Biocatalysis and Agricultural Biotechnology 17 (2019) 685–689

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Fibrinogenolytic activity of serine proteases(s) from *Cucumis dipsaceus*

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ARTICLE INFO

Keywords:
Cucumis dipsaceus
Hemostasis
Fibrinogen
Pro-coagulation

ABSTRACT

Cucurbitaceae species plants have been widely used in traditional medicine for wound care management and hemostasis. This study aims to explore the possible involvement of *Cucumis dipsaceus* fruit extract on the blood coagulation pathway. The *aqueous Cucumis dipsaceus dialysate fraction* (*AqCDF*) hydrolyzes casein and exhibiting a protease activity in a dose-dependent manner. Casein zymography further reveals the presence of high molecular weight protease(s). The inhibition of proteolytic activity by phenylmethanesulfonyl fluoride (PMSF) suggesting the presence of serine protease. The serine protease(s) fraction exhibiting a promising fibrinogenolytic activity at 25 μg and 50 μg concentration by hydrolyzing Aα and Bβ subunit of fibrinogen, whereas γ-subunit undergo partial degradation. The stability of protease activity against various parameters viz., temperature, pH has been evaluated and its optimum activity was found to be 60 °C & pH 7.0 respectively. The *AqCDF* shows a promising recalcification time up to 80% against trypsin. More interestingly *AqCDF* (300 μg) did not induce hemolysis even at higher concentration. These findings suggest the possible role of the dialyzed fraction of *Cucumis dipsaceus* in hemostasis and the wound healing process.

1. Introduction

Protease is a broad class of enzymes involved in various physiological and pathological conditions. Six different classes of proteases (Serine, Cysteine, Aspartic, Threonine, Glutamic, and Metallo) were found in animal, plant and microbial sources (Neurath, 1984; Barrett et al., 2003). Among them, plant and microbial proteases are widely used commercially for their wide therapeutic applications (Mahajan et al., 2010). These proteases are acting as molecular knives involve in several physiological processes, such as wound healing, hemostasis, apoptosis, zymogen's activation, cell proliferation, blood clotting and fibrin clots (Shen et al., 2009). Wound healing is associated with two important events such as coagulation and fibrinolysis (Walker et al., 1985). Series of reaction has been carried out during blood coagulation or clotting, that involves activation of fibrin from fibrinogen by the action of thrombin. Formation of fibrin mesh involves the activation of a series of zymogen's activation by protease(s) that leads to blood coagulation. This causes the platelet aggregation and forms a stable hemostatic plug which prevents the blood loss (Berkner et al., 2001). The fibrin matrix seals the wound region and thereby facilitating cell invasion and angiogenesis (Colette et al., 2004). The pharmacology and therapeutic applications of plant proteases action on a specific substrate that results in the altered physiological function (Rajesh et al., 2005).

From our ancient time's plants metabolites have been used for wound care and management. Plant metabolites have gained importance due to its time management and minimal infection complications (Hart, 2002; Thakur et al., 2011, Korpenwar et al., 2012). Therefore, a large number of plants have been used by folk practitioners to treat the wounds. Especially plant extracts have been directly applied to the burnt areas and fresh cut wounds to stop bleeding and to initiate the quick healing process. Earlier reports showed that the plant proteases found in various parts of medicinal plant species involved in hemostasis and interfered in blood coagulation cascade (Satish et al., 2012; Rajesh et al., 2006; Shivaprasad et al., 2009; Gomes et al., 2009). Earlier studies showed that serine and cysteine proteases isolated from various plant species involve in wound healing and hemostatic mechanism (Priyanka et al., 2017).

Cucumis dipsaceus belongs to the family Cucurbitaceae and commonly known as an Arabian cucumber, hedgehog cucumber. The various parts of the plant have been used in traditional treatment such as hemorrhoid, rabies, gallstone, constipation, diarrhea, hair loss, etc (Lata et al., 2015; Rainer et al., 2010). Earlier studies say that aerial & fruit part of the *Cucumis dipsaceus* exhibits various pharmacological actions, such as anti-bacterial (Vasantha et al., 2013), anti-inflammatory (Salama et al., 1999a,b), and anti-cancer (Salama et al., 1999a,b) activity. The leaf & fruit parts exhibit a trypsin inhibitory

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https://doi.org/10.1016/j.bcab.2019.01.041
Received 21 August 2018; Accepted 22 January 2019
Available online 26 January 2019
1878-8181/

activity has been reported (Sadasivam et al., 1992). The earlier reports showed that Cucurbitaceae is a potential source of protease enzyme (Zohara et al., 2015). An earlier report from our research group showed the hemostatic potential of cysteine proteases from *Sesbania grandiflora* leaf extract exhibits a promising fibrinogenolytic and procoagulant activity which helps to maintain in the hemostasis. This is the first report on the presence of serine proteases in *Cucumis dipsaceus* fruit involved in the wound care and healing management. Dialysate rich protease fraction exhibits a promising fibrinogenolytic & strong procoagulant activity. Further studies required to purify the protease(s) and evaluate its molecular mechanism involved in the blood coagulation cascade.

2. Materials and methods

2.1. Preparation of dialysate protease fraction

Cucumis dipsaceus fruit was collected from an authenticated source, Mysore. The leaves were cleaned by using double distilled water to remove the dust and another contaminant. The leaves were air dried in the shade and powdered, kept in an airtight jar at 40 °C prior to use. 50 g of fruit was homogenized with 100 mL distilled water and allowed to equilibrate at room temperature in a rotary shaker at 120 rpm for 4 h. The supernatant obtained by centrifugation at 10,000 g at 40 °C for 20 min was subjected to 80% ammonium sulfate precipitation and allowed to precipitate overnight at 40 °C. The precipitated protein was collected by centrifugation at 12,000 g for 20 min at 40 °C and the pellet was resuspended in a small volume of distilled water and was extensively dialyzed against double distilled water for 24 h by using dialysis bag with molecular cut-off 10 kDa and lyophilized, used for the further assays.

2.2. Protein estimation

The protein content was determined by using Bovine Serum Albumin as standard (Lowry et al., 1951). Different aliquots of the extract were made up to 100 µL with distilled water, to this of 900 µL of Bradford's reagent was added and the color developed was read spectrophotometrically at 595 nm.

2.3. Gel electrophoresis

Electrophoresis was carried out as previously described (Laemmli et al., 1970). Ten percentile gels SDS-PAGE (10%) was carried out for the aqueous extract. Later the gel was stained with Coomassie brilliant blue R-250, followed by destined by using destaining solution consists of 30% (v/v) methanol - 10% (v/v) acetic acid in water.

2.4. Caseinolytic activity

The Caseinolytic activity was carried out according to by using casein as a substrate (Murata et al., 1963). Briefly, the reaction mixture contains 0.4 mL casein (2%) in 0.2 M Tris-HCl buffer, pH 8.5, was incubated separately with different concentrations of *AqCDF* (20 µg-100 µg) in a final volume of 1 mL for 2.5 h at 37 °C. The reaction was stopped by adding 1.5 mL of 0.44 M trichloroacetic acid (TCA) and the mixture was allowed to stand for 30 min. The reaction mixture was centrifuged at 1500 g for 15 min. An aliquot (1 mL) of the supernatant was mixed with 2.5 mL of 0.4 M sodium carbonate and 0.5 mL of Folin–Ciocalteu reagent (1:2 v/v). The color developed was read spectrophotometrically at 660 nm. Activity was expressed as units/h. One unit of enzyme activity was defined as the amount of enzyme required to increase the absorbance by 0.01OD at 660 nm/h.

2.5. Casein zymography

Protease activity was further confirmed by Casein zymography described earlier (Satish et al., 2012). The 2% casein was incorporated into SDS-PAGE (10%). The different concentration of *AqCDF* (25 µg, 50 µg & 100 µg) was mixed with sample buffer and incubated at 37 °C for 30 min without subjected to heat treatment. Later, the electrophoresis gel was washed with Triton X-100 (2.5% in 10 mM Tris-HCl buffer, pH 7.5) and then the washed gel was incubated for 18 h in incubating buffer solution containing 50 mM Tris-HCl, 50 mM CaCl2, 0.15 mM NaCl, at 37 °C. After incubation, the gel was transferred to a staining solution (Coomassie brilliant blue R-250) and then destained the gel by using a destaining solution. The white, clear bands appeared on the gel indicating degradation of casein by the enzyme.

2.6. Effect of temperature & pH on protease activity of AqCDF

The effect of temperature on protease activity was tested by incubating at different temperature (20°- 100 °C) for 30 min. Later protease activity of *AqCDF* was determined as performed above (Murata et al., 1963). The effect of pH on the protease activity of AqCDF was examined at pH ranging between 2 and 12 for 30 min at room temperature using following buffers: glycine-HCl (pH 2–3), sodium acetate-acetic acid (4–5), Sodium phosphate buffer (pH 6), Tris-HCl (pH 7–9) and glycine-NaOH (pH 10–12). The residual activity was measured as described above (Murata et al., 1963).

2.7. Effect of inhibitors on protease activity of AqCDF

The nature of protease activity was determined by using various standard protease inhibitors such as HgCl2 (Cysteine protease), PMSF (Serine protease), EDTA (Metalloprotease), Pepstatin A (Aspartic protease) at 5 mM concentration. The 100 µg of *AqCDF* were pre-incubated with inhibitors for 2.5 h at 37 °C. Protease activity was measured as described earlier (Murata et al., 1963).

2.8. Fibrinogenolytic activity

The fibrinogenolytic activity of *AqCDF* was carried out according to the method described earlier (Condrea et al., 1983). Human fibrinogen (50 µg) was pre-incubated with different concentration of *AqCDF* in 10 mM Tris-HCl buffer pH 7.4 for 30 min at 37 °C. The reaction was terminating by adding sample buffer (4% β-mercaptoethanol, 1 M urea and 4% SDS and subjected to SDS-PAGE (10%). The hydrolyzing pattern of fibrinogen was visualized by staining with Commassie Brilliant Blue R-250.

2.9. Pro-coagulant activity

Plasma re-calcification time was determined according to the method described earlier (Lee et al., 2006). Blood was collected from the non-smoker healthy donor in a tube containing anti-coagulant (0.11 M trisodium citrate) in the ratio of 9:1. The reaction mixture was centrifuged at 2500 rpm for 15 min and collect the supernatant is considered as platelet poor plasma (PPP). Platelet-poor plasma (volume of 300 µl) was pre-incubated with different concentrations of the *AqCDF* (20–100 µg/mL) at 37 °C for 60 s. Clot formation was initiated by adding 25 µl of $CaCl_2$(0.25 M) and time is taken for visible clot was recorded from the time of adding $CaCl_2$. Without a sample, Tris-HCl buffer was used as a control. Clotting efficiency was expressed in percentage, as mentioned below:

$$\textit{Clotting efficiency}\ (\%) = 100 \times \left[1 - \frac{Tp}{Twp}\right]$$

Where Tp = Clotting time with protease and Twp = Clotting time

without protease.

2.10. Hemolytic assay

AqCDF induced hemolysis was studied by using sheep RBC's cells as described earlier (Madhu et al., 2014). Briefly, blood was collected in a tube containing alsevers' solution and subjected to centrifugation at 2500 rpm. The pellet was collected and washed by using the hyposaline solution. The process was repeated until the supernatant obtained was colorless. The 0.5% of RBC′ suspensions were made by using 0.15 M NaCl.

The suspension RBC's (0.5%) were incubated with different concentration of *AqCDF* ranging from 25 µg −500 µg/ml and were incubated at 37 °C for 1 h. Triton X-100 (1%) was used for lysed the RBC's cells and referred as a control (100%). The reaction mixture was subjected to centrifugation (300 g x for 5 min) and the absorbance of the supernatant was read at 540 nm.

3. Results and discussion

Let us start our discussion with the words of Hippocrates “Let food be thy medicine and medicine be thy medicine”, this study explores the medicinal importance of the Cucumis diapauses fruits, towards the wound healing mechanism. The crude dialyzed protein fraction *AqCDF* were obtained from an aqueous extract of Cucumis diapauses, later it is subjected to precipitation by using 80% ammonium sulfate, followed by dialysis using 2.5 KDa bag to remove secondary metabolites and other Phyto constituents (Sarrouh et al., 2012). The *AqCDF* and Trypsin were subjected to protease activity by using casein as a substrate in a dose-dependent manner. The Trypsin exhibited a higher activity when compared to *AqCDF* and activity was expressed in terms of units/hr as showed in Fig. 1A. Further casein zymography study confirms the presence of a protease in the *AqCDF*. The catalytic activity of the *AqCDF* depicted as white bands on blue background shown in Fig. 1B. On electrophoresis of *AqCDF* in 10% SDS-PAGE, resolves clear, distinguished protein bands present in the *AqCDF* (Data not shown here). The stability of the protease present in *AqCDF* was determined by using different parameters such as temperature, pH, and salt. The protease activity remained unchanged at 40 °C ± 1.5 temperature and pH 7.5 ± 0.5. This shows the protease present in the sample having an optimum temperature of 40 °C ± 1.5 and optimum pH 7.5 ± 0.5, depicted in Fig. 2A and B. A further effect of salt on *AqCDF* was determined and 3% NaCl that slightly affects the protease activity as shown in Fig. 2C. Due to its proteolytic nature and stability, protease(s) gain wide attention & application in the pharmaceutical and biotechnology industries (Laskowski et al., 1956).

The effect of standard protease as being determined by using various protease inhibitors such as phenylmethylsulfonyl fluroide (PMSF) for a serine protease, Ethylene diamine tetraacetic acid (EDTA) for metalloprotease, Mercury chloride ($HgCl_2$) for Cysteine protease, and Pepstatin A for Aspartic protease at 1 mM concentration. Among these inhibitors, PMSF exhibited a strong protease inhibitor activity, concludes the possible presence of Serine protease in the *AqCDF* was shown in Fig. 2D. Similar enzyme inhibition from PMSF results has been reported in *Cucumis sativus* (Zohara et al., 2015). Other inhibitor does not show any effect on the enzyme activity of *AqCDF*.

A plasma glycoprotein human fibrinogen (factor X) has only tyrosyl and glutamyl N-terminal residues, with a molecular weight 340-kDa, made up of three subunits Aα (63.5 kDa), Bβ (56.0 kDa) and γ (47.0 kDa), whereas fibrin having glycyl N-terminal residues instead of glutamyl residue (Komori et al., 1985). The formation of fibrin by thrombin-like enzymes breaks fibrinogen by hydrolyzing the N-terminal disulfide knot, which releases fibrinopeptide A &/or B (Imamura et al., 2001b). Serine proteases are the important enzymes involved in the mammalian blood coagulation pathway. The fibrinogenolytic activity of the *AqCDF* has been studied by incubating the 25 µg & 50 µg of *AqCDF* with fibrinogen at 37 °C and their action has been revealed on 10% SDS-PAGE shown in Fig. 3B. *AqCDF* exhibit a thrombin-like activity (Serine protease), which preferentially breakdown the Aα subunit of fibrinogen, whereas it partially hydrolyzes Bβ and γ subunits; this is due to extensive N-glycosylation in the Bβ & γ subunits, whereas, these carbohydrate content absent in Aα subunit. Most of the proteases(s) from plant sources lack the ability to hydrolyze the γ-subunit of the fibrinogen (Richter et al., 2002; Zauner et al., 2012). The different subunits of fibrinogen undergo partial hydrolysis in Aα > Bβ > γ manner. Similar results have been observed in our previous report done earlier (Madhu et al., 2017). This shows the fibrinogenolytic activity of *AqCDF* can also show the procoagulant activity which leads to clot formation in human citrate plasma induced by $CaCl_2$ in a dose-dependent manner shown in Fig. 3A. Upon treatment of plasma with *AqCDF* decreases the clotting time from 180sec to 37sec and shows 80.05% clotting efficiency, whereas Trypsin shows 23.87% of clotting efficiency lowers than the *AqCDF*. This clearly manifests the procoagulant activity of *AqCDF* involves the blood clotting mechanism. More interestingly, the toxic effect of *AqCDF* was determined by using human RBC's cells and *AqCDF* did not exhibit hemolysis even at higher concentration

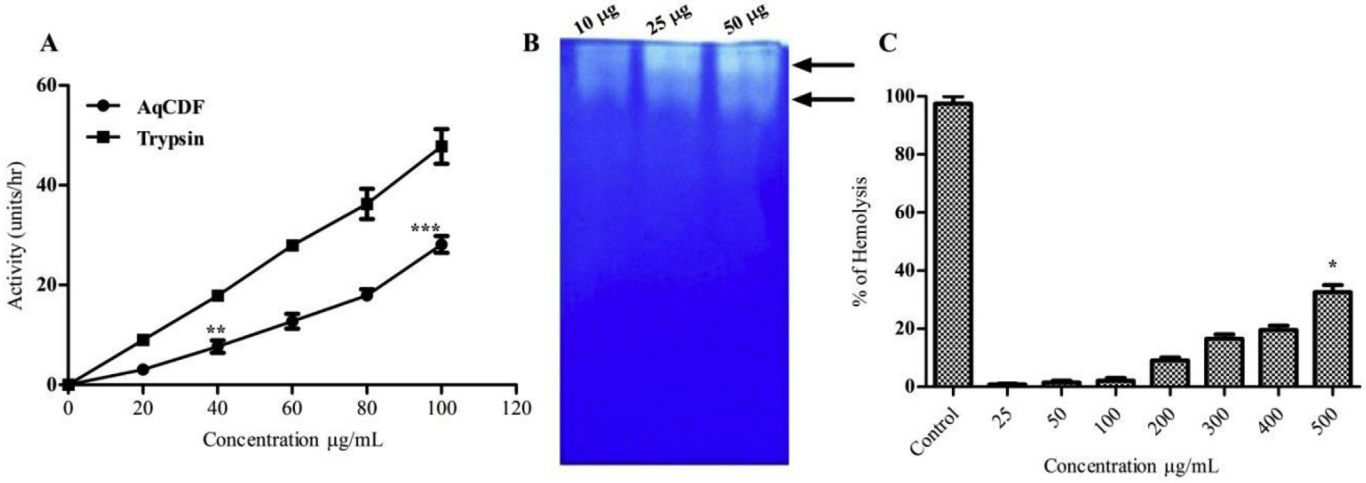

Fig. 1. : (A) Protease activity of *AqCDF* against trypsin: Protease activity of *AqCDF* was determined by using casein(2%) as a substrate in a dose dependent manner. One unit of enzyme activity was defined as the amount enzyme required to increase the absorbance by 0.01 O.D at 660 nm/h. **(B)** Casein zymography Lane 1: 10 µg *AqCDF*, Lane 2: 25 µg *AqCDF* & Lane 3: 50 µg *AqCDF*. White band (black arrow indicates) on blue background shows the protease activity of enzyme fraction **(C)** Hemolytic activity of *AqCDF* was performed by using 2% RBC's. Values represent mean ± SEM (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

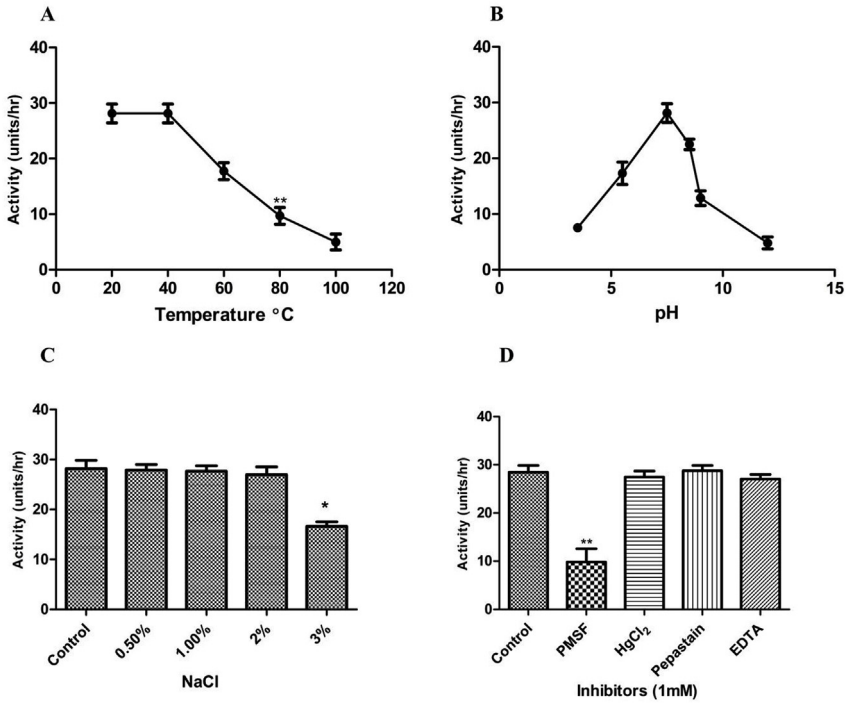

Fig. 2. (A) Effect of temperature **(B)** Effect of pH **(C)** Effect of salt (NaCl) **(D)** Effect of protease inhibitors (1 mM concentration) on protease activity was determined as described earlier. Values represent mean ± SEM (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$.

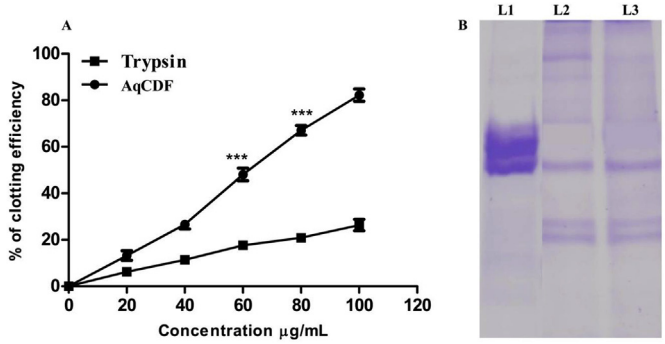

Fig. 3. A) Pro-coagulant activity of partially purified *AqCDF* against Trypsin. **B)** Fibrinogenolytic activity of partially purified *AqCDF*. L1: Control (50 µg Fibrinogen), L2: (50 µg Fibrinogen+ 25 µg *AqCDF*), L3: (Fibrinogen 50 µg + 50 µg *AqCDF*). Values represent mean ± SEM (n = 3). $^{***}p < 0.001$.

(Fig. 1C). These data suggest that further *in vivo* studies needed in this direction to elucidate the cellular and molecular mechanism of *AqCDF*.

4. Conclusion

Serine proteases are the important enzymes involved in the mammalian blood coagulation pathway. This is the first report of protease activity from *Cucumis dipsaceus* fruit, which revealed the presence of protease(s), which belongs to the Serine Protease family. Further study needed to identify the new Serine protease from *Cucumis dipsaceus* and providing scientific validation for *Cucurbitaceae* species use in traditional medicine in maintaining hemostasis. Further studies in this direction help to purify and characterize the protease present in the sample and further elucidate the molecular mechanism involved in the blood clotting cascade pathway.

Acknowledgment

The authors gratefully acknowledge the Teresian Research Foundation for providing the facility.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.01.041.

Conflicts of interest

Authors declares there is no conflict of interest.

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