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ORIGINAL ARTICLE

Procoagulant serine glycoprotease from *Cucumis sativus* L.: action on human fibrinogen and fibrin clot

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Abstract Upon examination of the fruit extract of *Cucumis sativus* L. for its pharmacological benefits, it was previously observed that it has potential proteolytic, fibrinogenolytic and procoagulant activities. These properties can be attributed to the presence of the protease. In this regard, the present study comprised of purification and characterization of protease. Purification of the enzyme involved ammonium sulfate precipitation followed by gel filtration and ion exchange chromatography. The purified cucumis protease (CPro) exhibits homogeneity as attested by SDS-PAGE and RP-HPLC with a retention time of 14.246 min with molecular mass ∼75.3 kDa. CPro was identified as a glycoprotein and serine protease. Azocasein is the preferred substrate for CPro as it showed low K_m value of 0.3809 mg/ml. Purified CPro exhibits optimum activity at 37 °C and pH 8. CPro shows its involvement in hemostasis—the very first step in wound healing. CPro degrades the subunits of human fibrinogen in the order $A\alpha > B\beta > \gamma$. It also hydrolyzes the subunits of the partially cross-linked fibrin clot in the order α-polymer $> \gamma-\gamma$ dimer $> \beta$-chain. CPro reduced the clotting time of citrated plasma, prothrombin time and activated partial thromboplastin time of plasma. CPro is neither hemorrhagic nor edema-inducing, thus considered to be a non-toxic protease. This work provides evidence for the use of cucumber extract in wound healing and authenticates its use in cosmetics.

Keywords Serine protease · Glycoprotein · Fibrin(ogen)olytic · Hemostasis · Procoagulant · Cucumis protease

Abbreviations

CPro	Cucumis protease
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
RP-HPLC	Reverse-phase high-performance liquid chromatography
AqFEC	Aqueous fruit extract of European cucumber
RFC	Relative clotting factor
PMSF	Phenyl methyl sulphonyl fluoride
EDTA	Ethylene diamine tetra acetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N*, *N*, *N′*, *N′*-tetraacetic acid
IAA	Iodoacetic acid
PT	Prothrombin time
APPT	Activated partial thromboplastin time
TFA	Trifluoroacetic acid
PAS	Periodic acid-Schiff

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Introduction

The family Cucurbitaceae includes large group of plants with medicinal properties either in their fruits or seeds. In folk medicine, plant parts such as fruits, seeds, leaves and roots from Cucurbit family are used in treating diseases such as external wounds, burns, cancer, diabetes, gastrointestinal disorders, goiter, heart disorders, helminthiasis, menstrual disorder and skin disorders (Rahman 2013). *Cucumis sativus* commonly known as cucumber is a member of Cucurbit family. Several active phytoconstituents present in cucumber are progressively being identified by researchers in order to scientifically validate its traditional use. The *C. sativus* lyophilized fruit juice is shown to possess potent DPPH-free radical, superoxide radical scavenging activity, anti-elastase and anti- hyaluronidase activity with IC_{50} values 14.73 ± 1.42, 35.29 ± 1.30, 6.14 ± 1.74 and 20.98 ± 1.78 µg/ml, respectively (Nema et al. 2011). Lutein, a xanthophyll, an active component extracted from leaves and stem of cucumber has been potentially useful as skin-whitening agent (Sotiroudis et al. 2010). An aspartyl proteinase of ~42 kDa was isolated from the seeds of cucumber and was identified glycoprotease (Wilimowska-Pelc et al. 1982). Many proteases form Cucurbitaceae family have been isolated, purified and characterized from the sarcocarp of *Cucumis melo* (var. Prince), *Cucumis trigonus* roxburghi, *Benincasa hispida* (var. ryukyu) with molecular weights ranging between 50 and 67 kDa (Kaneda and Tominaga 1975; Uchikoba et al. 1998; Asif-Ullah et al. 2006). However, their role in hemostasis has not been studied. In our study, the property of purified protease from *C. sativus* European variety was scientifically correlated with its traditional use and found to display hemostatic action. In folk medicine, the fresh fruit is used externally as a poultice for burns, sores and wounds (Lim 2012). Based on this practice, in our earlier study, we have reported the role of aqueous whole fruit extract of European variety cucumber (AqFEC) in blood coagulation cascade via fibrinogenolytic activity (Nafeesa et al. 2015). In the present study, we are reporting the purification and characterization of a glycoprotein involved in hemostasis with respect to human fibrinogen and fibrin clot hydrolyzing activity. A study on in vivo toxicity using mice model was also carried out.

Materials and methods

Fibrinogen from human plasma, trypsin, phenyl methyl sulphonyl fluoride (PMSF), iodoacetic acid (IAA), Sephadex G-100, G-75, CM-Sephadex C-50, azocasein, gelatin and glycine were procured from Sigma-Aldrich Corporation (St. Louis, MO, USA). Standard protein marker was from Bio-Rad. The fruits were purchased from Reliance market, Mysuru in the month of April 2014. Fresh human blood samples were collected from healthy volunteers for the experiments.

Collection and extraction of Cucumis protease from whole fruit

The enzyme was isolated from 4.5 kg fruits of *C. sativus* L. European variety. The fruits were washed with distilled water then homogenized in a blender. The dark green homogenate was filtered using cheese cloth and then centrifuged at 8000 rpm for 10 min. The clear supernatant was collected and the pellet was discarded. Ammonium sulfate crystals were added gradually with constant stirring to obtain 90% saturation. The resultant solution was centrifuged for multiple times and the pellet obtained was subjected to dialysis against distilled water. The dialysed sample was lyophilized and stored at -20 °C for further use. Protein concentration was quantitatively analyzed by Bradford method using BSA as standard (Bradford 1976).

Purification of a glycoprotein from *Cucumis sativus* L.

Step 1. Sephadex G-100 column chromatography

Lyophilized sample (100 mg) was dissolved in 1 ml of buffer A (50 mM sodium phosphate pH 5) and loaded on the Sephadex G-100 column. The column (1 × 152 cm) was pre-equilibrated with buffer A. The column was eluted with buffer A at the flow rate of 20 ml/h and 2 ml fractions were collected and protein elution was monitored at 280 nm using a Shimadzu spectrophotometer. Alternate tubes were assayed for protease activity. Protein peaks showing caseinolytic activity were pooled and lyophilized.

Step 2. CM-Sephadex C-50 column chromatography

The sample recovered from G-100 column was lyophilized and kept for dialysis overnight in buffer A before loading on the CM-Sephadex C-50 column (1.5 × 22 cm) which was pre-equilibrated with 20 mM sodium phosphate buffer pH 5.5. The bounded protein was eluted stepwise using sodium phosphate buffer ranging between 40 and 150 mM with varied pH ranging between 5.5 and 8.5. Fractions of volume 1.5 ml were collected at a flow rate of 15 ml/h. Alternate tubes were assayed for protease activity, pooled and lyophilized after confirmation.

Step 3. Sephadex G-75 column chromatography

The fractions obtained from CM-Sephadex C-50 were loaded on the Sephadex G-75 column. The column (1 × 152 cm) was pre-equilibrated with buffer A. The column was eluted with buffer A at the flow rate of 10 ml/h and 1 ml fractions were collected and protein elution was monitored at 280 nm using a Shimadzu spectrophotometer. Alternate tubes were assayed for caseinolytic activity. Protein peak showing protease activity was pooled and used for its biochemical characterization.

Molecular weight determination by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

For determining molecular mass, the purified CPro along with the standard protein ladder was subjected to SDS-PAGE (Laemmli 1970). The molecular mass standards ranging from 10 to 250 kDa was run with CPro in a 7.5% (w/v) acrylamide gel. CPro was boiled with reducing and non-reducing sample buffer for 5 min before loading on the gel. After electrophoresis, the gel was stained for 3 h with coomassie brilliant blue R-250 dye and then destained using methanol acetic acid solution until clear bands were seen. The molecular weight of the CPro was estimated by comparing the relative front value of the standard protein ladder using image lab software version 5.1. Glycoprotein staining was done using periodic acid-Schiff (PAS) stain according to the method of Deepak et al. (2007).

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

The purity of CPro was adjudged by RP-HPLC. The RP-HPLC unit composed of water LC pump (Waters, Model 1525, USA) with manual sampler injector port having binary gradient pumping system with dual lambda absorbance detector (Model 2487) having Empower2 data processor. Exactly, 20 μl of the purified CPro (lyophilized first peak of G-75 column, third step purification) was injected through manual injector port on a reverse phase symmetry shield C_{18} column (5 μm, 4.5 × 150 mm). The C_{18} column was pre-equilibrated with 95% of 0.1% TFA (solvent A) containing 5% solvent B (70% (v/v) acetonitrile with 0.1% TFA) for 10 min. The simple linear gradient was programmed with increasing quantity of 95% solvent B from 10 to 35 min at the flow rate of 1 ml/min. It was detected at 280 nm and eluted at 80% concentration of solvent B as a sharp single peak with the retention time of 14.246 min. After the run, the column was washed with 100% solvent B to remove all the residual components and re-equilibrated with 95% solvent A for 10 min.

Biochemical characterization

Protease assay of CPro using different substrate and inhibitors

The proteolytic assay was done according to the modified method of Shivalingu et al. (2015) with 2% casein and gelatin prepared in 0.2 M Tris–HCl buffer pH 8.5. Casein substrate (400 μl) was incubated with different concentrations of purified CPro (10–100 μg) at 37 °C for 2.5 h. The reaction was arrested by adding 1.5 ml of 0.44 M TCA to each test tube, and the test tubes were allowed to stand for 30 min. The reaction mixture was centrifuged at 2000 rpm for 10 min, and an aliquot of 1 ml supernatant was taken and mixed with 2.5 ml of 0.4 M sodium carbonate and 0.5 ml of 1:2 diluted Folin–ciocalteu's reagent. The chromophore developed by the reaction between free tyrosine and Folin's reagent was read at 660 nm. The control was prepared by the same procedure but TCA was added before the addition of enzyme. One unit of protease activity was defined as the amount of enzyme required to increase the absorbance by 0.01 unit at 660 nm at 37 °C and expressed as units per hour. Azocasein digesting activity was assayed by incubating the different concentration of purified CPro, 0.05 M Tris–HCl buffer, 400 μl azocasein for 2.5 h at 37 °C (Casano et al. 1989). The reaction was arrested by adding 2 ml of ice-cold 12% Trichloroacetic acid (TCA). The precipitated proteins were removed by centrifugation for 10 min at 3000 rpm and absorbance was measured at 340 nm in the supernatant. One unit of azocaseinolytic activity was defined as the amount of enzyme required to increase in absorbance of 0.01 at 340 nm at 37 °C. Absorbance was recorded immediately after stopping the reaction. For inhibition studies, before adding 0.4 ml of 2% casein CPro was pre-incubated with and without specific protease inhibitors for 30 min at 37 °C.

Michaelis–Menten constant (K_m) determination

To determine the kinetic parameters, V_{max} and K_m of purified enzyme, protease activity was measured at the various concentrations of casein, azocasein and gelatin (0–5 mg/ml) prepared in 50 mM Tris–HCl buffer (pH 7.4). K_m and V_{max} were calculated by Michaelis–Menten equation using Graph pad prism version 6.

Effect of variation in temperature and pH on CPro

To determine the effect of temperature on enzyme activity, the enzyme as well as substrate solution were incubated at a particular temperature (4–90 °C) and further protease assay was carried out. The effect of pH in the range of 4–10 and temperature in the range of 4 °C to 90 °C on the

activity of purified CPro was investigated. The buffers used for different pH were 0.05 M sodium acetate (pH 4.0–5.5), 0.05 M sodium phosphate (pH 6.0–7.5) and 0.05 M Tris–HCl (pH 8.0–10.0). A control assay was done at all temperature and pH without enzyme.

Coagulant activity

Clotting activity of purified CPro was studied using citrated human plasma. Recalcification time was determined according to the method of Condrea et al. (1983). One part of 0.11 M tri-sodium citrate and nine parts of fresh human blood were mixed. The mixture was centrifuged for 15 min. The collected supernatant was used as platelet poor plasma (PPP). Different concentrations of CPro and trypsin (10–100 µg) in 0.01 M Tris–HCl buffer (pH 7.4) were added to 300 µl of pre-warmed PPP (to 37 °C) and incubated for 1 min. Clot formation was initiated by adding 30 µl of 0.25 M calcium chloride ($CaCl_2$). Time taken for appearance of visible clot from the time of addition of $CaCl_2$ was recorded. For control, Tris–HCl buffer was added instead of CPro. Percent clotting efficiency was calculated according to Nafeesa et al. (2015).

Prothrombin time (PT) and activated partial thromboplastin time (APTT)

PT and APTT were performed using the method by Manjula et al. (2015). Platelet poor plasma was prepared from blood collected into citrated anticoagulant. The reagents were incubated for 1 min with CPro (0–16 µg). The clotting for PT activity was initiated directly by adding 200 µl PT reagent UNIPLASTIN (rabbit brain thromboplastin). For APTT 100 µl LIQUICELIN (activated cephaloplastin) reagent was activated for 3 min at 37 °C and clotting was initiated by 100 µl 0.25 M $CaCl_2$. In both the cases, the clotting time was recorded in seconds by observing the formation of visible clot against a light source.

Carbohydrate estimation of CPro by phenol–sulfuric acid method

This is a simple colorimetric method used to determine the carbohydrate content of the purified CPro which was carried out according to the method of Albalasmeh et al. (2013). The basic principle behind this method is that carbohydrates dehydrated by reaction with concentrated sulfuric acid, produce furfural derivatives. Further reaction between furfural derivatives and phenol develops detectable colored products. Absorption at 490 nm is read using a spectrophotometer. The carbohydrate content of the purified CPro was determined by comparing absorbance with glucose as standard.

Gelatin zymography

The gelatinolytic activity of purified CPro was determined by substrate gel assay according to the method of Yariswamy et al. (2013). Purified CPro (15 µg) was loaded onto a 10% polyacrylamide gel copolymerized with gelatin (2%) and subjected to electrophoresis.

Human fibrinogenolytic activity

Fibrinogenolytic activity was measured according to the method of Venkatesh et al. (2015). The 50 µl reaction mixture contained 50 µg of human plasma fibrinogen (prepared in 10 mM Tris–HCl buffer pH 7.6) was incubated at 37 °C for 2.5 h with different concentration ranging from 1 to 8 µg of purified CPro separately. The fibrinogenolytic activity method was followed for time-dependent assay by varying incubation time (0–120 min). For inhibition studies, 5 µg CPro was pre-incubated with and without specific protease inhibitors (5 mM of PMSF, EDTA, EGTA and 100 µM of IAA) for 30 min at 37 °C.

Fibrinolytic activity

Fresh human blood was used to prepare blood clot and plasma clot which served as substrates to study the fibrinolytic activity (Rajesh et al. 2005). Human blood (1 ml) was mixed with 2 mg of EDTA. Equal volume of treated blood and 100 mM $CaCl_2$ were mixed and allowed to stand for 30 min to obtain a hard blood clot. EDTA-treated blood was centrifuged for 15 min at 500 g to separate plasma. 100 µl of plasma and 30 µl $CaCl_2$ (250 mM) were mixed and allow to stand for 1 h to get soft fibrin clot. Blood clot and plasma clots were thoroughly washed in 50 mM PBS buffer pH 7.5 for 8–10 times as used as substrate for the assay.

Hard blood clot and plasma clot hydrolyzing activity

The washed hard blood clot and plasma clot (explained in fibrinolytic activity section) were suspended in 400 µl of 10 mM Tris–HCl buffer (pH 7.6) and incubated with different concentrations of purified CPro (10–100 µg) and trypsin (10–100 µg) separately for 2.5 h at 37 °C. The undigested blood clot was precipitated using 0.44 M TCA and hydrolyzed products were assayed as described in the section protease assay of CPro (data not shown).

Human plasma clot hydrolysing activity using SDS-PAGE

The plasma clot (prepared as explained in fibrinolytic activity section) was incubated with different doses (0–50 µg) with purified CPro in a 50 µl reaction mixture at 37 °C in the presence of 10 mM Tris–HCl buffer pH 7.6. For time-dependent activity, 7 µl of CPro was incubated for different time periods (0–18 h). For inhibition studies, purified CPro (40 µg) was pre-incubated with and without specific protease inhibitors (5 mM of PMSF, EDTA, and 100 µM of IAA) for 30 min at 37 °C. The reaction was initiated by adding plasma clot and the assay was carried out as explained earlier. The reaction was arrested by adding 20 µl denaturing sample buffer (4% SDS, 4% β-mercaptoethanol and 1 M urea), boiled for 5 min and centrifuged for 10 min at 800 g to settle the debris of plasma clot. An aliquot (30 µl) of the supernatant was used to analyze the hydrolyzing pattern of plasma clot in 7.5% SDS-PAGE.

In vivo studies of purified protease from *Cucumis sativus* L.

Hemorrhagic activity and histopathology studies

Hemorrhagic activity was assayed as described by Devaraja et al. (2008). The purified CPro with different concentration was injected intradermally at the dose of 10 and 50 µg, at a concentration of 10 mg/kg body weight independently into groups of five mice in 20 µl saline. Saline receiving group served as negative control. After 3 h, mice were killed as per the JSS University Animal Ethics Committee regulations. Injected patch of skin surface was removed carefully and observed for hemorrhage against saline-injected control mice. The group that received *Daboia russellii* venom served as positive control. Dissected parts of skin surfaces were fixed in formalin solution and subjected to dehydration by processing the tissues through different grades of alcohol and chloroform: alcohol mixture. The 5 µm thick sections were cut from the processed tissue embedded in paraffin. The hematoxylin–eosin-stained sections were observed through the microscope. The sections were observed under Olympus BX-41 microscope and photographs were taken using ProgRes speed XT core 3 Jenoptik camera attached to the microscope.

Edema-inducing activity

Six mice in each group were injected separately into the right foot pads with different doses (0–50 µg) of purified CPro in 20 µl saline. The left foot pads received 20 µl salines alone served as controls. After 1 h mice were anesthetized using diethyl ether and euthanized. Hind limbs were cut off at the ankle joints and weighed. Variation in weights was calculated as the edema ratio (Vishwanath et al. 1987).

Mice were killed as per the JSS University Animal Ethics Committee (Registration no 261/CPSCE, dated 16th October 2000; sanctioned letter no JSS MC/PR/IAEC/24/2091/2013-14, dated 29th July 2013) regulation.

Statistical analysis

All statistical analysis were performed using GraphPad Prism 6.01. The SDS-PAGE analysis was done using Image lab version 5.1. Data of four sets were expressed as the mean ± SEM.

Results

Based on previous studies, crude enzyme extract of *C. sativus* shows hemostatic properties. A novel serine glycoprotein is purified to homogeneity from the *C. sativus* European variety using various steps that include salting out using ammonium sulfate, gel filtration with G-100, cation exchange chromatography using CM-Sephadex C-50, and gel filtration chromatography using G-75. Purification index and the yield of CPro is given in (Table 1). The crude sample shows many protein bands under non-reducing condition of SDS-PAGE (Fig. 2d lane 1). The protein elution profile of the G-100 column shows unresolved three peaks, G1, G2, and G3 but G2 is proteolytic active peak (Fig. 1a). Proteolytic active sample from G-100 column was loaded on to CM-Sephadex C-50 cation exchange chromatography column. The proteolytic activity was observed only in CM 3 peak (Fig. 1b) of cation chromatography. SDS-PAGE shows the reduction of protein bands after CM-Sephadex column (Fig. 2d lane 2). The active fractions were collected, lyophilized, and loaded on the G-75 column (Fig. 2a). The CPro peak of G-75 column was homogeneous and shows a single peak in RP-HPLC (Fig. 2c) and a single stainable band in SDS-PAGE and the molecular mass was approximately 75.3 kDa (Fig. 2b). Carbohydrate content of 4% was estimated by the phenol–sulfuric acid method. It was further confirmed that CPro is a glycoprotein by Periodic acid-Schiff (PAS) staining as shown in the figure (inset Fig. 2c). Gelatinolytic activity of CPro is visualized in substrate gel assay (inset Fig. 2c lane 1). CPro cleaves azocasein, casein and gelatin in dose-dependent manner (****$p < 0.0001$, Fig. 3a). The kinetic parameter K_m is determined (Fig. 3b) using three different substrates like azocasein, casein and gelatin. Azocasein is the preferred substrate with V_{max} 9.5 units/mg/min and K_m as low as 0.3809 mg/ml towards CPro. The pH and temperature kinetics of CPro are shown in Fig. 3c

Table 1 Purification by sequential multi-step process of CPro from *Cucumis sativus* L. European variety

Purification Step	Total protein obtained (mg)	Total Activity (units)	Specific Activity (units/mg)	Yield %	Fold purification
Crude whole Fruit extract	880	149600	170	100	1
90% Ammonium sulfate ppt.	527	131750	250	88	1.47
G-100	58	49300	850	33	5.0
CM C-50	16	15936	996	11	5.86
G-75	6.14	7368	1200	4.92	7.1
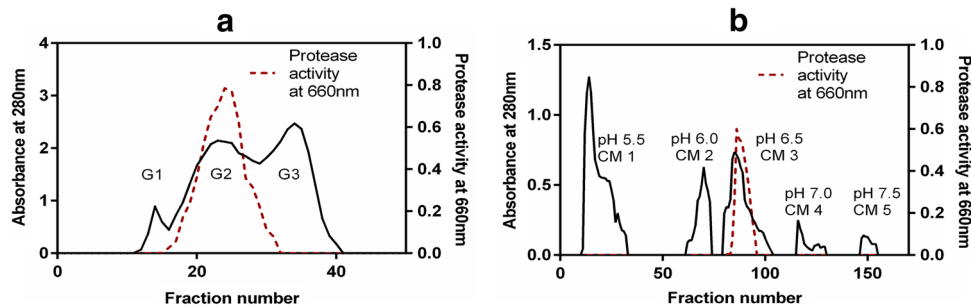
Fig. 1 Purification of *C. sativus* protease. **a** Sephadex G-100 elution profile of *Cucumis sativus* fruit extract shows G1, G2 and G3 peaks. Protein elution of G2 peak was monitored at 280 nm (−) and corresponding protease activity from the fraction. **b** CM-Sephadex C-50 elution profile of column chromatography. Protease active pooled fraction was subjected to CM- Sephadex chromatography, fractions were collected by changing the pH of the buffer CM 1 peak constitutes the unbound fraction and CM 2 to 5 constitutes the bound fractions alternative fractions were subjected to protease activity and CM 3 was identified to be only protease active peak

and d, respectively. Purified CPro exhibits optimum activity at 37 °C and pH 8. CPro shows decrease in recalcification time, prothrombin time and activated partial thromboplastin time of citrated human plasma, (Fig. 4a–c), respectively. CPro reduced the clotting time of citrated plasma by 88.8 ± 0.98%, prothrombin time by 56.85 ± 0.76% and activated partial thromboplastin time of plasma by 47 ± 1.7%. Effect of CPro on human fibrinogen incubated with different concentration (Fig. 5a), at different period of time (Fig. 5b) and in the presence of different inhibitors is show in (Fig. 5c). CPro degrades the subunits of human fibrinogen and the extent of cleavage is in the order $A\alpha > B\beta > \gamma$. Fibrinolytic activity was checked with different concentration of CPro (Fig. 5d), incubated with different inhibitors (Fig. 5e) and with variation of incubation time as shown in Fig. 5f. It also hydrolyses the subunits of partially cross-linked fibrin clot in the order α-polymer $> \gamma$–γ dimer $> \beta$-chain. CPro incubated with 5 mM PMSF showed no activity on human fibrinogen and plasma clot (Fig. 5c, e).

Edema of hind limbs with varying concentration of CPro was checked (Fig. 6a) and found that CPro does not induce edema. Dose-dependent hemorrhagic activity of CPro was examined in vivo with *Daboia russellii* venom as positive control. Skin patches of mice injected with Russell viper (*Daboia russellii*) venom (8 μg) after dissection showed hemorrhage, and those injected with CPro (50 μg in 20 μl saline), and the negative control group that received only Saline were found to be in no hemorrhagic condition (Fig. 6b). Histopathology of skin hemorrhage in mice stained with Hematoxylin–Eosin is shown in Fig. 6c. Skin sections of saline and CPro (50 μg) treated groups shows no significant pathology.

Discussion

A glycoprotein enzyme from aqueous fruit extract of *C. sativus* was purified to homogeneity by conventional methods. The homogeneity and intactness of the protein was affirmed by SDS-PAGE under reducing and non-reducing conditions and the molecular mass was found to be ~75.3 kDa. The purified protein is named as Cucumis protease (CPro). The homogeneous fractions were pooled, dialyzed and stored to conduct studies on biochemical and pharmacological characterization. CPro is found to be a glycoprotein. The estimated carbohydrate content of CPro was 4 %. Hexose content of cucumisin from *C. melo* was
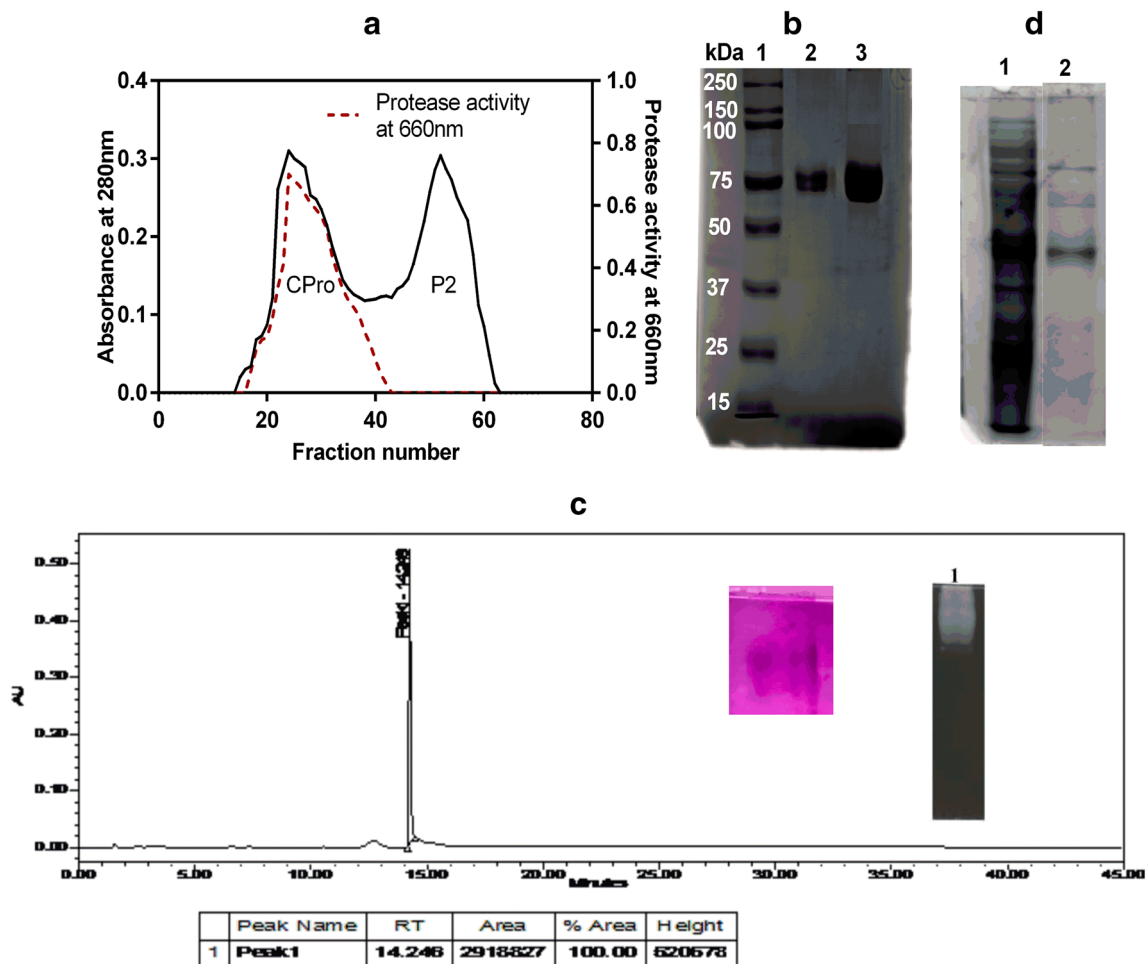
Fig. 2 Purification of *C. sativus* protease. **a** Sephadex G-75 elution profile of peak CM 3 obtained from CM-Sephadex C-50. **b** Purified CPro was run along with standard protein marker on 7.5% SDS-PAGE *Lane 1* 5 µl of protein ladder, *Lane 2* 20 µg of protein without β-mercaptoethanol, *Lane 3* 25 µg of protein with β-mercaptoehtanol. **c** RP-HPLC profile of CPro 20 µl of purified CPro from G-75 column was subjected to RP-HPLC The *inset* electrophoretogram shows the PAS staining of CPro, Gelatinolytic activity band *lane 1* 15 µg of CPro. **d** The electrophoretogram of crude sample before subjecting it to G-100 chromatography lane 1. *Lane 2* is the peak CM 3 from CM-Sephadex C-50 column chromatography

reported to be 2.4% and seven proteases from snake gourd varied from 0.3 to 1.1% (Kaneda and Tominaga 1975; Uchikoba et al. 1990). The protein surface attached with carbohydrate moieties is important to regulate the stability of the protein (Pande et al. 2006). Green asparagus, kiwi, and miut fruit contain high proteolytic and gelatinolytic activities (Yamaguchi et al. 1982). CPro with concentration as low as 15 µg showed clear gelatinolytic band against dark blue background. The properties of CPro can be compared with other sarcocarp of Cucurbitaceae family like *C. melo, C. trigonus roxburghi, Trichosanthes cucumeroides maxim* (snake gourd), *Benincasa hispida var.* ryukyu and it showed homology in many characters like pH specificity, inhibition with specific protease inhibitors, molecular weight and hexoses content (Albalasmeh et al. 2013; Kaneda and Tominaga 1975; Uchikoba et al.

1990, 1998; Asif-Ullah et al. 2006). Protease known at present, are widespread among all plant parts, but seems to be more abundant in fruits. Generally, plant serine proteases vary from 19 to 110 kDa and the majority lie between 60 and 80 kDa. The optimum pH for their activity lies in the alkaline range (pH 7–11) and many of them are active at 40 °C and above (Antao and Malcata 2005).

CPro works well at pH 8 and on either side of pH scale caseinolytic activity decreases. The CPro showed activity at 4 °C but the optimum temperature is found to be 37 °C. It loses stability as temperature increase and hence is not a thermostable protease. CPro incubated with 5 mM PMSF showed no activity with human fibrinogen and plasma clot hence it is a serine protease, similar to enzyme cucumisin from sarcocarp of melon fruit (Kaneda and Tominaga 1975). CPro hydrolyses different substrates like azocasein,

Fig. 3 Caseinolytic activity, and k_m of CPro. **a** Caseinolytic activity of CPro with different substrates like casein, azocasein and gelatin. ****$p < 0.0001$. **b** Lineweaver–Burk plot of the CPro. **c** Effect of pH on the activity of CPro with different buffers. All the buffers used were 50 mM concentration. **d** Effect of temperature from 4 to 90 °C on the activity of CPro was displayed in the graph
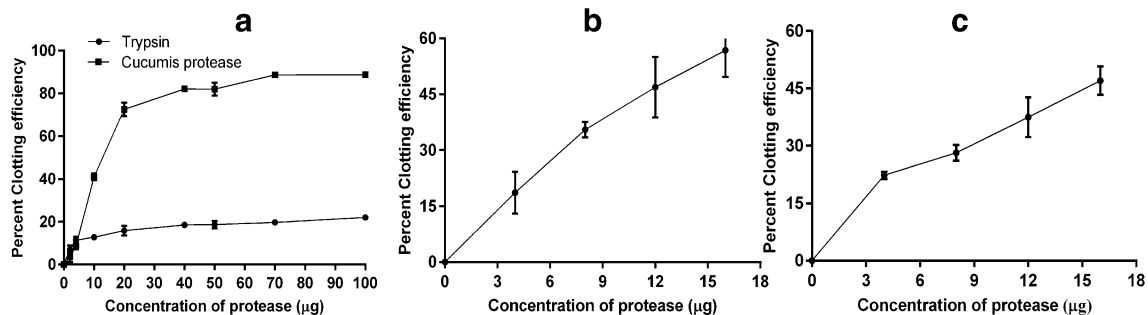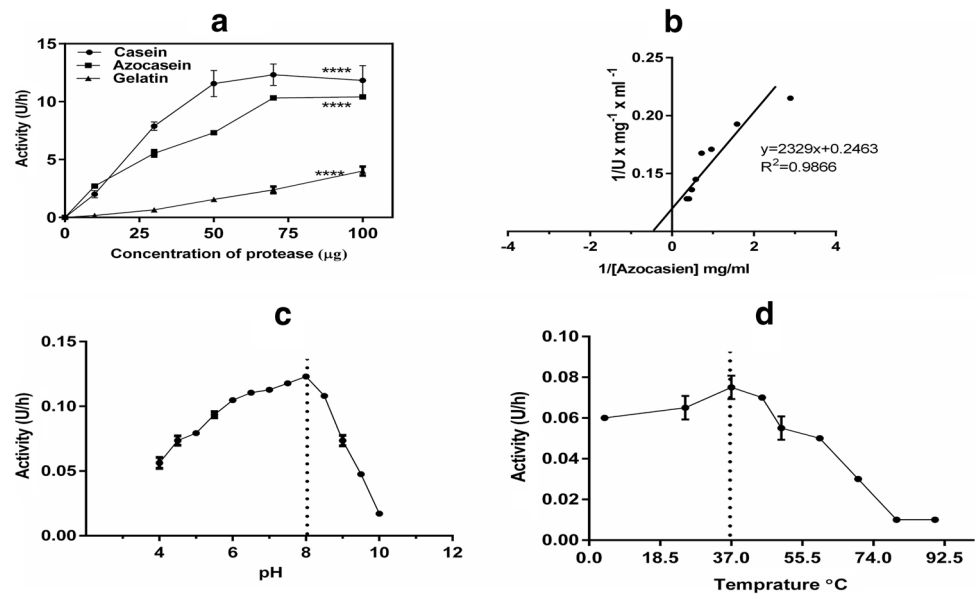

Fig. 4 a Effect of CPro on recalcification time of citrated human plasma. CPro ranging from 0 to 100 μg. **b** Effect of CPro (0–16 μg) on citrate human plasma with prothrombin time reagent and the values were given in percent clotting efficiency. **c** Effect of CPro (0–16 μg) on citrate human plasma with activated partial thromboplastin reagent

casein and gelatin with substrate specificity in the order gelatin (V_{max} 2.31 units/mg/min, K_m 1.197 mg/ml) <casein (V_{max} 3.864 units/mg/min, K_m 0.7765 mg/ml) <azocasein (V_{max} 9.5 units/mg/min, K_m 0.3809 mg/ml) based on K_m values obtained from Michaelis–Mentens equation. The V_{max} is 9.5 units/mg/min, and K_m value is 0.3809 mg/ml for azocasein. Azocasein is the preferred substrate with lowest K_m value towards CPro than casein and gelatin. It reduces the recalcification time by 88.8 ± 0.98%. CPro reduced the clotting time of citrated human plasma by 56.85 ± 0.76% and 47 ± 1.7% for prothrombin time and activated partial thromboplastin time respectively and values are expressed in percent clotting efficiency. The coagulation cascade of blood is a highly regulated pathway involves several coagulation factors with or without proteolytic activity. Many proteases are found to interfere in hemostasis and they have been isolated and well characterized from various plant lattices and snake venom

(Rajesh et al. 2006). Proteases obtained from Kiwi, pineapple and raspberries were reported to be procoagulant (Torres-Urrutia et al. 2011). CPro shows involvement in extrinsic as well as intrinsic pathway of blood coagulation cascade. CPro showed both fibrinogenolytic and fibrinolytic activities in a dose-dependent manner, while PMSF hinders both the activities. The action of CPro on extracellular proteins such as fibrinogen, fibrin were studied in vitro. Human fibrinogen incubated with 4 μg cleaves Aα chain and 8 μg of CPro cuts the Bβ chain. 5 μg of CPro cleaves both Aα and Bβ chain at 120 min as time of incubation. γ chain was not cleaved even at 24 h of incubation. In hemostasis along with clot formation, the dissolution of blood clot is a key step in wound healing. The clot-dissolving property of CPro was better than the trypsin enzyme. Alpha polymer and gama dimer of human plasma clot were degraded by 50 μg of CPro and gamma chain cleavage was also observed when incubation time was
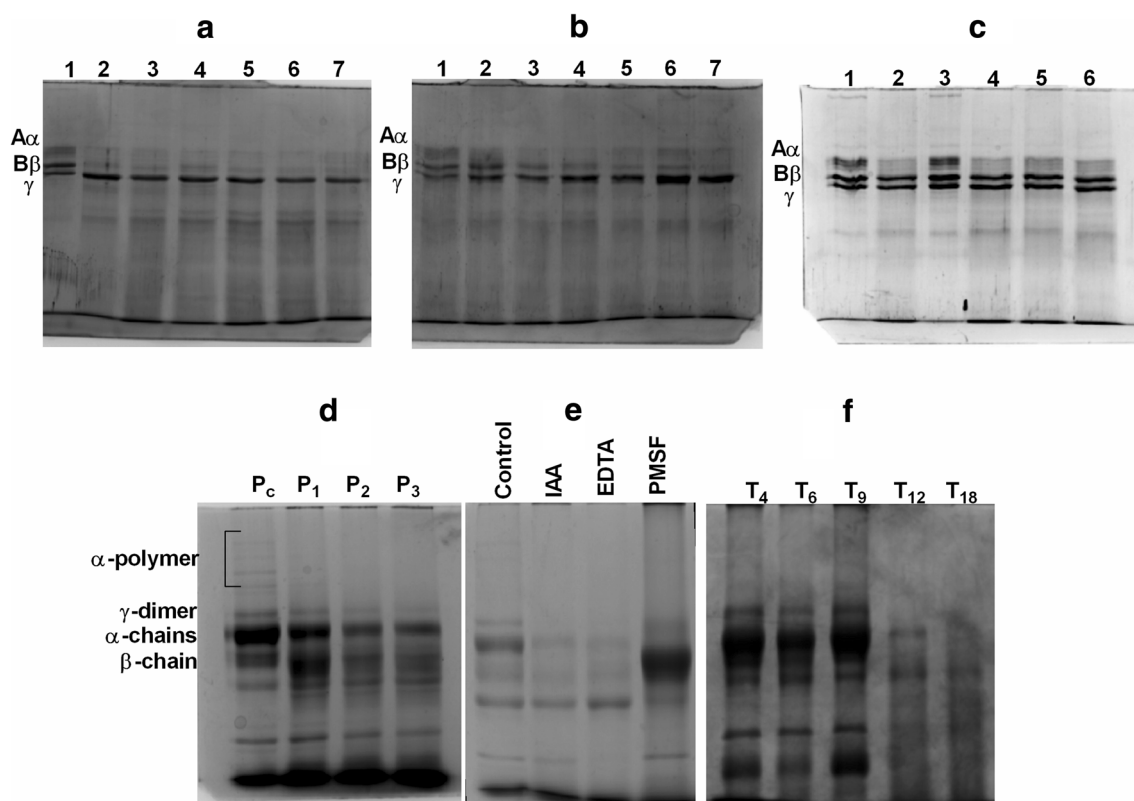
Fig. 5 Fibrinogenolytic activity of CPro on human fibrinogen. **a** Dose-dependent activity: *lane 1* 50 μg of fibrinogen without CPro, *lane 2* 1 μg, *lane 3* 2 μg, *lane 4* 4 μg, *lane 5* 5 μg, *lane 6* 6 μg, and *lane 7* 8 μg of CPro was incubated for 2.5 h at 37 °C. **b** Time-dependent activity: 1: 50 μg of human fibrinogen 0 min incubated with 5 μg of CPro, 2:5 min, 3:15 min, 4:30 min, 5:60 min, 6:90 min and 7:120 min. **c** Inhibition of fibrinogenolytic activity by CPro.1:50 μg fibrinogen, 2:50 μg fibrinogen with 5 μg CPro (R_x), 3:R_x with 5 mM PMSF, 4:R_x with 100 μM IAA, 5, 6:R_x with 5 mM of EDTA, and EGTA, respectively. Fibrinolytic activity of CPro on human plasma clot. **d** Dose-dependent activity: Washed plasma clot was incubated with CPro for 7 h at 37 °C in the presence of 10 mM Tris-HCL buffer pH 7.6. *Lane P_c* plasma clot without CPro, *lane P_1* plasma clot with 25 μg CPro, *lane P_2* plasma clot with 40 μg CPro, *lane P_3* plasma clot with 50 μg CPro. **e** Inhibition of fibrinolytic activity: *lane control* plasma clot witout CPro, *lane IAA* plasma clot with 100 μM IAA, *lane EDTA* plasma clot with 5 mM of EDTA *lane PMSF* plasma clot with 5 mM PMSF, respectively. **f** Time-dependent activity: *Lane T_4* plasma clot incubated with 50 μg of CPro for 4 h, *lane T_6* plasma clot incubated with CPro for 6 h, T_9 plasma clot incubated with CPro for 9 h, T_{12} Plasma clot incubated with CPro for 12 h, T_{18} plasma clot incubated with CPro for 18 h

prolonged to 18 h. To check edema and hemorrhage inducing activities of CPro, different concentrations of CPro were administered intradermally along with positive control *Daboia russellii* venom. CPro in 20 μl saline was injected separately into the right foot pads. The left foot pads received 20 μl saline alone which served as control. Hind limbs were cut off at the ankle joints and weighed. Edema formation is reflected through gain in weight. There is no significant difference in the weights of limbs with CPro treated and saline treated, which indicates non-toxic nature. Section treated with 8 μg of venom confirmed hemorrhage having denuded epidermis with thinning. Dermis showed edema and destruction of extracellular matrix with inflammation. No significant pathology in the skin of mice was observed even with 50 μg of CPro hence this proves CPro to be a nonedematous and nonhemorrhagic.

Conclusion

CPro is a novel serine glycoprotease purified from *C.sativus* European variety. The purity was adjudged by RP-HPLC as it shows a single peak at retention time of 14.246 min along with, single stainable band in both denaturing and reducing conditions of SDS-PAGE. The purified enzyme is a glycoprotein with molecular mass of ∼75.3 kDa. The optimum pH and temperature was 8, 37 °C respectively. Azocasein was found to be the preferred substrate with lowest Km (0.3809 mg/ml) value. CPro shows primary and secondary hemostatic activities as it cleaves both fibrinogen as well as fibrin. In addition CPro reduces prothrombin time and activated partial thromboplastin time which suggests the involvement of CPro in both intrinsic and extrinsic pathways. Reduction in recalcification time of human blood suggests CPro to be a potent
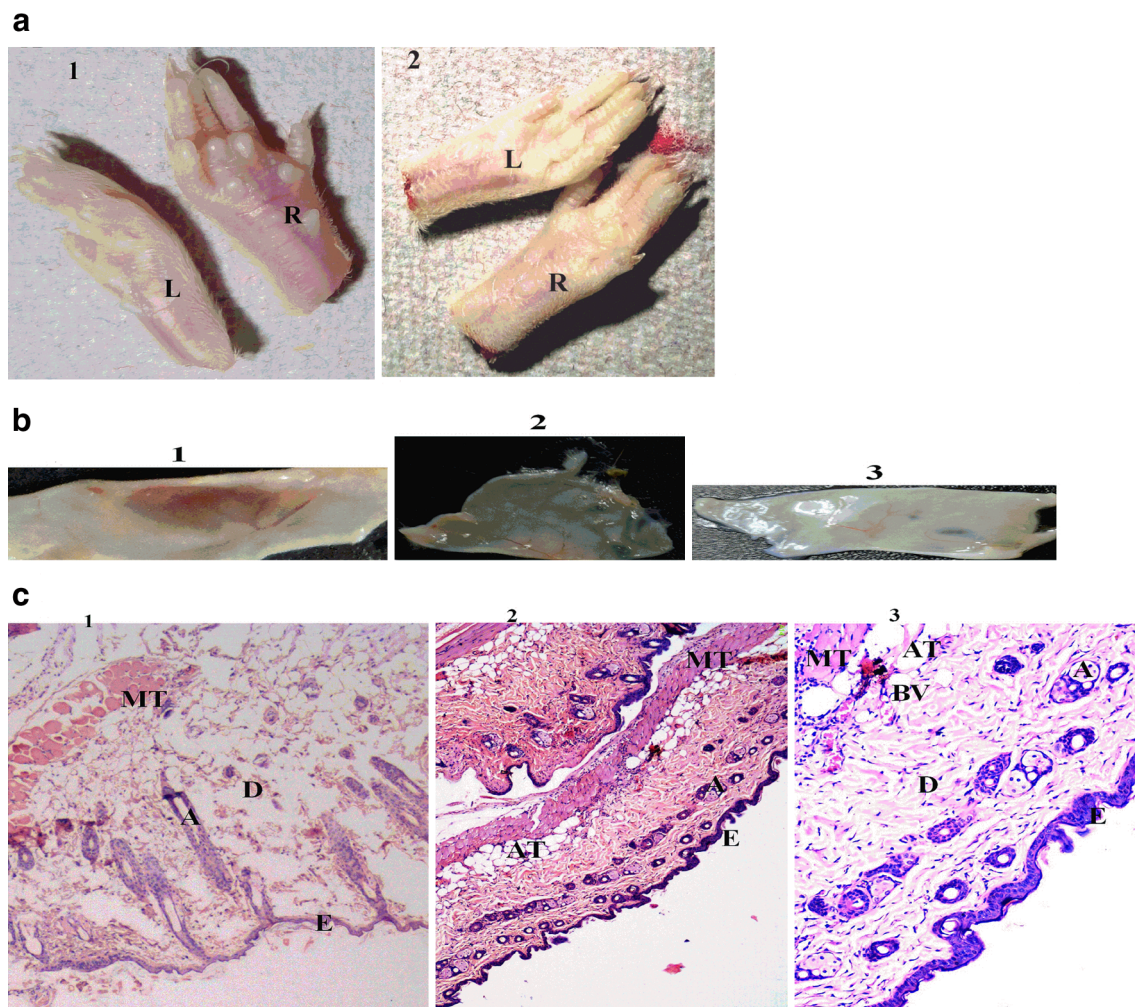
Fig. 6 Edema-inducing and hemorrhagic activity of CPro. **a** Edema inducing activity (*1*) Mice injected with *Daboia russellii* venom 8 µg, (*2*) 50 µg of CPro in 20 µl saline separately into the right foot pads (*R*). The left foot pads (*L*) received 20 µl saline served as controls. **b** Skin patches of mice after dissection showed with hemorrhage (*1*) injected with *Daboia russellii* venom 8 µg and without hemorrhage (*2*) 20 µl saline (*3*) 50 µg of CPro in 20 µl saline. **c** Histopathology of skin stained with Hematoxylin–Eosin. *1 Daboia russellii* venom 8 µg was used as positive control. *2* 20 µl saline as negative control. *3* Different concentration of CPro was injected intradermally. Images were taken under microscope and photographed in ×20. *A* skin adnexa, *D* dermis, *E* epidermis, *BV* blood vessel, *AT* adipose tissue, *MT* muscle tissue

procoagulant enzyme. As CPro shows remarkable hemostatic activity its use in traditional medicine is thus validated, and found to be an agent acting in the very first step of wound healing. The non-toxic nature of protease is also ascertained in the study through in vivo assays.

Compliance with ethical standards

Conflict of interest The authors confirm that this article content has no conflict of interest.

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