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Biochemical characterization of proteases isolated from the latex of *Tabernaemontana divaricata* L. and *Carissa carandas* L.: Their role in hemostasis

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Abstract
In the present investigation, comparative profile of proteases isolated from the latex of *Tabernaemontana divaricata* L. and *Carissa carandas* L. of Apocynaceae and their role in hemostasis was studied. The crude enzyme was prepared from the latex of *Tabernaemontana divaricata* L. and *Carissa carandas* L. by repeated freeze-thaw followed by centrifugation. The protein yield was 30mg/ml for *Tabernaemontana divaricata* L. and 66mg/ml for *Carissa carandas* L. Sephadex G-75 fractionation yielded major peak TDP4 (19kDa) from *Tabernaemontana divaricata* L. and CCP1 (22kDa) from *Carissa carandas* L. with significant proteolytic activity. The enzymes showed dose-dependent caseinolytic, fibrinogenolytic, blood and plasma clot activities. TDP4 and CCP1 exhibited optimum pH at 6.8 and 7.8 & temperature at 40^0C $\pm2^0$C and 25^0C $\pm2^0$C respectively. TDP4 hydrolyzed blood clot, while CCP1 hydrolyzed plasma clot. Caseinolytic activity of TDP4 and CCP1 increased by 4-folds upon β-mercaptoethanol treatment and inhibited by iodine suggesting that the enzymes are serine proteases.

Keywords: *Tabernaemontana divaricata* L., *Carissa carandas* L., Serine Protease, Plant Latex, Fibrinogen, Caseinolysis

Introduction
Medicinal plants have become a vital module of today's' research activities. Be it food sector, cosmetic sector, fertilizers or pharmaceutical industries, the applications of products derived from medicinal plants are growing exponentially [1]. Medicinal plants are considered as a rich source of ingredients finding applications in drug development. Among the other ingredients, plant latex, which shows antibacterial, antifungal, antiviral, antitumor activities, is a rich source of various secondary metabolites. It shows toxicity to insects, act as growth and reproductive cycle inhibitor. It is reported to have anti-arthritic property and is widely used in various cosmetic products [2]. Plant latex is a rich source of hydrolytic enzymes, more profoundly proteases, which play a major role in host pathogen interaction [3, 4].

The plant latex derived proteases have gained importance due to their broad substrate specificity, stability and activity in extreme pH and temperature. Thiol and serine proteases are two rich classes of proteases found in plant latex. The limitation is using thiol proteases are that their proteolytic activity is readily inhibited by presence of metal ions. Thus, these proteases require mild reducing and chelating agents for activation and optimum activity and, are not suitable economically. On the other hand, serine proteases are much stable in extreme pH and temperatures ranging from 60-85 ^{0}C. Thus there is a continuous search for new potent serine proteases from plant latex having wider industrial applications than thiol proteases. *Tabernaemontana divaricata* L. (Apocynaceae), commonly called crape jasmine is an evergreen shrub native to India and now cultivated throughout South East Asia and the warmer regions of continental Asia. It is cultivated for the flowers and foliage. The stem exudes latex, which is used in Ayurvedic medicine. The plant has been reported to contain a variety of alkaloids, such as tabernaemontanine, coronaridine and dregamine [5]. *Carissa carandas* L. is a flowering shrub belonging to the family Apocynaceae. It produces berry-sized fruits that are commonly used as a condiment in Indian pickles and spices. It is reported that the plant is a rich source of sesquiterpenes namely carissone and carindone. The fruits of the plant have a sour and astringent taste and widely used to cure anemia [6]. In the present investigation, comparative profile of proteases isolated from the latex of *Tabernaemontana divaricata* L. and *Carissa carandas* L. and their role in hemostasis is studied.

Materials and methods

All chemicals (AR grade only) were procured from HiMedia Pvt. Ltd. India. Sephadex G-75 was procured from Sigma Aldrich Inc. USA.

Plants for the study

The plants for the study *Tabernaemontana divaricata* L. and *Carissa carandas* L was collected locally.

Preparation of the crude enzyme

Latex from the flowers of *Tabernaemontana divaricata* L. and fruits of the *Carissa carandas* L. was collected into glass tubes containing 1 ml of 10% sodium metabisulphite. The latex was subjected to repeated freezing and thawing, centrifuged at 7000 rpm at 4 °C for 30 min to remove suspended coarse inert materials. The supernatant was collected and used as the crude enzyme.

Total protein estimation

Total protein content was estimated according to the method of Bradford [7] using bovine serum albumin as internal standard.

Caseinolytic activity

The caseinolytic activity was assayed according to the method of Murata *et al* [8]. Briefly, 0.4 ml of 2% casein in 0.2M Tris-HCl buffer (pH 8.5) was incubated with different concentrations of crude enzyme of *Tabernaemontana divaricata* L. and *Carissa carandas* L. at 37 ^{0}C for 120 min. The reaction was stopped by adding 1.5 ml of 0.44M TCA and the mixture was allowed to stand for 30 min. The reaction mixture was centrifuged at 1500g for 15 min. An aliquot (1ml) of the supernatant was mixed with 2.5 ml of 0.44M sodium carbonate and 0.5 ml Folin and Ciocalteu's phenol (FC) reagent (1:2v/v). The intensity of the colour developed was read at 660 nm. One unit of enzyme activity was defined as the amount of enzyme required to increase the absorbance of 0.01 at 660nm/h at 37^{0}C. Activity was expressed as units/h at 37 ^{0}C

Fractionation on Sephadex G-75

The crude enzyme of *Tabernaemontana divaricata* L. and *Carissa carandas* L. were fractionated on Sephadex G-75. The total protein content of 10mg and15 mg/ml of *Tabernaemontana divaricata* L. and *Carissa carandas* L. was loaded on to the column pre-equilibrated with 0.1M phosphate buffer, pH 7.4. The fractions were eluted at a flow rate of 1.0 ml/5 min and monitored at 280nm. Each fractions with intense peaks were collected and lyophilised. The lyophilized fractions were tested for caseinolytic activity by dissolving 1mg of each of the lyophilised fractions in 1 ml of 0.1M phosphate buffer pH 7.4 separately. The fractions from *Tabernaemontana divaricata* L. and *Carissa carandas* L. showing maximum caseinolytic activity were named as TDP4 and CCP1 respectively.

Polyacrylamide gel electrophoresis of TDP4 and CCP1 enzyme fractions

Sephadex G-75 purified lyophilized fractions of *Tabernaemontana divaricata* L. and *Carissa carandas* L. TDP4 and CCP1 showing significant caseinolytic activity was tested for purity on 12.5% SDS-PAGE according to the method of Laemmli [9]. 20µg of each of TDP4 and CCP1 was loaded onto the gel along with protein marker ranging from 11kDa to 245kDa. The electrophoresis was carried out at 80V for 5% stacking gel and 40V for 12.5% resolving gel. After electrophoresis the gel was stained with 0.05% (w/v) Coomassie blue R250.

Determination of Optimum pH and temperature of TDP4 and CCP1 enzyme fractions

Optimum pH and temperature of TDP4 and CCP1 were determined according to the method of Kunitz [10]. The enzyme solution was incubated with the substrate (2% casein in 0.2M Tris-HCl buffer) with pH values ranging from 4.5 to 8.0 at 50 °C for 120min and the enzyme activity were measured. Similar experiment was done to determine the optimum temperature by incubating the enzyme solution with the substrate (2% casein in 0.2M Tris-HCl buffer, pH 7.0) at temperatures ranging from 40 to 70 ^{0}C for 10 min and the enzyme activity were measured.

Fibrinogenolytic activity TDP4 and CCP1 enzyme fractions

Fibrinogen hydrolyzing activity of TDP4 and CCP1 enzyme fractions was determined according to the method described by Ouyang and Teng [11]. Briefly 50µg of fibrinogen in 10mM Tris- Hcl buffer, pH 7.6 were incubated for 30 min at 27±2 ^{0}C with different concentrations (1-5µg) of TDP4 and CCP1. The reaction was terminated by adding 20µl of buffer containing 4% β-mercaptoethanol, 1M urea and 4% SDS and subjected to 12.5% SDS-PAGE. The electrophoresis was carried out at 80V for 5% stacking gel and 40V for 12.5% resolving gel. The fibrinogen-hydrolyzing pattern of the crude enzyme was visualized by staining the gel with coomassie brilliant blue R250 [12].

Human blood clot hydrolyzing activity of TDP4 and CCP1 enzyme fractions

The assay was carried out according to the method described by Rajesh *et al* [13]. Briefly, 100µl of citrated human blood obtained from the healthy male donor were mixed with the 30µl of 0.25M CaCl2 and allowed to stand for 30 min to form hard clot. The hard clot was thoroughly washed 4-5 times with 10mM phosphate buffered saline, pH 7.4. To this, 400µl of 10mM Tris -HCl buffer pH 7.4 was added, the reaction was intiated by adding different concentrations (10-100µg) of TDP4 and CCP1 in 0.01M Tris-HCl buffer, pH7.4. The undigested blood clot was preceipted by adding 1.5ml of 0.44M TCA and and allowed to stand for 30 min and centrifuged for 15 min at 1500 *g*. The aliquots of 0.5 mL supernatant were transferred to clean glass tubes, followed by the addition of 1.25 mL of 0.4 M sodium carbonate and 0.25 mL of 1:3 diluted Folin and Ciocalteu's phenol (FC) reagent. The color developed was read at 660 nm after allowing the tubes to stand for 30 min. One unit of activity is defined as the amount of enzyme required to increase the absorbance by 0.01 at 660 nm/h (37 °C). The enzyme inhibition studies of TDP4 and CCP1 were performed by pre-incubated the enzyme with or without specific protease inhibitor (20µg/ml) for 15 min.

Human plasma clot hydrolyzing activity of TDP4 and CCP1 enzyme fractions

The assay was carried out according to the method described by Rajesh *et al* [13]. Briefly, 100µl of citrated human blood obtained from the healthy male donor were mixed with 30µl of 0.25 M $CaCl_2$ and allowed to stand for 30 min to form a soft plasma clot. The fibrin clot was thoroughly washed with with 10mM phosphate buffered saline, pH 7.4 and

colorimetric assay was carried out. For analyzing the cleavage pattern of the plasma clot the washed clot (fibrin incubated with different concentration of TDP4 and CCP1 were incubated in a reaction volume of 40µl in the presence of 10mM Tris-HCl buffer pH 7.6 for 1 h at 37 ^{0}C. The reaction was terminated by adding 20µl of buffer containing 4% β-mercaptoethanol, 1M urea and 4% SDS and subjected to 7.5% SDS-PAGE. The electrophoresis was carried out at 80V for 5% stacking gel and 40V for 7.5% resolving gel. The fibrinogen-hydrolyzing pattern of the crude enzyme was visualized by staining the gel with coomassie brilliant blue R250 [12].

Statistical Analysis

All data obtained were computed from the mean of atleast three independent experiments and expressed as mean ±SD.

Results and discussions

The present study showed the protease activity of crude and purified enzyme of *Tabernaemontana divaricata* L. and *Carissa carandas* L. The plants represent rich source of proteases, which may act as key regulators of a striking variety of biological processes, including meiosis, gametophyte survival, embryogenesis, seed coat formation, cuticle deposition, epidermal cell fate, stomata development, chloroplast biogenesis, and local and systemic defense responses [14]. Proteases are specifically expressed in time and space and accumulate in different subcellular compartments. Their substrates and activation mechanisms are elusive, however, and represent a challenging topic for further research. Fractionation on Sephadex G-75

The protein yield was 30mg/ml for *Tabernaemontana divaricata* L. and 66mg/ml for *Carissa carandas* L. The crude enzyme of *Tabernaemontana divaricata* L. when fractionated on Sephadex G-75 yielded four peaks (Fig. 1), of which only peak 4 showed significant caseinolytic activity thus named as TDP4 *(Tabernaemontana divaricata* L. *peak 4)*. Fractionation of *Carissa carandas* L. gave one single major peak (Fig. 2) with significant caseinolytic activity and thus named as CCP1 *(Carissa carandas* L. *peak 1)*.

Polyacrylamide gel electrophoresis of TDP4 and CCP1 enzyme fractions

TDP4 and CCP1 were subjected to electrophoresis under 12.5% SDS-PAGE. In both the cases, clearly distinguishable protein bands were resolved in the gel after staining with Coomassie brilliant blue R250 (Fig. 3). The relative molecular mass of TDP4 and CCP1 was 19 and 22kDa respectively when compared to the protein marker ranging from 11kDa to 245kDa. Kinetic properties of the enzyme such as optimum temperature and optimum pH were determined. TDP4 and CCP1 exhibited optimum pH at 6.8 and 7.8 & temperature at 40 ^{0}C ±2 ^{0}C and 25 ^{0}C ±2 ^{0}C respectively. The proteolytic activity of TDP4 and CCP1 was inhibited by iodine, cobalt chloride and silver nitrate and activated by β-mercaptoethanol and hydrogen sulfide. Hence the proteases may belong to the family of serine proteases. The reported molecular mass of several plant proteases range from 19-110kDa. In general majority of the plant proteases show a molecular mass between 60 and 80kDa. The low molecular weight proteases TDP4 and CCP1 could prove to be easy in isolation, purification and characterization for biotechnological applications. The optimum pH for serine proteases lies in the alkaline range, pH 7–11; with few exceptions as reported in the literature such as hordolisin and SEP-1 from barley (optimum pH of 6 and 6.5, respectively), as well as Ara12 from *A. thaliana* RSIP from maize and protease C1 from soybean (which act best in the pH range 3.5–6.5). The optimum temperature is rather variable among these enzymes, from 30 up to 80 °C, but most plant serine proteases usually show maximum activity in the range 20–50 °C [15, 16].

Fibrinogenolytic activity TDP4 and CCP1 enzyme fractions

Fibrinogenolytic activity of TDP4 and CCP1 was assessed as mentioned in methods. They exhibited dose-dependent fibrinogenolytic activity (data not shown). The proteases completely hydrolyzed Aα subunit and to a lesser extent the β and γ subunits following incubation at 37^{0}C (Fig. 4). The hydrolysis pattern may be due to release of fibrinopeptide, as is true with many proteases showing fibrinogenolytic activity [17].

Human blood and plasma clot hydrolyzing activity of TDP4 and CCP1 enzyme fractions

There are two types of clots, the blood clot which is a hard clot and the plasma clot known as a soft clot. The TDP4 proteases hydrolyzed crude fibrin of blood clot to a greater extent when compared to CCP1, while the activity of CCP1 was greater with respect to plasma clot. Caseinolytic activity of both TDP4 and CCP1 increased to 4 folds when treated with β-mercaptoethanol and inhibited with iodine suggesting that the proteases are serine proteases exhibiting clot enhancing and clot dissolving properties.

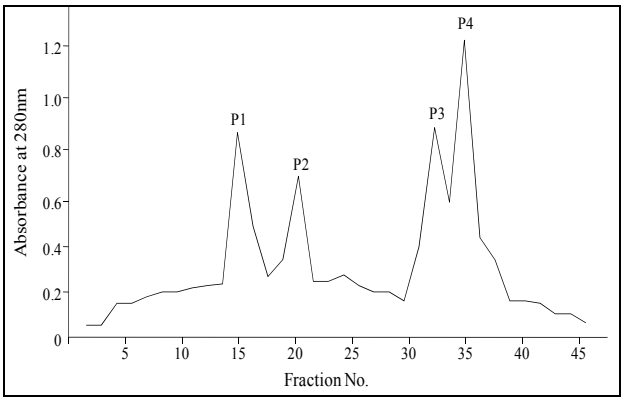

Fig 1: Fraction of crude enzyme of *Tabernaemontana divaricata* L. on Sephadex G-75

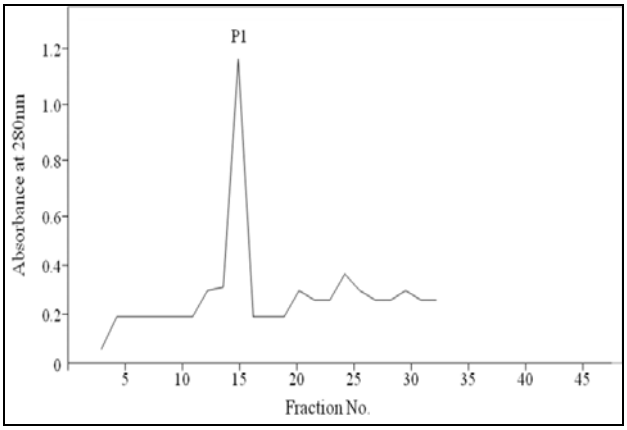

Fig 2: Fraction of crude enzyme of *Carissa carandas* L. on Sephadex G-75

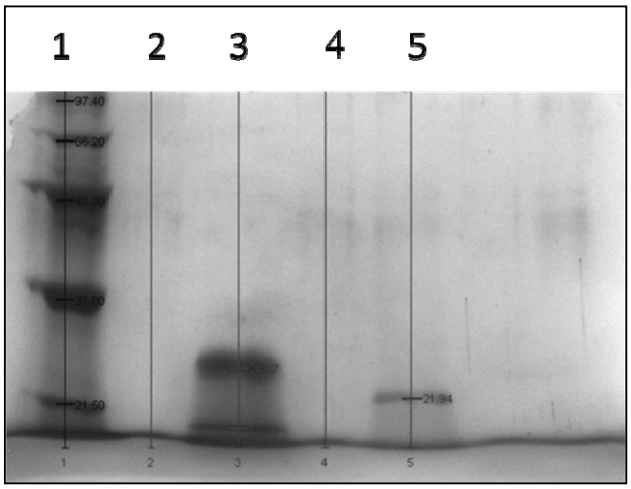

Fig 3: Polyacrylamide gel electrophoresis of TDP4 and CCP1 enzyme fractions

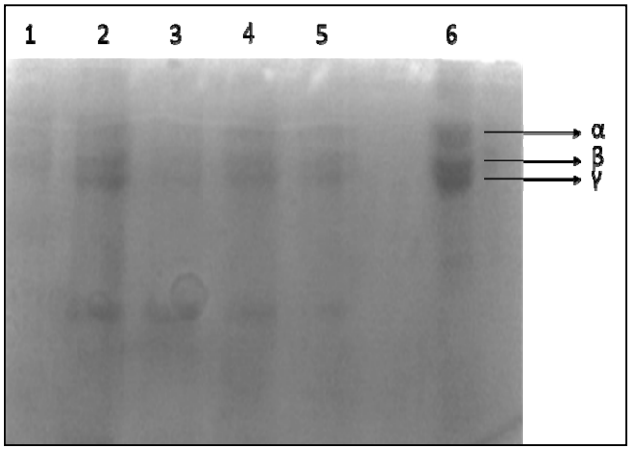

Fig 4: Fibrinogenolytic activity TDP4 and CCP1 enzyme fractions

Conclusion

In the present investigation, proteases from the latex of *Tabernaemontana divaricata* L. and *Carissa carandas* L. have been purified to homogeneity and characterized. The proteases are serine proteases, responsible for clot inducing and clot dissolving properties. This study supports the traditional use of plant latex to stop bleeding and wound healing. Further investigation into the structural aspects of these enzymes would enhance our knowledge regarding therapeutic applications and understanding of coagulation physiology. This would certainly be a boon to hemophilic patients as these proteases enhance coagulation process.

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