulose chromatography. The purified protease was found to be a homodimeric protein with a native molecular weight of 36 kDa. The enzyme acting on azocasein as its substrate had a specific activity of 71 units/mg protein, and it exhibited hyperbolic kinetics with K_m and V_{max} values of 26.7 µM and 2.23 units/mL respectively. The enzyme was also characterized by optimum pH of 6, optimum temperature of 50°C, and $T_{½}$ of 64°C. Further, specific inhibitory studies revealed the enzyme to be a cysteine protease, and the enzyme exhibited fibrinolytic activity. Peptide mass fingerprinting analysis showed that the protease was not identical with any protein characterized earlier.

Keywords: *Thevetia peruviana*; cysteine protease; fibrinolytic activity.

INTRODUCTION

Proteases are hydrolytic enzymes that catalyze the breakdown of proteins or peptides by cleaving the peptide bonds (Barrett & McDonald, 1986). They are found in all forms of organism, *viz*. animal, plant, and microorganism, and are involved in different complex physiological processes essential for their survival, including protein catabolism, cell division, fertilization, developmental morphogenesis, apoptosis, zymogen activation, germination, senescence and defence against pathogens (Liggieri et al., 2009; Badgujar & Mahajan, 2010). Proteases are abundant in occurrence. They correspond to 1 to 5% of the total gene content in different organisms and represent about 2% of all gene products in higher organisms (Puente et al., 2003). About 10% of the enzymes listed in the enzyme database of the **International Union of Biochemistry and Molecular Biology (IUBMB)** are proteases (Sorokhaibam et al., 2015). Among these, the proteases occurring in plants contribute the highest proportion (43.85%), followed by bacteria (18.09%), fungi (15.08%), animals (11.15%), algae (7.42%) and viruses (4.41%). Apart from the different biological roles they play, several proteases find applications in detergent, textile, leather, food and pharmaceutical industries (Badgujar & Mahajan, 2010). They account for about 65% of the total worldwide sale of enzyme (Jaouadi et al., 2011). Though most commercial proteases are obtained from microbial sources, proteases from plant sources, because of their marked stability behaviour, are also becoming increasingly important for industrial application. Among these plant proteases, cysteine proteases, viz. papain, bromelain and ficin, are often used in different industries (González-Rábade et al., 2011). As the demand for industrial proteases increases, there is an expanding need for the purification and characterization of such proteases from different sources. The lattices of different plants belonging to the families, including Apocynaceae, Asclepiadaceae, Caricaceae, Euphorbiaceae and Moraceae, are rich sources of protease (Domsalla & Melzig, 2008). In the present investigation, a new cysteine protease exhibiting fibrinolytic activity was purified from the latex of *Thevetia peruviana* (Pers.) Merr. and was characterized with respect to some of its physicochemical properties.

MATERIALS AND METHODS

Plant Material

The plant, the latex of which was used as a source of the protease under the present investigation, was *Thevetia peruviana* (Pers.) Merr. (commonly known as yellow

oleander or lucky nut) grown in the Manipur University Campus, Manipur, India. It is a small evergreen tree belonging to the Apocynaceae family.

Chemicals

Azocasein, human fibrin, Sephadex G-75, native protein molecular weight markers (bovine serum albumin, M_r 67 kDa; egg albumin, M_r 45 kDa; carbonic anhydrase, M_r 29 kDa; lysozyme, 14 kDa), blue dextran (M_r 2000 kDa), phenylmethyl sulphonyl fluoride (PMSF), pepstatin A, 2,6-pyridine dicarboxylic acid, E-64, 1,10-phenanthroline hydrate were obtained from Sigma-Aldrich Company, St. Louis, MO, USA. Protein molecular weight markers (Medium Range, M_r 6.5 - 97.4 kDa) were purchased from Merck Limited, Mumbai, India. All the other chemicals were of analytical reagent grade. Purified water was obtained through the TKA Smart2PureUV/UF (Niederelbert, Germany) water purification system.

Collection of Plant Latex

The milky white latex was allowed to exude when the pedicel of the fresh and tender fruit of *Thevetia peruviana* was chopped off at its base using a scalpel. It was then collected in a pre-chilled test tube for use in the present experimental investigation.

Protease Assay

Proteolytic activity was determined using azocasein as the substrate by a slight modification of the method of Sorokhaibam et al. (2015). The standard protease assay mixture (1.2 mL) was constituted in a microfuge tube by mixing 0.1 mL of the appropriately diluted protease sample and 0.2 mL of 0.5% azocasein (w/v) in an assay buffer of 0.1 M sodium phosphate buffer pH 6. The enzymatic reaction was started with the addition of the substrate solution. After incubating for 30 min at 37ºC, the reaction was stopped by adding 0.3 mL of 10% TCA (w/v); the resulting mixture was kept in ice-cold condition for 5 min to allow complete protein precipitation, and then it was centrifuged at 12,000g for 10 min to collect the supernatant containing TCA-soluble peptides. 0.8 mL of the supernatant was mixed with 0.4 mL of 1.0 M NaOH, and then the absorbance of the mixture was read at 440 nm. A blank was always run by adding TCA before the addition of the substrate. One unit of the protease activity was defined as the amount of protease, which gave an absorbance increase of 0.1 per min under the standard assay conditions. Protein concentration was estimated according to the method of Lowry et al. (1951) using crystalline bovine serum albumin (BSA) as the standard. The corresponding specific activity of the protease sample was expressed in terms of protease activity units/mg of protein.

Purification of the Enzyme

The protease was purified from the latex of *Thevetia peruviana* by a procedure involving pre-treatment of latex followed by DEAE-cellulose chromatography. The freshly collected latex was thoroughly mixed with three volumes of pre-chilled 0.2 M sodium phosphate buffer pH 7, and the resulting suspension was centrifuged at 21,000g for 30 min at 4ºC to collect a clear supernatant discarding the sticky white rubbery pellet. The supernatant was then brought to 4% ammonium sulphate saturation, and the resulting suspension was centrifuged as before to collect a clear supernatant, discarding the white non-sticky pellet. The supernatant was dialyzed exhaustively against 0.01 M imidazole-HCl buffer pH 6.2 under ice-cold condition. The dialyzate obtained was collected as the latex protease extract for further purification. The extract was then loaded onto a DEAE-cellulose column (size, 1.5x4 cm, bed volume 5 mL) pre-equilibrated with 0.01 M imidazole-HCl buffer pH 6.2 containing 0.01 M NaCl. The unbound materials were washed from the column by eluting five bed-volumes of the equilibration buffer, and then the ionically bound proteins were eluted by passing 0.01 M imidazole-HCl buffer pH 6.2 having a linear gradient of NaCl ranging from 0.01 - 0.4 M at a flow rate of 0.3 mL/min. The protein elution was monitored by absorbance at 280 nm, whereas the protease elution was monitored by its activity. The fractions showing protease activity were pooled and dialyzed extensively against 0.1 M sodium phosphate buffer pH 6. The dialyzate obtained was collected as the purified protease preparation for further physico-chemical characterization.

SDS-PAGE and Determination of Subunit Molecular Weight

The purified protease preparation was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE, 13.5% gel) with reduction according to the method of Laemmli (1970) coupled with silver staining by following the method of Merril (1990). The standard proteins (Merck, Medium Range), viz. phosphorylase b (M_r 97.4 kDa), bovine serum albumin (M_r 66 kDa), ovalbumin (M_r 43 kDa), carbonic anhydrase (M_r 29 kDa), lactoglobulin (M_r 18.4 kDa) and aprotinin (M_r 6.5 kDa) were used as reference proteins. Using the standard plot of log M_r *vs* R_f prepared based on the migration of standard proteins in the gel, the relative subunit molecular weight of the purified protease was determined.

Determination of the Native Molecular Weight

The native molecular weight of the purified protease was determined by gel filtration through a column of Sephadex G-75 by following the method of Devi et al. (2011). A Sephadex G-75 column (size,1x42 cm) was pre-equilibrated with 0.1 M sodium phosphate buffer pH 6 containing 0.2 M NaCl. The void volume (V_o) of the gel column was determined by using blue dextran (M_r 2,000 kDa), and the column was calibrated by using protein standards, viz. bovine serum albumin (M_r 67 kDa), egg albumin (M_r 45 kDa), carbonic anhydrase (M_r 29 kDa), lysozyme (M_r 14.3 kDa) and recombinant insulin (M_r 5.6 kDa). Each of the

protein standards (0.5 mL containing 0.5 mg protein) or the purified protease sample (0.5 mL containing 106 µg) was loaded separately onto the column and eluted with 0.1 M sodium phosphate buffer pH 6 containing 0.2 M NaCl at a flow rate of 9 mL/hr. The elution of protein standards was monitored by absorbance at 280 nm, and the elution of the purified protease by proteolytic activity. The native molecular weight of the purified protease was estimated from the standard plot of log M_r *vs* V_e/V_o prepared based on the data obtained from the gel filtration experiment.

Effect of pH on the Proteolytic Activity

Proteolytic activity of the purified protease was determined at different pH values in the otherwise standard assay system described in *Protease Assay*. The pH was varied from 3.0 to 9.0 using different assay buffers, viz. 0.1 M glycine-HCl buffer (pH 3.0 - 3.5), 0.1 M sodium acetate buffer (pH 4.0 - 5.5), 0.1 M sodium phosphate buffer (pH 6.0 - 8.0), and 0.1 M Tris-HCl buffer (pH 8.5 - 9.0). The pH profile was obtained by plotting pH against the percentage of the residual protease activity, taking the highest activity as the 100% value.

Effect of Temperature on the Proteolytic Activity

Proteolytic activity of the purified protease was determined at different incubation temperatures in the otherwise standard assay system as described in *Protease Assay*. The assay mixture was maintained at different designated temperatures ranging from 10 to 80ºC using a digital circulatory water bath (accuracy ± 0.01ºC). The temperature profile was obtained by plotting temperature against the percentage of protease activity, taking the highest activity as the 100% value.

Thermal Stability of the Enzyme

Thermal stability behaviour of the purified protease was studied by following the method of (Sorokhaibam et al., 2015). Aliquots of the enzyme sample (21.3 µg/mL), dissolved in 0.1 M sodium phosphate buffer pH 6, were taken in microfuge tubes and incubated at different designated temperatures ranging from 10 to 90ºC for 10 min using a digital circulatory water bath (accuracy ± 0.01ºC). At the end of incubation, the protease sample was cooled in ice-cold condition for 1 min, followed by standing for 4 min at room temperature. Then, the protease sample was assayed for its residual activity as usual by the standard *Protease Assay.* The thermal stability profile was obtained by plotting incubation temperature against the percentage residual protease activity, taking the highest activity as the 100% value.

Effect of Substrate Concentration on Proteolytic Activity

The effect of substrate concentration on the activity of the purified protease was determined using azocasein as the substrate. The substrate concentration was varied in the otherwise standard assay system as described in *Protease Assay.* The protease activity in terms of rate of absorbance increase at 440nm was plotted against the substrate concentration.

Effect of Protease Inhibitors

The effect of some possible inhibitors on the activity of the purified protease was analysed using azocasein as the substrate. The inhibitors tested were E-64 (N-[N-(L-3-trans-carboxirane-2-carbonyl)-L-leucyl]-agmatine, specific for cysteine proteases), mercuric chloride ($HgCl_2$, specific for cysteine proteases), pepstatin-A (a hexapeptide with the sequence Iva-Val-Val-Sta-Ala-Sta, specific for aspartic proteases), PMSF (phenylmethylsulfonyl fluoride, specific for serine proteases), 1,10-phenanthroline (specific for metalloproteases) and pyridine-2,6-dicarboxylic acid (specific for metalloproteases). The protease was pre-incubated with each of the inhibitors in the assay buffer within its known effective concentration range for 30 min at 37ºC (Beynon & Bond, 1990; Windle & Kelleher, 1997). Then, the residual protease activity was determined using the otherwise standard protease assay mixture described in *Protease Assay*. The protease activity in the absence of inhibitor was taken as the control having 100% activity.

Determination of Fibrinolytic Activity

The fibrinolytic activity of the purified protease was determined by the fibrin-agarose gel plate method and also by the gel filtration method described below:

Fibrinolytic activity by fibrin agarose gel plate method: The fibrinolytic activity of the purified protease was determined by a slight modification of the method of Astrup & Müllertz (1952) using a 1% (w/v) agarose gel plate containing 0.2% (w/v) fibrin. A mixture containing 0.2 g of agarose powder, 4 mL of 1% (w/v) fibrin solution and 16 mL of 0.1 M sodium phosphate buffer pH 6 was warmed in a hot water bath. The resulting clear suspension was poured into a petri dish (10 cm in diameter), and the gel was allowed to set by standing at room temperature. Three wells, each of 0.5 cm diameter, were punched. Two appropriately diluted purified protease samples, one containing 0.65 µg protein and another containing 1.30 µg protein, were loaded into two of the wells. The third well was loaded with heat-inactivated protease sample containing 1.30 µg protein as the control. Then, the gel plate was incubated at 37ºC for 6 hr. At the end of the incubation period, the gel plate was treated with 10% (w/v) TCA. The fibrinolytic action of the enzyme was confirmed by the appearance of a clear zone around the sample well against the opaque background.

Fibrinolytic activity by gel filtration method: The fibrinolytic activity of the purified protease was double-checked by a method involving gel filtration of the post-fibrinolysis reaction mixtures through a Sephadex G-75 column. A fibrinolytic assay mixture (1.2 mL) was constituted in a microfuge tube by mixing 0.1 mL of the appropriately

diluted purified protease sample and 0.2 mL of 1% fibrin (w/v) as the substrate in an assay buffer of 0.1 M sodium phosphate buffer pH 6. The reaction was started with the addition of fibrin solution. After incubating for 2 hr at 37ºC, the reaction was stopped by heating the assay mixture in a boiling water bath for 5 min. The resulting reaction mixture was spun down briefly and then subjected to gel filtration through a Sephadex G-75 column (size, 1x42 cm). The assay buffer containing 0.2 M NaCl was passed through the column at a flow rate of 9 mL/hr and the elution of the proteins and fibrin fragments was monitored by absorbance at 280 nm. The corresponding experimental control was run by taking the heat-inactivated protease sample.

Peptide Mass Fingerprinting by LC-MS/MS

The peptide mass fingerprinting of the purified protease was carried out in the mass spectrometry facility at Shantani Proteome Analytics Pvt. Ltd., Pune, India. The purified protease preparation was subjected to SDS-PAGE by following the method of Laemmli (1970) followed by Coomassie blue staining as described by (Neuhoff et al., 1988). The gel plug containing the stained protein band was excised, and then it was subjected to in-gel protein reduction, alkylation, trypsin digestion, and peptide extraction according to the standard technique of Bringans et al. (2008). The resulting peptides were separated by using Agilent 1260 infinity HPLC-Chip/MS system. Then, the charged peptides emerging from the HPLC-Chip system were directly infused into the mass spectrometer. Agilent Mass Hunter software was used for data acquisition and analysis of the total ion chromatograms obtained. Protein searches were carried out using Morpheus software (Wenger & Coon, 2013) (http://cwenger.github.io/Morpheus/) against plant proteome database obtained from Swiss-Prot (as of July 24, 2020).

Statistical Analysis

The experimental data were collected as mean ± standard deviation (SD) of at least three determinations. Statistical analysis was performed using Microsoft Excel 2010 (Microsoft, WA, USA). The data points in graphical illustrations having zero-SD values were shown without error bars.

RESULTS AND DISCUSSION

Enzyme Purification

In the present investigation, a cysteine protease was purified from the latex of *Thevetia peruviana* by a procedure involving successive steps of latex pre-treatment and DEAE-cellulose chromatography. During the latex pre-treatment, the freshly collected latex was mixed with three volumes of 0.2 M sodium phosphate buffer pH 7 and the resulting suspension was centrifuged to collect a clear supernatant. It was brought to 4% ammonium sulphate saturation and then centrifuged to collect a clear supernatant. The supernatant was then dialyzed extensively against 0.01 M imidazole-HCl buffer pH 6.2 at ice-cold condition. The dialyzate obtained was collected as the latex protease extract. Its proteolytic activity was determined by the standard *Protease Assay*. Protein was determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as the standard. The specific activity of the protease sample was found to be 12.0 units/mg protein. When subjected to SDS-PAGE, the latex protease extract was found to exhibit four distinct protein bands as shown in Figure 1 (A) Lane 1, indicating the presence of only a few protein species in the latex of *Thevetia peruviana.* To achieve purification of the latex protease, the protease extract was subjected to DEAE-cellulose chromatography. The corresponding protein elution profile is shown in Figure 1 (B). Three protein peaks labelled I, II and III were obtained. The protease activity was detected only in the slowest eluting peak (peak III). The fractions having protease activity were pooled and dialyzed extensively against 0.1 M sodium phosphate buffer pH 6. The dialyzate thus obtained was found to migrate as a single homogeneous protein band on SDS-PAGE performed according to the method of Laemmli (1970) coupled with the silver staining procedure of Merril (1990) as shown in Figure 1 (A) Lane 2. The resulting purified protease was found to have a specific activity of 72 units/mg protein. It was purified 6-fold with 53% recovery. Its specific activity compares well with that of procerain, a latex cysteine protease from *Calotropis procera* (65 units/mg protein, calculated from the reported activity and protein values) (Dubey & Jagannadham, 2003). The purification of the protease from the latex of *Thevetia peruviana* is summarised in Table 1.

Molecular Weight

The relative subunit molecular weight of the protease purified from the latex of *Thevetia peruviana* was determined by SDS-PAGE (13.5% gel) according to the method of Laemmli (1970) coupled with silver staining by the method of Merril (1990) using molecular weight markers (Merck, Medium Range). It was found to be 18.4 kDa when deduced from the standard plot R_f vs log M_r shown in Figure 2 (A) prepared based on the data of migration of the standard proteins on the gel shown in Figure 1 (A) Lane 3. Furthermore, the native molecular weight of the purified protease was found to be 36 kDa as estimated from the standard plot V_e/V_o vs log M_r shown in Figure 2(B) prepared from the experimental results obtained by gel filtration of the standard proteins and the purified protease through a Sephadex G-75 column. By considering the result of native molecular weight determination together with that of the molecular weight determination by SDS-PAGE, the purified protease was found to be a dimeric protein made up of apparently chemically identical subunits, each with a molecular weight of 18.4 kDa. The subunit molecular weight was found to be relatively smaller than that of cysteine proteases reported from other members of the Apocynaceae family (23.2 kDa

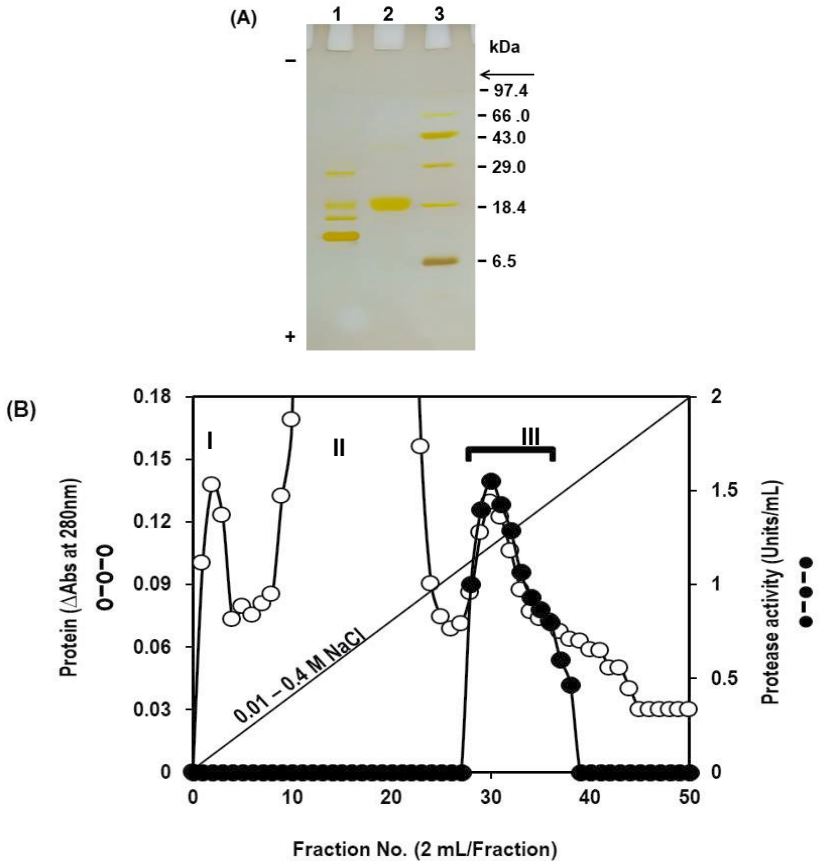

Figure 1: (A) SDS-PAGE (13.5% gel) with reduction of the latex protease extract and the protease purified from the latex of *Thevetia Peruviana* carried out according to the method of Laemmli (1970) coupled with silver staining procedure of Merril (1990). The arrow indicates the origin of electrophoretic migration. Lane 1: latex protease extract (0.105 µg), Lane 2: the purified protease (0.06 µg) and Lane 3: marker proteins (Merck, Medium Range). **(B)** Elution profile of DEAE - cellulose chromatography for purification of the protease. Proteins bound in the column were eluted by a linear NaCl gradient (0.01 - 0.4 M) in 0.01 M imidazole-HCl buffer pH 6.2 at a flow rate of 0.3 mL/min. The plot with the unfilled circles (o) represents protein elution profile, whereas the plot with filled circles (●) represents the elution of protease activity.

Table 1: Summary of the purification of the protease from the latex of *Thevetia peruviana.*

Purification step	Volume (mL)	Total protein (mg)*	Total activity (units)**	Specific activity (units/mg)	Purification fold	Recovery (%)***
Latex protease extraction	21.4****	3.64	43.5	12.0	1	100
DEAE- cellulose chromatography	15.0	0.32	23.0	71.0	6	53

* Protein concentration was estimated according to the method of Lowry et al. (1951) using crystalline bovine serum albumin as standard.

** One unit of enzyme activity was defined as the amount of protease, which gives an absorbance increase of 0.1 per min under the conditions of standard *Protease Assay* described under *Materials and Methods.*

*** Recovery (%) was calculated with reference to the total activity of the latex protease extract taken as 100%.

**** Volume of the protease extract obtained after pre-treatment of 4 mL of freshly collected latex from fruit of the plant.

- 28.8 kDa) (Sundd et al., 1998; Kundu et al., 2000; Dubey & Jagannadham, 2003; Vairo Cavalli et al., 2003).

Effect of Substrate Concentration on the Enzyme Activity

The effect of substrate concentration on the steady-state rate of the reaction catalyzed by the protease purified from the latex of *Thevetia peruviana* was determined using azocasein as the substrate. The substrate concentration was varied in the otherwise standard reaction mixture described in *Protease Assay.* The substrate saturation curve of the enzyme is shown in Figure 3(A), and the corresponding double reciprocal plot is shown in Figure 3(B). The substrate saturation curve indicated that the enzyme follows hyperbolic kinetics. This result was further confirmed by the linearity in the corresponding double reciprocal plot. The K_m and V_{max} values estimated from the double reciprocal plot were found to be 0.63 mg/mL (26.7 µM taking the molecular weight of the azocasein as 23.6 kDa) and 2.23 units/mL respectively under the experimental conditions. At this juncture, it has to be mentioned that Michaelis-Menten kinetics applies to an enzyme-substrate system where the substrate has a single cleavage or bonding site per molecule. Besides, the substrate should have a well-defined molecular weight much smaller than that of the enzyme. Therefore, there is a need for taking caution in

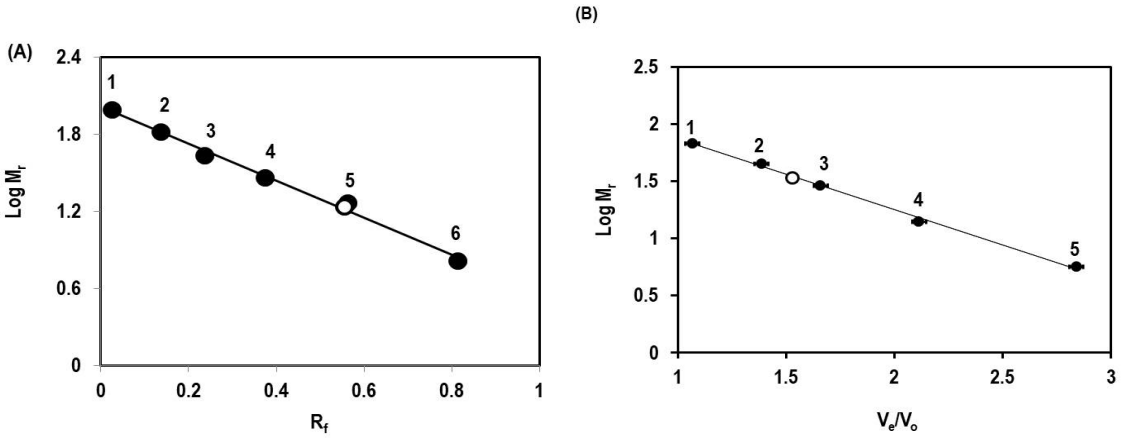

Figure 2: (A) Determination of relative subunit molecular weight of the purified protease based on SDS-PAGE in **Figure 1 (A) Lane 3**. The filled circles (●) labelled 1 to 6 represent the experimental points corresponding to standard proteins, viz. phosphorylase b (M_r 97.4 kDa), bovine serum albumin (M_r 66 kDa), ovalbumin (M_r 43 kDa), carbonic anhydrase (M_r 29 kDa), lactoglobulin (M_r 18.4 kDa) and aprotinin (M_r 6.5 kDa), respectively. The unfilled (o) circle represents the experimental point corresponding to the purified protease.

(B) Determination of native molecular weight of the purified protease based on gel filtration through a Sephadex G-75 column (size, 1x42 cm). The filled circles (●) labelled 1 to 5 represent experimental points corresponding to the standard proteins, *viz*. bovine serum albumin (M_r 67 kDa), egg albumin (M_r 45 kDa), carbonic anhydrase (M_r 29 kDa), lysozyme (M_r 14 kDa) and recombinant insulin (M_r 5.6 kDa), respectively. The unfilled circle (o) represents the experimental point corresponding to the purified protease.

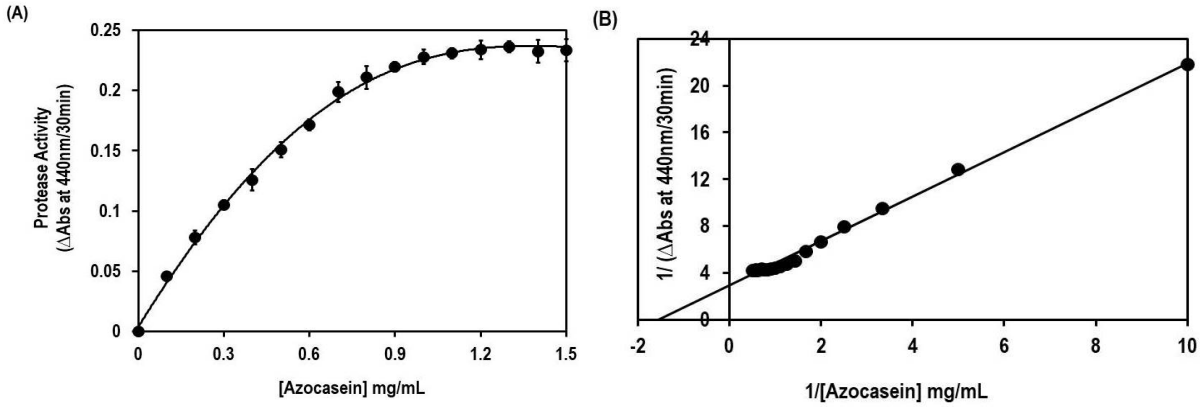

Figure 3: Substrate saturation kinetics behaviour of the purified protease using azocasein as the substrate **(A)** Hyperbolic substrate saturation curve of the protease (0.89 µg/mL protein in test). **(B)** The corresponding double reciprocal plot of the data of **Figure 3(A)**.

describing the kinetics behaviour of the protease acting on the substrate azocasein having multiple cleavage sites per molecule (Sorokhaibam et al., 2015). The K_m value of the cysteine protease purified in the present investigation was found to be more or less comparable with those of other latex cysteine proteases from the plants belonging to the same Apocynaceae family reported in the literature, e.g. 25 µM for ervatamin B (*Ervatamia coronaria*) (Kundu et al., 2000), 22 µM for procerain A and 31 µM for procerain B (*Calotropis procera*) (de Freitas et al., 2016).

Effect of pH

The effect of pH on the activity of the protease purified from the latex of *Thevetia peruviana* was determined using azocasein as the substrate as described in *Protease Assay* by varying the pH in the otherwise standard assay mixture from 3.0 to 9.0 using different designated buffers. The results are shown in Figure 4. The protease was found to exhibit optimal activity at pH 6. It is within the optimum pH range of 5 to 10, exhibited by other latex cysteine proteases from different plants belonging to the same Apocynaceae family (Sundd et al., 1998; Priolo et al., 2000; Kundu et al., 2000), and it is more or less same with that of ervatamin B (pH 6 - 6.5) from *Ervatamia coronaria* (Kundu et al., 2000).

Optimum Temperature

The effect of temperature on the activity of the protease purified from the latex of *Thevetia peruviana* was determined using azocasein as the substrate by varying the incubation temperature from 10 to 90ºC in the otherwise standard *Protease Assay*. The results are shown in Figure 5. The enzyme was found to exhibit optimal activity at 50ºC. It compares well with the temperature optima of other latex cysteine proteases from different plants belonging to the same Apocynaceae family, e.g. 50-55ºC for ervatamin from *Ervatamia coronaria* (Sundd et al., 1998; Kundu et al., 2000) and 55-60ºC for procerain from *Calotropis procera* (Dubey & Jagannadham, 2003).

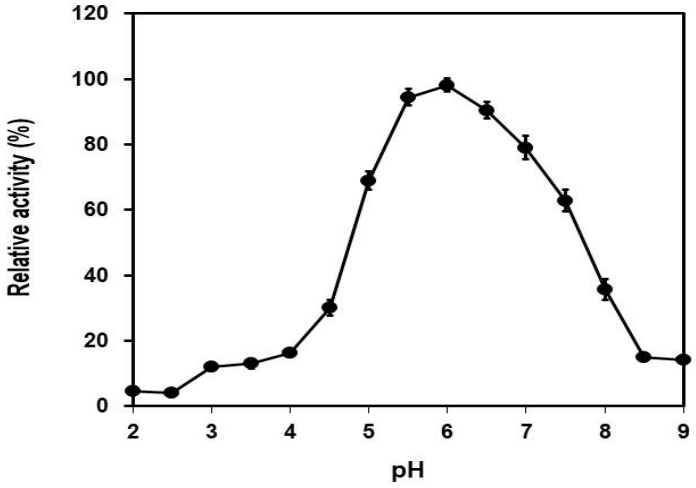

Figure 4: Effect of pH on the proteolytic activity of the purified protease (1.1 µg in test). The proteolytic activity was determined using azocasein as the substrate by varying the incubation pH in the otherwise standard *Protease Assay*. The buffers used were 0.1 M glycine-HCl (pH 3.0 - 3.5), 0.1 M sodium acetate (pH 4.0 - 5.5), 0.1 M sodium phosphate (pH 6.0 - 8.0), and 0.1 M Tris-HCl (pH 8.5 - 9.0).

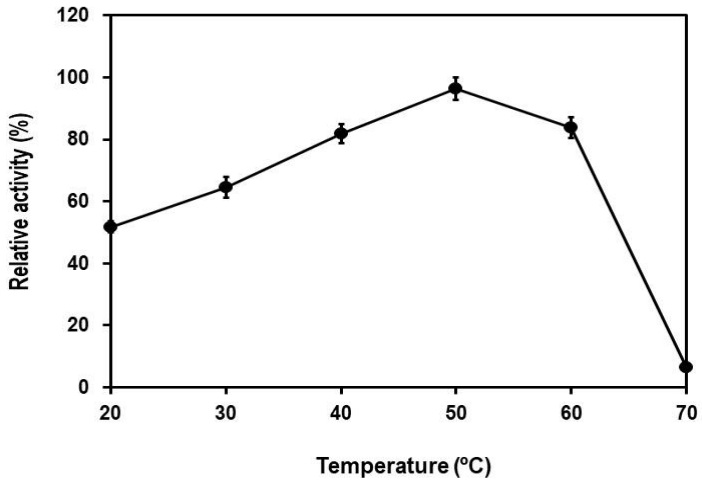

Figure 5: Effect of temperature on the proteolytic activity of the purified protease (1.1 µg in test). The proteolytic activity was assayed using azocasein as the substrate by varying the incubation temperature in the otherwise standard *Protease Assay*.

Effect of Temperature on the Enzyme Stability

Thermal stability behaviour of the protease purified from the latex of *Thevetia peruviana* was studied by following the method of (Sorokhaibam et al., 2015). Aliquots of the enzyme sample in 0.1 M sodium phosphate buffer pH 6 at 21.3 µg/mL were incubated at different designated temperatures ranging from 10 to 90ºC in microfuge tubes for 10 min using a digital circulatory water bath (accuracy ± 0.01ºC). At the end of incubation, the protease sample was cooled in ice-cold condition for 1 min followed by 4 min standing at room temperature, and then the sample was spun down by brief centrifugation. The residual activity of the protease sample was then determined by following the standard *Protease Assay* as usual. It was then plotted as a function of temperature, as shown in Figure 6. The enzyme retained full activity up to the incubation temperature of 50ºC. Beyond this, the enzyme activity was decreased. The residual activity decreased to 76% at the incubation temperature of 60ºC, and to 0% at the incubation temperature of 70ºC under the experimental conditions. The $T_{½}$ for the thermal inactivation of the enzyme was found to be 64ºC. The thermostability behaviour exhibited by the purified protease compares well with the latex cysteine proteases from different plants belonging to the same Apocynaceae family, *viz*. miswak protease P1 from *Salvadora persica* (Abdulaal, 2018) and procerain from *Calotropis procera* (Dubey & Jagannadham, 2003).

Determination of Protease Class

Based on the active site amino acid residue or metal ion dependency, proteases are currently classified into six broad classes: serine proteases, threonine proteases, cysteine proteases, aspartate proteases, glutamic acid proteases, and metalloproteases (López-Otín & Bond, 2008). To determine the class to which the protease purified from the latex of *Thevetia peruviana* belongs, it was subjected to inhibition

by known class-specific standard protease inhibitors. The results are shown using the bar diagram in Figure 7. Among the inhibitors studied, only the cysteine protease specific inhibitors, viz. E-64 and $HgCl_2$ at their effective concentration range were found to exhibit significant inhibition of the protease activity. Thus, the results of the inhibition assay showed that the purified protease belongs to the cysteine protease class. This finding is consistent with the literature report on the presence of cysteine proteases in the latex of many plants belonging to the same Apocynaceae family, e.g. procerain from *Calotropis procera* (Dubey & Jagannadham, 2003), ervatamin A and B from *Ervatamia coronaria* (Sundd et al., 1998; Kundu et al., 2000), araujiain h l from *Araujia hortorum* (Priolo et al., 2000), solanain from *Vallaris solanacea* (Somavarapu et al., 2017) and morrenain b I from *Morrenia brachystephana* Griseb., (Vairo Cavalli et al., 2003).

Fibrinolytic activity

The fibrinolytic activity of the protease purified from the latex of *Thevetia peruviana* was determined by fibrin agarose gel plate method as described in *Fibrinolytic activity by fibrin agarose gel plate method*. The fibrin dissolved in the agarose gel was subjected to the action of the purified protease loaded into the wells punched in the gel plate and subsequently subjected to TCA treatment. The fibrinolytic action of the protease was then confirmed by the appearance of clear zones around the sample wells against the opaque background formed by the precipitated unhydrolyzed fibrin. There was no clear zone formation around the control well loaded with heat-inactivated purified protease sample. Furthermore, the clear zone formed around the sample well loaded with higher amount of protease was more significant. The results are shown in Figure 8(A). The appearance of a clearer zone around the sample well loaded with more amount of protease confirmed that the fibrinolytic activity was solely due to the purified protease. The fibrinolytic activity of the protease was double-checked by a method involving gel filtration of the post-fibrinolysis reaction mixture through a Sephadex G-75 column as described in *Fibrinolytic activity by gel filtration method*. The fibrinolysis reaction mixture obtained following the action of the purified protease on fibrin was subjected to gel filtration through the Sephadex G-75 column. The control experiment was run by taking the heat inactivated purified protease. The two corresponding gel filtration profiles obtained by plotting absorbance at 280 nm against elution volume are shown together for comparison in Figure 8 (B). The elution profile obtained by taking the active protease was characterized by a smaller protein peak followed by a broad tailing peak of low absorbance, the latter being due to the elution of fibrin fragments formed by fibrinolysis. These experimental results re-confirmed that the protease purified from the latex of *Thevetia peruviana* exhibits fibrinolytic activity. Fibrinolytic activities of other latex cysteine proteases were also reported from a few plants belonging to the Apocynaceae family, viz. *Asclepias curassavica* L., *Calotropis gigantea* R.Br., *Pergularia extensa* R.Br. and *Cynanchum puciflorum* R.Br. (Shivaprasad et al., 2009).

Peptide Mass Fingerprinting

The protease purified from the latex of *Thevetia peruviana* in the present investigation was subjected to peptide mass fingerprinting by LC-MS/MS. Protein searches based on the data thus acquired were carried out using Morpheus software (Wenger & Coon, 2013) (http://cwenger.github.io/Morpheus/) against plant proteome database obtained from Swiss-Prot (as of July 24, 2020). The tryptic peptides were found to have insignificant matches with the peptides derived from eleven proteins listed in the database. However, these peptides matched up to a sequence coverage of 20.79% with the peptides derived from a 202-amino acid-residue fragment obtained from a germin

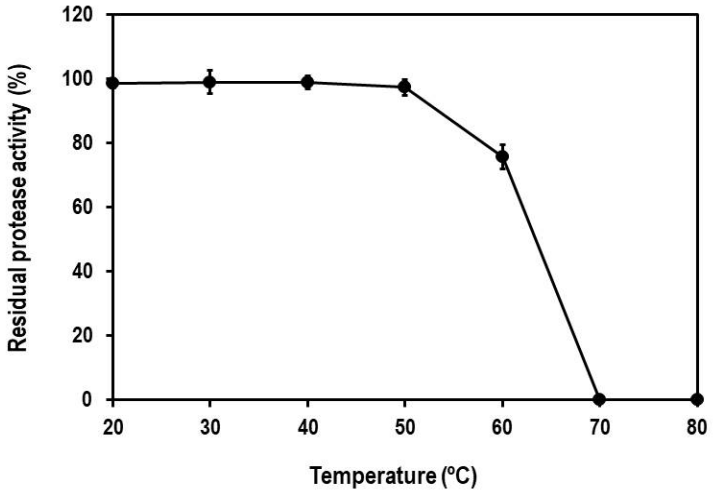

Figure 6: The thermostability behaviour of the purified protease. The protease solution in 0.1 M sodium phosphate buffer pH 6 at 21.3 µg/mL was incubated for 10 min at the designated temperatures. The rest of the procedure is described as in *Thermal Stability of the Enzyme* under MATERIALS AND METHODS.

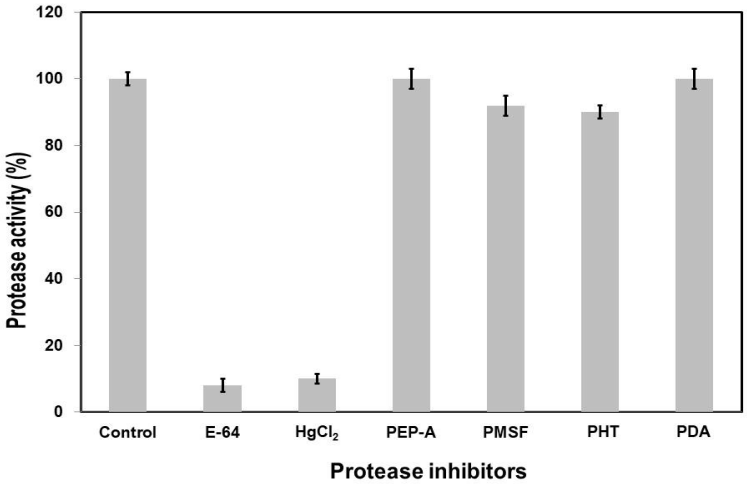

Figure 7. Effect of different protease inhibitors on the activity of the purified protease (3.2 µg in test). The inhibitory effect was determined by pre-incubating each of the inhibitors with the enzyme. The residual protease activity was determined by the otherwise standard *Protease Assay*. The corresponding activity obtained in the absence of inhibitor was taken as the control with 100% activity. Inhibitors and their concentrations tested were: E-64, 300 µM; $HgCl_2$, 2.5 mM; PEP-A (Pepstatin A), 10 µM; PMSF (Phenyl methyl sulphonyl fluoride), 10 mM; PHT (1,10 - Phenanthroline), 10 mM and PDA (Pyridine 2,6-dicarboxylic acid),10 mM.

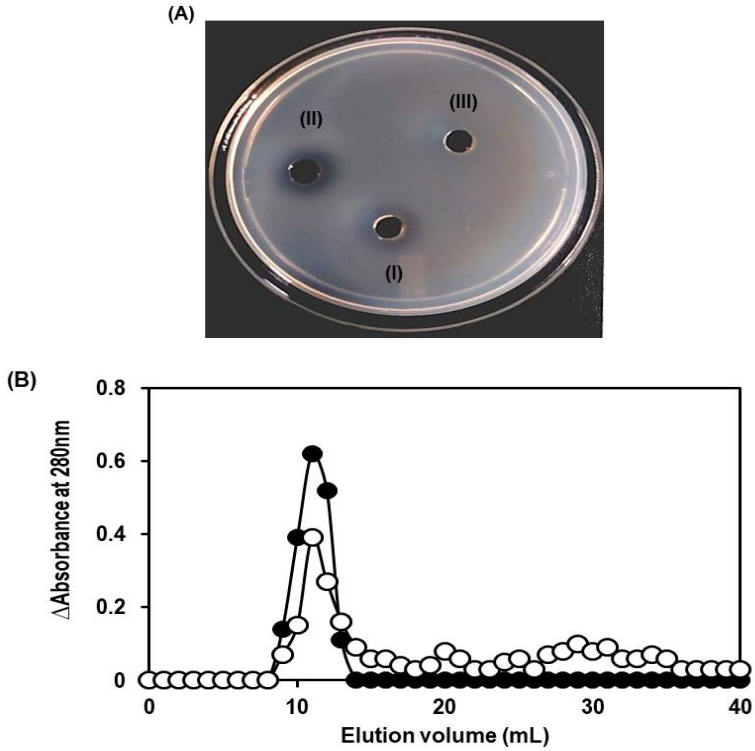

Figure 8: Determination of fibrinolytic activity of the purified protease. **(A)** Fibrinolytic activity by fibrin-agarose gel plate method as described in *Fibrinolytic activity by fibrin agarose gel plate method*. Sample well I, 0.65 µg purified protease; sample well II, 1.3 µg purified protease; control well III, 1.3 µg heat-inactivated purified protease. **(B)** Fibrinolytic activity by gel filtration method as described in *Fibrinolytic activity by gel filtration method*. The plot with the unfilled (o) circles represents the elution profile of the reaction mixture of fibrin and purified protease. On the other hand, the plot with the filled circles (●) represents the elution profile of the reaction mixture of fibrin and heat-inactivated purified protease.

like protein listed in the database (data not shown in the text but included as supplementary files, Annexure I & II). This germin like protein was also reported as a cysteine protease designated peruvianin-I by de Freitas et al. (2016). As the subunit molecular weight of the protease purified in the present investigation was determined to be 18.4 kDa, the number of amino acid residues it contains must be less than that of peruvianin-I. Thus, the two proteases, despite their similarity with 20.79% sequence coverage on peptide mass fingerprinting analysis, must be different. Moreover, a difference was also observed between the two proteases with respect to their ionic behaviour. In the present investigation, the unpurified latex protease extract was resolved into three distinct protein peaks when subjected

to DEAE-cellulose chromatography and the slowest eluting protein peak corresponded to the purified protease. On the other hand, de Freitas et al. (2016) found the protease peruvianin-I to elute together with other proteins as a single protein peak on DEAE-cellulose chromatography, and therefore, the investigators resorted to gel filtration for purifying the protease. Differences between the two proteases were also observed with respect to some other physico-chemical characteristics under similar experimental conditions, *viz*. native molecular weight, subunit structure, K_m value and optimum temperature. The purified protease was a homodimer with a native molecular weight of 36 kDa, whereas peruvianin-I was a homohexameric protein with a native molecular weight of 120 kDa. The K_m value of the purified protease (26.7 µM) was higher than that of peruvianin-I (17.6 µM) when azocasein was used as the same substrate. Further, the temperature for optimal activity of the purified protease (50ºC) was higher than that of peruvianin-I (25-37ºC). Based on these differential experimental observations obtained by mass spectrometric and physico-chemical analyses, it can be safely concluded that the cysteine protease purified in the present investigation is distinct from the cysteine protease peruvianin-I reported by de (Freitas et al., 2016).

CONCLUSION

A homodimeric cysteine protease with a native molecular weight of 36 kDa was purified from the latex of *Thevetia peruviana* (Pers.) Merr. to electrophoretic homogeneity. The purified protease acting on azocasein as its substrate exhibited hyperbolic kinetics, and it was characterized by specific activity of 71 units/mg protein, K_m value of 26.7 µM, V_{max} value of 2.23 units/mL, optimum pH of 6, optimum temperature of 50°C, and $T_{½}$ of 64°C. The enzyme also showed fibrinolytic activity. Peptide mass fingerprinting analysis showed that the protease purified in the present investigation was not identical with any protein characterized earlier.

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