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Original article

Purification and characterization of novel fibrin(ogen)olytic protease from *Curcuma aromatica* Salisb.: Role in hemostasis

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ABSTRACT

Background: The proteases from turmeric species have procoagulant and fibrinogenolytic activity. This provides a scientific basis for traditional use of turmeric to stop bleeding and promote wound healing processes.

Purpose: Our previous studies revealed that fibrinogenolytic action of crude enzyme fraction of *Curcuma aromatica* Salisb., was found to be more influential than those of *Curcuma longa* L., *Curcuma caesia* Roxb., *Curcuma amada* Roxb. and *Curcuma zedoria* (Christm.) Roscoe. Hence, the purpose of this study is to purify and characterize protease from *C. aromatica* and to explore its role in wound healing process.

Methods: The protease was purified by Sephadex G-50 gel permeation chromatography. Peak with potent proteolytic activity was subjected to rechromatography and then checked for homogeneity by SDS-PAGE and native PAGE. Furthermore purity of the peak was assessed by RP-HPLC and MALDI-TOF. The biochemical properties, type of protease, kinetic studies, fibrinogenolytic, coagulant and fibrinolytic activities were carried out.

Results: The two proteolytic peaks were fractionated in gel permeation chromatography. Among these, the peak-II showed potent proteolytic activity with specific activity of 10 units/mg/min and named as *C. aromatica* protease-II (CAP-II). This protein resolved into a single sharp band both in SDS-PAGE (reducing and non-reducing) as well as in native (acidic) PAGE. It is a monomeric protein, showing sharp peak in RP-HPLC and its relative molecular mass was found to be 12.378 kDa. The caseinolytic and fibrinolytic activity of CAP-II was completely inhibited by phenylmethylsulfonylfluoride (PMSF). The CAP-II exhibited optimum temperature of 45 °C and optimum pH of 7.5. The Km and Vmax of CAP-II was found to be 1.616 µg and 1.62 units/mg/min respectively. The CAP-II showed hydrolysis of all three subunits of fibrinogen in the order Aα > Bβ > γ. The CAP-II exhibited strong procoagulant activity by reducing the human plasma clotting time. It also showed fibrinolytic activity by complete hydrolysis of α-polymer and γ-γ dimer present in fibrin.

Conclusion: The CAP-II is a novel serine protease from *C. aromatica,* which has been demonstrated to stop bleeding and initiate wound healing through its procoagulant and fibrin(ogen)olytic activities. Our study demonstrates the possible role of CAP-II, as therapeutic enzyme to stop bleeding at the time of wounding.

Introduction

Wound healing is a physiological process of restoration of tissue integrity and functional anatomy after an injury (Nagori and Solanki, 2011). It is a complex coordinated process, which involves four overlapping phases such as hemostasis, inflammation, proliferation and remodeling (Steenkamp et al., 2004). Among these,

Abbreviations: CAP-II, *C. aromatica* protease-II; RP-HPLC, Reverse phase high performance liquid chromatography; MALDI-TOF, Matrix-assisted laser desorption ionization-time of flight; PMSF, Phenylmethylsulfonylfluoride; IAA, Iodoaceticacid; EGTA, Ethylene glycol-N,N,N',N'-tetraacetic acid; EDTA, Ethylene diaminetetraacetic acid; CEF, Crude enzyme fraction.

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hemostasis plays a very important role in stopping bleeding and initiation of wound healing process by activation of two important proteolytic events such as blood coagulation by fibrinogenolytic and hydrolysis of hemostatic plug by fibrinolysis (Lijnen, 2002).

Blood coagulation cascade involves a series of inactive precursors of coagulation enzymes and factors. Most of the coagulation factors are synthesized by the liver and few are from blood cells, which are present as zymogens. Once activated, each factor further activates subsequent factors of coagulation cascade by proteolysis. Depending on the initial triggering of coagulation factors upon injury, blood coagulation cascade operates *via* two distinct pathways namely, intrinsic and extrinsic pathways (Shivaprasad et al., 2010). Later, this was followed the common pathway by the formation of factor V complex or prothrombin activator, which consists of factor Xa with factor V, Ca^{2+} and platelets derived phospholipids (Riddel et al., 2007). The factor V complex initiates the conversion of inactive prothrombin into active thrombin enzyme. This thrombin, a serine protease acts on fibrinogen and converts it into insoluble fibrin clot. The fibrin monomers undergo polymerization, retract with platelet contractile protein and stop bleeding by the formation of strong hemostatic plug. Furtherly, the serine protease plasmin dissolves thrombi and hemostatic plugs through the action on fibrin, facilitating wound healing and promote flow of blood (Cesarman-Maus and Hajjar, 2005). Hence, protease plays a pivotal role in stopping bleeding and promoting wound healing process with relevance to hemostasis.

Therefore, the isolation and characterization of highly specific fibrin(ogen)olytic protease from natural sources is a pre-requisite study in wound healing and also for the treatment of thrombotic disorders. Till date proteases from snake venoms, leeches, annelids, insects, algae, caterpillars, microbes and plants have been isolated, characterized and shown to be useful for blood coagulation and fibrinolysis (Rajesh et al., 2006). Further, the research on traditional knowledge based drugs from plant origin has gained considerable importance due to the reduction in time required for wound healing and minimizing incidences of infection (Hart, 2002).

In Indian ayurvedic system of medicine, few medicinal plants such as *Ficus bengalensis, Cynodon dactylon, Symplocos racemosa, Rubia cordifolia, Pterocarpus santalinus, Ficus racemosa, Glycyrrhiza glabra, Berberis aristata, Curcuma longa, Centella asiatica, Euphorbia nerifoli* and *Aloe vera* are effectively used for the treatment of different types of wounds (Biswas and Mukherjee, 2003). Recently, we have showed that the procoagulant and fibrinogenolytic activity of crude protease fractions from different turmeric species *viz*., *C. aromatica, C. longa, C. caesia, C. amada* and *C. zedoria* to prevent blood loss upon injury (Shivalingu et al., 2015). Among these, *C. aromatica* showed potent procoagulant and fibrinogenolytic activity by hydrolyzing all the subunits of fibrinogen. Hence, these results led us to further explore the potential of *C. aromatica* protease in blood coagulation followed by fibrinolysis. In the present study for the first time, we have purified and characterized the protease from *C. aromatica,* which effectively stops bleeding.

Material and methods

Plant material

C. aromatica rhizome was collected from Indian Institute of Spice Research (IISR) Centre, Kozhikode, Kerala, India and was authenticated by Dr. D. Prasath, Senior Scientist, Department of Crop Improvement and Biotechnology, IISR, Kozhikode, Kerala, India. A voucher specimen (14SJCE051) is deposited at Department of Biotechnology, Sri Jayachamarajendra College of Engineering, JSS Technical Institutions Campus, Mysuru 570006, Karnataka, India.

Purification of protease from C. aromatica

Crude enzymatic fraction from *C. aromatica* was prepared as reported earlier by Shivalingu et al. (2015) and used as primary extract for purification of protease. The protein concentration was estimated according to the method of Bradford (1976) using bovine serum albumin as standard.

The protease from *C. aromatica* was purified by using Sephadex G-50 gel filtration column chromatography. The crude protein was loaded onto the Sephadex G-50 column (1.0 cm x 90 cm) equilibrated with 50 mM Tris-HCI buffer, pH 7.4. The elution was performed using the same buffer with the flow rate of 15 ml/h and 1.5 ml fractions were collected. These fractions were monitored at 280 nm and assayed for proteolytic activity. The peak that showed significant proteolytic activity was pooled, concentrated and subjected to rechromatography under the same condition and stored at 4 °C for further analysis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the method of Laemmli (1970) for crude enzyme fraction (50 µg) and purified protease (15 µg) from peak –II on 10% polyacrylamide gel containing 0.1% SDS using 50 mM Tris-glycine buffer pH 8.8 at constant voltage for 2 h along with the molecular marker (97.4–14.3 kDa). Native PAGE was carried out according to the method of Davis (1964) for purified protease on 10% polyacrylamide gel at constant voltage for 4 h under acidic (pH 4.3) and basic (pH 8.8) conditions using β-alanine acetic acid buffer and Tris glycine buffer respectively. The bands were visualized by staining with coomassie brilliant blue R-250.

Reverse-phase high-performance liquid chromatography (RP-HPLC)

Lyophilized Peak-II sample was analysed in RP-HPLC system consisting of Water LC pumps (Waters, Model 1525, USA) with manual sample injector port consisting of binary gradient pumping system, dual λ absorbance detector (Model 2487) with Empower 2 data processor. 20 μg of protease containing fraction was injected via manual injector port on a reverse-phase Symmetry shield C_{18} column (5 µm, 4.6 × 150 mm) to pre-equilibrated column with 95% of solvent A (0.1% Trifluroacetic acid (TFA)) for 10 min containing 5% of solvent B [(70% (v/v) acetonitrile containing 0.1% TFA]. Linear gradient from 10 to 35 min was programmed with increasing amounts of 95% solvent B for 35 min at the flow rate of 1 ml/min. A single sharp protease peak was detected at 280 nm eluted at about 80% concentrations of solvent B. After the run, a washing step with 100% of solvent B was included to remove residual components and re-equilibrate the column with 95% solvent A for 10 min. The program for the gradient elution was controlled and analyzed by an Empower 2 analytical programmer. SDS-PAGE (non-reducing and reducing) pattern was confirmed by single band, which was further confirmed by MALDI- TOF/TOF.

Mass spectrometry

The molecular mass of CAP-II (10 µg) was determined on a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry by using 0.5 µl α-cyano-4-hydroxycinnamic acid matrix in 50% acetonitrile +0.1% trifluoroacetic acid. Eluate was allowed to dry and directly introduced into the mass spectrometer (Bruker Daltonics Ultraflextreme). The samples were analyzed in positive voltage polarity mode to obtain the mass spectrum.