Hemostatic, milk clotting and blood stain removal potential of cysteine proteases from *Calotropis gigantea* (L.) R. Br. Latex

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ABSTRACT

Introduction: Plant latex is a natural source of biologically active compounds and several hydrolytic enzymes responsible for their diverse health benefits. Recent past has witnessed substantial progress in understanding their supplementary industrial and pharmaceutical utility. *Calotropis gigantea* is one of the important latex producing plants belonging to asclepediaceae family with wide ethnopharmacological applications and is rich in proteolytic enzymes. Present study investigates hemostatic, milk clotting and blood stain removal potential of *C. gigantea* latex proteases. **Materials and Methods:** The protease activity of crude enzyme (CE), obtained by centrifugation followed by ammonium sulphate precipitation and dialysis, was assayed using casein as the substrate. Effect of pH, temperature and specific inhibitors on protease activity was determined. Native PAGE and in gel protease activity of CE was performed. Hemostatic (Fibrinogen polymerization, fibrinogen agarose plate and blood clot lysis assays), milk clotting and blood stain removal efficacies of CE were determined. **Results:** CE exhibited high caseinolytic activity. Enzyme activity was optimum at 37-50°C and pH 8.0. Fibrinogen polymerization assay showed concentration dependent increase in turbidity indicating thrombin like activity which was further confirmed by fibrinogen agarose plate assays. Clot lysis assay indicated 92.41% thrombolysis by CE in 90 min. CE also revealed significantly high ratio of milk clotting to protease activity (Milk Clotting Index, MCI = 827.59 ± 1.52). Complete destaining of blood stained fabric was observed when incubated with 1% detergent incorporated with 0.1mg/ml CE. The study highlights and validates the compound application potential of latex cysteine proteases from *C. gigantea.*

Key words: Latex proteases, milk clotting, pharmaceutical, procoagulant, stain removal

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INTRODUCTION

Proteases constitute one of the most important groups of industrial enzymes, accounting for about 60% of the total enzyme market.[1] Recent years have envisaged a surge in enzyme market growth due to diverse key factors including cost effectiveness and productivity.[2] Plant proteases have been implicated in the design and synthesis of therapeutic agents.[3] Accumulating data sheds light into their possible suitability in other applications such as food and detergent industries. Proteolytic enzymes from plant latexes are of widespread interest due to their involvement in various physiological functions and economic benefits. They receive added attention due to broad substrate specificity and activity in wide range of pH, temperature, in presence of organic compounds and other additives.[4]

Presence of latex, a complex milieu of several hydrolytic enzymes, is one of the characteristic features of plants belonging to the families Euphorbiaceae, Asclepiadaceae, Moraceae and Apocyanaceae.[5] Family Asclepediaceae includes more than 2,000 species classified under 280 genera that are distributed worldwide in the tropical and sub-tropical regions. Most of the Asclepediaceae members are perennial herbs and their latex is exclusively used as a common remedy for wound healing and to stop bleeding on fresh cuts by traditional healers.[6-8] *Calotropis gigantea* commonly known as milkweed is one of the important latex producing plants belonging to Asclepediaceae family and is mostly spread across in the tropics and sub tropics.[9] The latex of this plant has been described for exhibiting diverse pharmacological properties including antimicrobial activities.[10,11] Saratha *et al.* has extracted and characterized a

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pentacyclic triterpenoid, Lupeol, from the latex of *C. gigantea* which may account for various biological activities exhibited by this plant.[12] The search for novel proteases from medicinal plants continues to be of extreme importance globally because of the increased multidrug resistance and toxicity associated with the existing remedies. Procoagulant and thrombolytic effects of proteases from many medicinal plants and their hemostatic utility are receiving much attention in the recent past. Proteases interfering in blood coagulation and fibrin hydrolysis have been isolated and characterized from several plant latexes.[13-15] Apart from its medicinal utility, proteases have also contributed significantly in food and detergent industry. The increasing demand for cheese, its insufficient supply, high cost of rennet and associated ethical issues have led to the search for a suitable alternative to rennin.[16,17] Vegetable extracts have been used as milk coagulants since ancient time inspite of poor scientific validation about their action. Several plant extracts (latex, leaves, fruits, flowers and seeds) having the capacity to coagulate milk with high proteolytic activity have been isolated. Ficin from *Ficus sp.*, papain from *Carica papaya* etc., are some of the early established vegetable milk coagulants from latex.[18,19] One of the largest applications of proteases is in detergent industry, where they help removing protein based stains from clothing by improving the cleaning efficiency of detergents.[20,21] Plant latex, with its easy availability and wealthy proteolytic enzyme load, may also serve as a possible source of cost-effective detergent enzyme/s.[22]

There appears to be a growing research interest in identifying and providing evidence of novel proteases with multiple industrial applications prospective owing to their escalating demand and production cost. Present paper reports the proteolytic behavior of *C. gigantea* latex. The present investigation reports the biochemical evaluation and potential application prospective of *Calotropis gigantea* latex proteases in wound healing, milk clotting and strain removal.

MATERIALS AND METHODS

Materials

Human fibrinogen and human thrombin were purchased from Sigma Aldrich, St Louis, MO, USA. Casein, Bovine Serum Albumin, papain, trypsin (RM612), rennin, TEMED, phenylmethylsulphonyl fluoride (PMSF) and PAGE chemicals from HI–MEDIA, Mumbai, India. Ammonium persulphate, Sodium dodecyl sulphate, Folin–Ciocalteu (FC) reagent, ethylene diaminetetracetic acid (EDTA) and 1,10- Phenanthroline purchased from Merck Speciality Pvt. Ltd, Worli, Mumbai. Iodoacetic acid (IAA) from Spectrochem Pvt. Ltd, India. All the chemicals and reagents used were of analytical grade. Plant latex was collected from in and around Bangalore, India. Fresh human blood samples were collected from healthy volunteers after obtaining their consent.

Plant material and its processing

The latex was obtained from the tender parts of *Calotropis gigantea*. A specimen voucher was deposited at the National Institute of Ayurveda and Dietetics, Jayanagar, Bangalore for plant identification and authentification (RRCBI/MCW/06). The latex was collected in clean glass beaker by breaking tender parts of *Calotropis gigantea*. This latex was diluted with equal volume of phosphate buffer (pH = 7.0) and kept overnight at 4°C. The supernatant was decanted and centrifuged at 12,000 g for 20 min at 4°C. The clear supernatant was decanted and dialyzed against 10mM phosphate buffer. The supernatant was subjected to protein precipitation by 80% ammonium sulphate. After ammonium sulphate precipitation, the sample was subjected to centrifugation for 10 min at 10,000g. The precipitated pellet was dissolved in 10mM phosphate buffer and dialyzed against the same buffer to remove ammonium sulphate.[23] The protein concentration of CE was measured at 660nm using Folins reagent.[24]

Caseinoytic activity

Caseinoytic activity was assayed by the method of Murata *et al.*[25] Briefly, casein 0.4ml was incubated with different concentration (20-100 μg) of crude extract trypsin and papain at 37°C separately for 2 h. The reaction was stopped by adding 1.5ml of the 0.44M TCA and allowed to stand for 30 min followed by centrifugation at 1500g for 15 min. An aliquot (1ml) of the supernatant was mixed with 2.5 ml of the 0.4M sodium carbonate and 0.5 ml of FC reagent (1:2) followed by reading absorbance at 660nm. One unit of enzyme activity was defined as the amount of the enzyme required to increase in absorbance of 0.01 at 660 nm/h at 37°C. Activity expressed as units/h at 37°C. Inhibition studies were carried out after preincubating the latex enzyme fractions with or without specific protease inhibitors separately for 15 min. Further, the assay was carried out as described above.

pH and temperature optima

The proteolytic activity of CE of *C.gigantea* was examined in the pH range of 2.0-12 to determine the optimum pH. The buffers used were 0.05 M KCl-HCl (pH 1.0-2.0), 0.05 M glycine-HCl (pH 2.0-3.0), 0.05 M sodium acetate (pH 4.0-5.0), 0.05 M sodium phosphate (pH 6.0-7.), 0.05 M Tris-HCl (pH 8.0-10.0), and 0.05 M sodium carbonate (pH 11-12). Substrate solution of casein (1% w/v) was prepared in the respective pH buffers and activity was taken at the same pH as per the method described earlier at 37°C.

The effect of temperature (in the range of 10-90°C) on the activity of CE of *C.gigantea* was also studied using casein as the substrate. Prior to the assays, the substrate solution was also equilibrated at the corresponding temperature in the same buffer. At each temperature, a control assay was carried out without the enzyme and used as a blank.

Electrophoresis

Native PAGE was carried out according to the method of Davis *et. al.,*[26] for CE under basic (pH 8.3) condition using Tris - HCl buffer. The bands were visualized by staining with Coomassie brilliant blue R-250.

Zymography

In-gel protease assay was performed using 0.1% gelatin co-polymerised with the resolving gel (10% SDS-PAGE).[27] Gel was stained with 0.15% CBB R-250 in water: Methanol: Acetic acid (50:40:10) and destained with same solution without dye to visualize the clear hydrolytic zone.

Haemostatic effect

Procoagulant assays

Fibrinogenolytic effect of CE was studied via fibrinogen polymerizing assay spectrophotometrically at 540 nm[28] and subsequent fibrinogen agarose plate assay.[29]

Blood clot lysis activity

Venous blood drawn from healthy volunteers was transferred in different pre weighed sterile microcentrifuge tube (500 μl/tube) and incubated at 37°C for 45 minutes. After clot formation, serum was completely removed and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube - weight of tube alone). Each micro-centrifuge tube containing clot was properly labeled and various concentrations of crude enzyme was added to the tubes (20-100 μg). Distilled water was added to one of the tubes containing clot and this served as a negative control. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. Fluid obtained after incubation was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis.[30]

Milk clotting activity

Milk-clotting activity was determined according to the method described by Arima, Ya, and Iwasaki (1970) with a slight modification.[31] The substrate (10% skim milk, w/v in 0.01 M $CaCl_2$) as prepared and pH was adjusted to 6. The substrate (2.0 ml) was pre-incubated for 5 min, at 37°C, 0.2 ml of enzyme was added and the curd formation was observed at 37°C, while manually rotating the test tube from time to time. The end point was recorded when discrete particles were discernible. One milk-clotting unit is defined as the amount of enzyme that clots 10ml of the substrate within 40 min. Milk clotting activity (MCA) U/ml = (2400/clotting time in sec) X dilution factor

From the ratio of MCA to proteolytic activity (PA), the milk clotting index (MCI) was calculated.

Detergent stability and blood stain removal studies

The compatibility of the partially purified protease with three commercially available detergents (Surf Excel, Tide and Ariel, Proctor and Gamble, India) was studied. The enzyme was incubated in 1% of above detergent (w/v) solutions at pH 7.5 at room temperature for overnight before measuring the enzyme activity. Enzyme activity without any detergent was considered as 100%. To evaluate the stain removal, clean cotton cloth pieces (5 × 5cm) were soiled with blood, dried and incubated in 1% detergent with 0.1 mg/ml of crude enzyme for 5 h, same size cotton cloth with blood stain soaked in 1% detergent alone served as the control.[22]

Statistical analysis

The data obtained from five independent experiments were analyzed using Graph Pad Prism (CA92037, USA). Each value represents the mean of five independent experiments performed in triplicates, with average SD of <5% and wherever applicable, the data were analyzed by students t-test and $P < 0.05$ were considered statistically significant.

RESULTS

The dialysed crude latex enzyme of *C.gigantea* resolved into three major bands when subjected to Native PAGE under non reducing conditions. The data generalizes the fact that crude enzyme contained three major proteins [Figure 1a]. CE it exhibited a strong caseinolytic activity when 2% casein was used as substrate. The

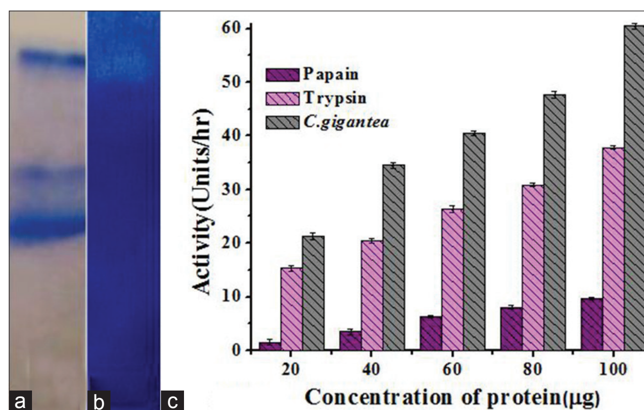

Figure 1: (a) Native gel electrophoresis of *C.gigantea* CE on 10% polyacrylamide gel, (b) Zymography (c) Caseinolytic activity of CE of *C. gigantea*, trypsin and papain

activity was progressively increasing with increase in the concentration of the latex protein. 20 to 100 μg of latex protein concentration gave a mean activity of 21.33 ± 0.57 to 60.5 ± 0.5 units/hour respectively. The proteolytic activity of CE was significantly higher than both the positive controls, papain ($P < 0.05$) and trypsin ($P < 0.05$) [Figure 1c]. In gel protease activity of CE by zymography (nondenaturing SDS PAGE) showed a clear zone of proteolytic activity against dark blue background [Figure 1b]. The proteolytic effect was completely inhibited by IAA, a specific cysteine protease inhibitor. There was no inhibition observed with any of the other specific protease inhibitors. The pH optimum for caseinolytic activity of CE of *C.gigantea* was 8 with a relatively higher activity from pH 7 to 9. The temperature optimum of CE of *C.gigantea* was between 37 to 50ºC. There is a sudden decrease in the activity on either side of this optimal range. procoagulant effect of CE was analyzed by spectrophotometric fibrinogen polymerization and fibrinogen agarose plate assays. Fibrin polymerization effect was quantitatively assayed by taking absorbance of fibrinogen solution at 540nm. There was an increase in the absorbance values of fibrinogen solution with increase in the protein concentration of CE, suggesting the ability of the enzyme/enzymes in hydrolyzing fibrinogen and its subsequent polymerization to form fibrin threads [Figure 2a]. The results of the fibrinogen agarose plate assay were also supporting the spectrophotometric results. With 10μg protein, CE produced a zone of precipitation having 1.1cm diameter which was comparable to the one given by 0.2 units of human thrombin [Figure 2b]. Negative control well showed complete absence of precipitation zone, suggesting the thrombin like activity associated with the latex of *C. gigantea*. However, prolonged incubation of CE (4 hours) resulted in clearance of the precipitation zone [Figure 3b]. Assay on blood clot dissolution property of CE exhibited approximately 92.41% ±0.29 of clot lysis with 100μg of protein [Figure 3a inset].

The clearing of fibrin precipitation area by CE of *C.gigantea* on prolonged incubation in fibrinogen agarose plate also substantiates the enzymes thrombolytic property. The procoagulant, as well as clot lytic effect of *C. gigantea* latex point towards its fitness as a haemostatic agent. Apart from the pharmaceutical suitability, the biotechnological applications of *C.gigantea* latex CE such as milk clotting and detergent stability and blood stain removal activity were also explored.

The CE could coagulate skimmed milk to white and firm curd [Figure 4a]. A mean MCA of 480.33 ± 1.52 U/ml was exhibited by CE whereas the standard milk coagulant, rennin, gave 282.35 ± 1.1 U/ml. Milk clotting index (MCI), a ratio of milk-clotting activity to proteolytic activity, was found to be higher than that of [Table 1]. CE showed very high stability in all detergents used for the study. It showed 100% proteolytic activity in all detergents (1% w/v) used. CE along with the detergents enhanced blood destaining than the detergent alone, supporting its significance in detergent industry [Figure 4b].

Table 1: Milk clotting activity, proteolytic activity and MCI of *C. gigantea* and rennin

Source of milk coagulant	Milk clotting activity (U/ml)	Proteolytic activity(OD at 660nm)	Milk clotting index (MCA/PA-U/OD 660nm)
C. gigantea	480.33±1.52	0.58	827.59±1.52
Rennin	282.35±1.1	0.06	4705.88±0.23

MCI: Milk clotting index; MCA: Milk clotting activity; PA: proteolytic activity

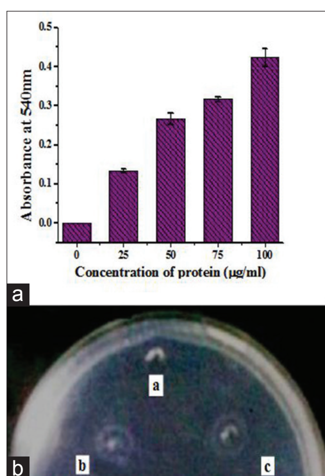

Figure 2: (a) Spectrophotometric analysis of fibrin clot formation by CE of *C.gigantea* (b) Results of Fibrinogen agarose plate assay: A-control b- 0.2 unit Thrombin, c- 10 μg of crude enzyme of *C. gigantea*

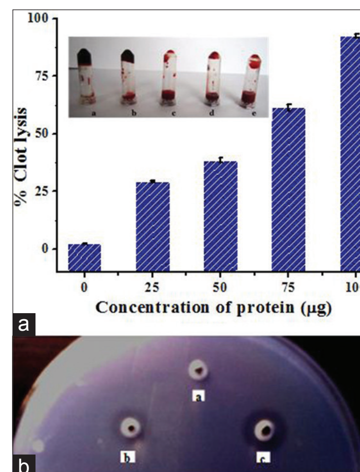

Figure 3: (a) % Clot lysis of blood samples by different concentration of CE of *C. gigantea* (Inset: Clot lysis of blood samples by different by different concentration of CE of *C.gigantea*) (b) Fibrinogen agarose plate upon prolonged incubation, (a) negative control (buffer), (b) and (c) 5 μg and 10 μg of CE of *C.gigantea*

DISCUSSION

Interest in proteolytic enzymes, particularly those from plant lattices, is gaining attention in the pharmaceutical industry and biotechnology because of their stability over wide ranges of temperature and Ph.[32] *C.gigantea* from family Asclepiadaceae has gained more attention for its wound healing properties. Present study, reports the haemostatic, milk clotting and stain removal properties of crude enzyme from *C.gigantea* latex. The results show that three industrially important functional properties are associated with the latex proteases of this plant species.

Proteolytic activity of *C.gigantea* latex was found to be significantly higher than that of papain and trypsin. Zymogram of CE invariably revealed a smudged clearance zone supporting the possibility of cysteine protease multiplicity which has been earlier demonstrated in the latices of several plants.[33] IAA completely inhibited caseinolytic activity supporting the cysteine protease nature of *C.gigantea* CE. Earlier studies have shown the presence of only cysteine proteases in latices of Asclepiadaceae family.[14,34] Our observation is also in accordance with these reports. Purification of proteases from *C.gigantea* has shown existence of four to five cysteine proteases.[33] The high proteolytic activity of CE in the pH range of 7-9 with optimum at pH 8 and temperature range of 37-50°C shows its suitability for many industrial applications.

Fibrinogen polymerization assay and fibrinogen agarose plate assay were carried out to assess thrombin like activity of CE. This observation supports the earlier reports on thrombin like activity by *C.gigantea latex* proteases.[13,28] Apart from procoagulant nature, proteases from plant latices have been shown to exhibit blood clot dissolution (Plasmin like activity). Latex proteases efficiently hydrolyzed and dissolved fibrin.[28,29] These dual activities by some latex proteases have projected them as good alternatives in the management of fresh cuts and wounds.[35,36] Results from the present study show CE of *C.gigantea* also possesses significantly high clot lytic potential. A new simplified test to evaluate clot lysis developed by Swetha *et. al.,* was used in the study to assess fibrinolytic activity. Comparision of extent of clot lysis by CE (100 μg of protein) with their results using streptokinase (100ul of undiluted streptokinase, 30000 I.U), demonstrates the paramount degree of thrombolytic value possessed by this plant candidate.[30] The formation of zone clearance by CE during an extended incubation period in fibrinogen agarose plate supports its thrombolytic action. Similar studies on various latex producing plants showed the involvement of their latex proteases in both blood coagulation and fibrinolysis.[12,15] Cysteine proteases present in the latex of a closely related species, *C. procera*, exhibited thrombin- and plasmin-like activities *in vivo* suggesting its therapeutic potential in various conditions associated with coagulation abnormalities[37]

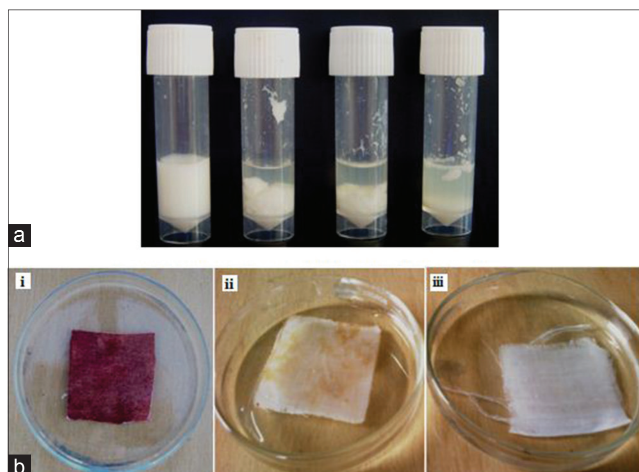

Figure 4: (a) Milk clotting activity (Representative picture) (b) Evaluation of stain removal activity of *C. gigantea* latex protease, (i) - Cloth piece of 5 × 5 cm soaked only in 1% detergent (ii) 2b - Incubated with 1% detergent (iii) Incubated with 1% detergent incorporated with 0.1 mg/ml CE

Plant enzymes are relatively safe, inexpensive, readily available and are generally acceptable for applications. Efficiency of milk coagulant enzymes from plants including *Calotropis procera* have been attempted by previous investigators.[38,39] CE coagulated skimmed milk into a white and firm curd. MCI value observed in the present study is comparable to earlier reported MCI values of religiosin, rennin, papain and trypsin.[40] With its high MCI value CE could serve as a vegetable rennet source and a probable rennin substitute. However, its use in cheese making would need further investigations on quality of both milk curds and the cheese formed.

Proteases also do find huge application in laundry industries, where they help removing protein based stains from clothing. Current study gives evidence for the presence of such proteases in the CE of *C. gigantea*. The enzyme enhanced blood stain removal efficiency of commercially available detergents supporting its suitability as a source for detergent enzyme. Procerain B, a purified cysteine protease, from *Calotropis procera* efficiently hydrolyzed blood stain and was found to have potential application in detergent industries.[22] Similar results have also been reported for microbial proteases from *B. subtilis* PE-11 and *Pseudomonas aeruginosa*.[20,21,41]

The hemostatic and milk clotting property along with stain removal capacity of crude enzyme from *C.gigantea* latex specify that this plant latex could serve as a new protease source for pharmaceutical and biotech industries.

CONCLUSION

Industrial applications of proteases have posed several problems and challenges for their further improvements. A recent trend has involved conducting industrial reactions with enzymes reaped from plants. Plant latex represents an invaluable resource for pharmaceutical and biotechnological innovations. Present study validates that the proteases in CE of *C. gigantea* latex is likely to mimic some of the unnatural properties that are desirable for their commercial applications particularly in food, pharmaceutical and detergent industry.

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