Jackfruit (*Artocarpus heterophyllus*) seed extract exhibits fibrino(geno)lytic activity

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ABSTRACT

Objective: The current study assesses the fibrinogen and fibrin clot hydrolyzing activities of aqueous seed extract of Jackfruit (AqSEJ). **Methods:** The protein banding pattern of AqSEJ (100 μg) was analyzed on SDS-PAGE. The proteolytic activity of AqSEJ was confirmed by spectrophotometer and zymography experiments. Fibrinogen, fibrin and plasma protein hydrolyzing activities of AqSEJ were analyzed on SDS-PAGE under reduced conditions. Plasminogen activation and indirect hemolytic activities was analyzed using spectrophotometer. The non-toxic property of AqSEJ was tested by edema, hemorrhage in experimental mice. **Results:** AqSEJ exhibited proteolytic activity and the specific activity was found to be 1.04 units/mg/min. Furthermore, AqSEJ non-specifically hydrolyzed Aα, followed by Bβ and γ chains of human fibrinogen and specifically hydrolyzed α polymer and α chain of partially cross linked human fibrin clot without affecting β chain and γ-γ dimer even up to the tested dose of 30 μg for the incubation period of 8 hours. Importantly, AqSEJ did not hydrolyze other plasma proteins and devoid of plasminogen activation property. The proteolytic activity of AqSEJ was completely neutralized by PMSF and IAA, while EDTA, EGTA, 1,10-Phenanthroline did not, suggesting the presence of serine and cysteine family proteases. Moreover, AqSEJ did not cause edema and hemorrhage in experimental mice up to the tested dose of 200 μg and non-toxic to RBC cells. **Conclusion:** AqSEJ hydrolyzes fibrinogen and fibrin clot and non-toxic in nature. Hence, this work showcases the potential applications of Jack fruit seed proteases in the treatment of thrombotic disorders.

Key words: Fibrino(geno)lysis, Hemostasis, Jackfruit, Moraceae, Non-hemorrhagic, Serine/cysteine protease.

INTRODUCTION

Proteases are ubiquitous hydrolytic enzymes involved in several physiological reactions including digestion, blood coagulation cascade, compliment system, apoptosis pathways and invertebrates prophenoloxidase activating cascade. Proteases have been extensively used in food industry, leather industry and pharmaceutical industries. In addition, proteolytic enzymes could be used as therapeutic agents to treat cancer (L-asparaginase), thrombotic disorder (nattokinase) and wound healing (papain).[1-4] The Jackfruit is popularly known as a seasonal fruit that belongs to the family of Moraceae and genus *Artocarpus* Lam. Though it is native to India, it is widely distributed especially in the tropical areas of East Asia and South America. Generally, the fruit part will be consumed due to its delectability; however, many times the seed will be discarded. While, in the southern part of India seeds are consumed as food. Jackfruit seeds are rich sources of both macro and micronutrients.[5-8] According to the recent studies Jackfruit seeds were found to contain anticancer, antihypertensive, antiulcer, antioxidant, antifungal, and antimicrobial

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properties.[9,10] In addition, the Jackfruit seeds are beneficial in blood purification and curing pancreatic ailments.[11] The large array of therapeutic applications of Jackfruit seeds is attributed to the major biochemical constituents such as enzymes/peptides/proteins and phytochemicals. Jackfruit seeds are reported to contain the highest amount of trypsin, chymotrypsin, elastase inhibitors and lectin family glycoprotein's (Jacalin).[12-14] For instance, Jacalin (immuno modulatory glycoprotein) is one of the most important lectin characterized so far from Jackfruit seeds, however, the proteolytic enzymes were least explored.[15] However Siritapetawee *et al* (2012) reported on antimicrobial protease (AMP48) and novel serine protease having fibrino (geno) lytic activities from *Artocarpus heterophyllus* latex. Therefore, the current study reports the fibrinogen and fibrin hydrolyzing properties of Jackfruit seed aqueous extract (AqSEJ).

MATERIALS AND METHODS

Materials

Fat free casein, phenyl methyl sulphonyl fluoride (PMSF), ethylene diamine tetra acetic acid (EDTA), ethylene glycol-N, N, N', N'- tetra acetic acid (EGTA), iodoacetic acid (IAA), 1,10-Phenanthroline were purchased from Sigma Chemicals Co. (St.Louis, USA). Molecular weight markers were from Bangalore Genei Private limited, India. Human plasma fibrinogen was purchased from Sigma Chemicals Co. (St Louis, USA). All other chemicals used were of analytical grade. Fresh human blood was collected from healthy donors for the platelet rich plasma (PRP). Swiss albino mice weighing 20-25 g (from the central animal house facility, Department of Zoology, University of Mysore, Mysore, India) were used for pharmacological studies. Animal care and handling compliance with the National Regulations for Animal Research. The animal experiment was carried out after reviewing the protocol by the animal ethical committees (UOM/IAEC/06/2011 dated 12/07/2011) of the University.

Plant material

Artocarpus heterophyllus Lam (Jackfruit) seeds were obtained in the month of April and May from Pittenahalli village, Tumkur district and identified by Dr. P.Sharanappa, University of Mysore. A voucher specimen (PS/52/18 FEB 2012) has been preserved at the herbarium of Bioscience Department, University of Mysore, Hassan PG campus, Hassan for future allusion.

Preparation of Jackfruit seed extract

Brown coat was removed from the Jackfruit seeds thoroughly chopped and homogenized using double distilled water and centrifuged at 2000 g for 20 min at 4°C. The supernatant was collected and proteins were precipitated using 30% of ammonium sulphate. The precipitated protein sample was again centrifuged at 3,500 g for 20 min; pellet was dissolved and dialyzed overnight. The protein sample obtained was stored at -20°C until use. This extracted protein sample was used throughout the study and referred as Jackfruit seed aqueous extract (AqSEJ).

Protein estimation

Protein concentration was determined as described,[16] using Bovine serum albumin (BSA) as standards.

Proteolytic activity

Colorimetric estimation

Proteolytic activity was assayed as described.[17] Briefly, fat free casein (2%) was incubated with AqSEJ (60 µg) for 2.5 hours at 37°C. Sodium carbonate (0.4 M) and Folin-Ciocalteu reagent were added sequentially and the color development was read at 660 nm. One unit of enzyme activity was defined as the amount of the enzyme required to cause an increase in OD of 0.01 at 660 nm/min at 37°C. The specific activity was expressed as the units/min/mg of protein. For inhibition studies, similar reaction was performed independently after preincubating the AqSEJ (60 µg) for 30 min with 5 mM each of EDTA, 1,10-Phenanthroline, EGTA, PMSF and IAA.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Zymogram

SDS-PAGE (10%) and zymogram were carried as described previously.[18] Breifly, the AqSEJ (100 µg) prepared under reducing and non-reducing condition was used for SDS-PAGE. For zymogram, the AqSEJ (60 µg) prepared under non-reduced condition was loaded on to polymerized 2% casein/gelatin in resolving gel. After electrophoresis, protein bands were detected using Coomassie brilliant blue R-250. Molecular weight standards from 200 to 14.3 kDa were used to compare the protein bands of AqSEJ. For zymogram, after electrophoresis gels were washed with 10 mM sodium phosphate buffer containing 2.5% of Triton X-100 with constant agitation for 1 hour to remove SDS. The gel was incubated overnight at 37°C in Tris–HCl buffer (50 mM) pH 7.6 containing 10 mM $CaCl_2$ and 150

mM NaCl. Gel was then stained to observe the translucent activity bands.

Periodic acid-Schiff (PAS) staining

SDS-PAGE was carried out as mentioned above. PAS staining was done as described.[19] After the electrophoresis gel was fixed in 7.5% acetic acid solution and stored at room temperature for 1 hour. Then gel was washed with 1% nitric acid solution and kept in 0.2% aqueous periodic acid solution and stored at 4°C for 45 min. Then the gel was placed in Schiff's reagent at 4°C for 24 hours and was destained using 10% acetic acid to visualize pink color band.

Fibrinogenolytic activity

Fibrinogenolytic activity was determined as described previously.[20] AqSEJ (0-20 µg) was incubated with the human plasma fibrinogen (50 µg) in a total volume 40 µL of 10 mM Tris-HCl buffer pH 7.4 for 4 hours at 37°C. After an incubation period, reaction was terminated by adding 20 µL denaturing buffer containing 1 M urea, 4 % SDS and 4 % β-mercaptoethanol. It was then analyzed by 10% SDS-PAGE. For inhibition studies, AqSEJ (20 µg) was preincubated for about 15 min with 5 mM each of PMSF, IAA, EDTA and 1,10-Phenanthroline.

Fibrinolytic activity

Colorimetric estimation

Briefly, 100 µL of citrated human plasma was mixed with 30 µL of 0.2 M $CaCl_2$ and incubated for 2 hours at 37°C. The clot obtained was washed thoroughly for 5-6 times with phosphate buffer saline (PBS) and suspended in 400 µL of 0.2 M Tris–HCl buffer pH 8.5. The reaction was initiated by adding varied concentration of AqSEJ (0-30 µg) in 100 µL of saline and incubated for 2 hours 30 min at 37°C. The undigested clot was precipitated by adding 750 µL of 0.44 M trichloroacetic acid (TCA) and allowed to stand for 30 min and centrifuged for 15 min at 1,500 g. The aliquots of 0.5 mLsupernatant were transferred to clean glass tubes and followed by the addition of 1.25 mL of 0.4 M sodium carbonate and 0.25 mL of 1:3 diluted Folin-Ciocalteu's phenol (FC) reagent. The color developed was read at 660 nm after allowed to stand for 30 min. One unit of activity is defined as the amount of enzyme required to increase in absorbance of 0.01 at 660 nm/hour at 37°C.

Protein banding pattern on SDS-PAGE

Fibrinolytic activity was determined as described.[21] Tri-sodium citrate (3.2%) treated blood was centrifuged for 15 min at 500 g to separate platelet poor plasma. Plasma (100 µL) was mixed with an equal volume of 0.25 M $CaCl_2$ for 15 min at 37°C to get the soft fibrin clot. The fibrin clot was washed thoroughly 5–6 times with phosphate buffered saline (PBS) and suspended and incubated with AqSEJ (0–30 µg) in a final volume of 40 µL 10 mM Tris–HCl buffer pH 7.4 at 37°C for 8 hours. For inhibition studies, AqSEJ (30 µg) was pre incubated for about 15 min with 5 mM each of PMSF and IAA. The reaction was stopped by adding 20 µL of sample buffer containing 4% SDS, 1M urea and 4% β-mercaptoethanol. The samples were kept in a boiling water bath for 5 min and centrifuged to settle the particulate of the plasma clot. An aliquot of 20 µL supernatant was analyzed in 7.5% SDS-PAGE for fibrin degradation.

Plasminogen activation

The plasminogen activation was assayed as described.[22] Briefly, the samples of 1 mg of plasminogen contaminated fibrinogen or 20 µL of citrated human plasma with 200 IU of streptokinase with 50 µg of AqSEJ and 200 IU of streptokinase alone were independently mixed with 500 µL of azocasein and incubated for 3 hours at 37°C. The reaction was terminated by adding 400 µL of 25% trichloroacetic acid. The supernatant (600 µL) was diluted with an equal volume 0.5 N NaOH and absorbance was read at 440 nm. One unit of activity was defined as the amount of enzyme yielding an increase in absorbance of 0.01/hour at 440 nm.

Indirect hemolytic activity

Indirect hemolytic activity was determined as described previously[23] using washed human erythrocytes in presence of 0-200 µg of AqSEJ. The amount of hemoglobin released in the supernatant was measured at 540 nm.

Degradation of human plasma proteins

Degradation of human plasma protein was assayed as described previously.[24] The AqSEJ (0-50 µg) was incubated with the 100 µg of plasma proteins for 12 hours at 37°C in a reaction volume of 40 µL 10 mM Tris–HCl buffer pH 7.4 containing 10 mM NaCl and 0.05 % sodium azide. The reaction was terminated by the addition of 20 µL denaturing buffer containing 4% SDS and boiled for 5 min and analyzed on 7.5% SDS-PAGE under non-reduced condition.

Edema inducing activity

Edema inducing activity was done as described earlier.[25]

The groups of five mice were injected separately in to the right foot pads with different doses (0-200 µg) of AqSEJ in 20 µL saline. The left foot pads received 20 µL saline alone and served as controls. After 1 hour mice were anaesthetized by diethyl ether inhalation. Hind limbs were removed at the ankle joint and weighed. Weight increase was calculated as the edema ratio, which equals the weight of an edematous leg×100/weight of a normal leg. Minimum edema dose (MED) was defined as the amount of protein required to cause an edema ratio of 120 %.

Hemorrhagic activity

Hemorrhagic activity was assayed as described.[26] A different concentration of AqSEJ (0-200 µg) was injected (intradermal) independently into the groups of five mice in 30 µL saline. The groups receiving saline alone served as a negative control and the group receiving Daboia resselli venom (2 MHD) served as the positive control. After 3 hours, mice were anaesthetized by diethyl ether inhalation. Dorsal patch of skin surface was carefully removed and observed for hemorrhage against saline injected control mice. The diameter of hemorrhagic spot on the inner surface of the skin was measured. The minimum hemorrhagic dose (MHD) was defined as the amount of the protein producing 10 mm of hemorrhage in diameter.

Statistical Analysis

The data are presented as mean s ± S.E.M of at least five animals in each group. Difference among the data were determined by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT).Data were considered different at $P < 0.01$.

RESULTS AND DISCUSSION

Characterization of proteins and identification of proteolytic activity of AqSEJ

AqSEJ exhibited varied protein bands in the broad molecular weight range from 200 kDa to 14.3 kDa under both reduced and non-reduced conditions (Figure 1a). AqSEJ hydrolyzed casein with the specific activity of 1.04 units/mg/min suggesting the proteolytic activity. The proteolytic activity of AqSEJ was further confirmed by casein and gelatin zymogram, it showed two similar translucent activity bands in the higher molecular weight region (Figure1b & c). In order to identify the nature of protease present in the AqSEJ inhibition studies were carried out using specific protease inhibitors such as PMSF, IAA, EDTA, EGTA and 1,10-Phenanthroline. Interestingly, proteolytic activity of AqSEJ was completely neutralized by PMSF, a serine protease inhibitor and IAA a cysteine protease inhibitor, while metalloprotease inhibitors such as, 1,10-Phenanthroline and EDTA did not inhibit the proteolytic activity (Figure 2a & b). (Table 1) represents the effect of protease inhibitors on the proteolytic activity of AqSEJ. In order to identify the presence of glycoproteins in the extract PAS staining was done, the AqSEJ showed glycoprotein band at 14.3 kDa region on SDS-PAGE (Figure 1d).

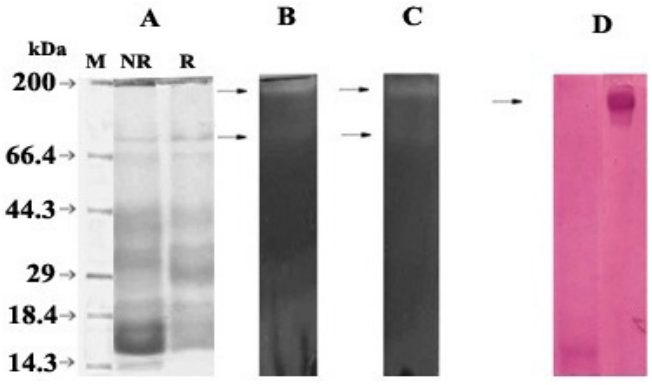

Figure 1: (a) AqSEJ as shown in SDS-PAGE (10 %)

(1) AqSEJ (100 µg) under non-reduced and (2) reduced conditions; (b) Caseinolytic activity staining (zymography) of AqSEJ (60 µg) resolved by SDS-PAGE (10 %) under non-reduced condition; (c) Gelatinolytic activity staining (zymography) of AqSEJ (60 µg) resolved by SDS-PAGE (10 %) under non-reduced condition; (d) PAS staining of AqSEJ (1) and PAS staining of positive control fibrinogen (2). M represents the molecular weight marker in kDa from top to bottom: myosin-H-chain (200), BSA (66.4), ovalbumin (44.3), carbonic anhydrase (29), β lactalbumin (18.4) and lysozyme (14.3).

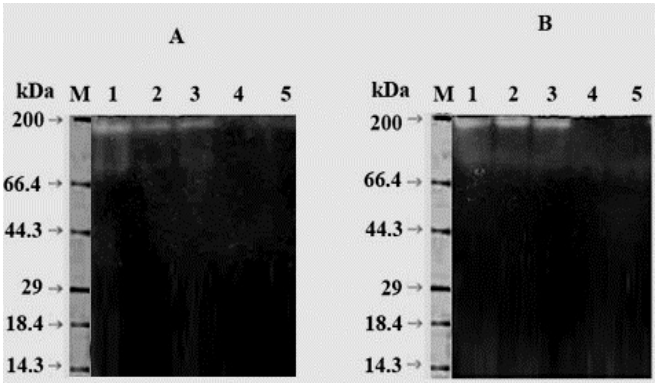

Figure 2: (a) Casienolytic activity of AqSEJ resolved in SDS-PAGE (10 %)

(1) AqSEJ 60 µg alone,(2) AqSEJ 60 µg was pretreated with 5 mM EDTA,(3) AqSEJ 60 µg was pretreated with 5 mM 1,10-Phenanthroline,(4) AqSEJ 60 µg was pretreated with 5 mM PMSF, (5) AqSEJ 60 µg was pretreated with 5 mM IAA under non-reduced condition; (b) Gelatinolytic activity of AqSEJ resolved in SDS-PAGE (10 %), (1) AqSEJ 60 µg alone,(2) AqSEJ 60 µg was pretreated with 5 mM EDTA,(3) AqSEJ 60 µg was pretreated with 5 mM 1,10-Phenanthroline,(4) AqSEJ 60 µg was pretreated with 5 mM PMSF,(5) AqSEJ 60 µg was pretreated with 5 mM IAA under non-reduced condition. M represents the molecular weight marker in kDa from top to bottom: myosin-H-chain (200), BSA (66.4), ovalbumin (44.3), carbonic anhydrase (29), β lactalbumin (18.4) and lysozyme (14.3).

Table 1: Effect of Inhibitors on the Proteolytic Activity of AqSEJ

Inhibitor (5 mM each)	Activity/residual activity
None	100
EDTA	98.95
EGTA	90.20
1,10-Phenanthroline	74.50
IAA	15.40
PMSF	01.80

Values are average of three independent experiments

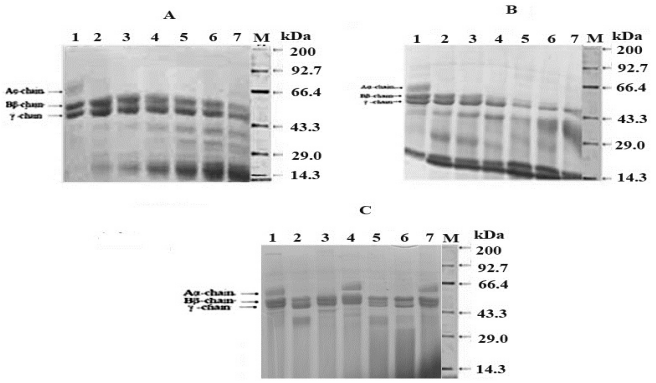

Figure 3: Fibrinogenolytic activity

(a) Dose dependent effect: Fibrinogen alone (1) and fibrinogen treated with 2 µg (2), 4 µg (3), 8 µg (4), 12 µg (5)16 µg (6) and 20 µg (7) of AqSEJ; **(b) Time dependent effect:** AqSEJ (16 µg) was incubated with fibrinogen (50 µg) at 37°C for 0 hour (1), 2 hours (2), 4 hours (3), 8 hours (4), 14 hours (5),18 hours (6) and 24 hours (7); **(c) Inhibition of fibrinogenolytic activity:** AqSEJ (16 µg) was preincubated with and without protease inhibitors for 30 min at 37°C.

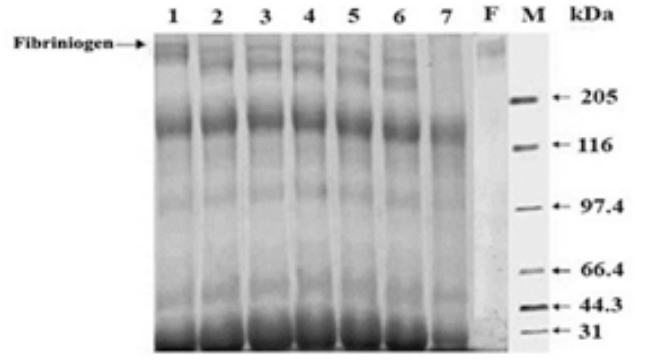

Figure 5: Degradation of plasma proteins:

Plasma protein (100 µg) is incubated with AqSEJ in 40 µL of 10 mM Tris-HCl buffer pH 7.4 at 37°C and then analyzed on 7.5% SDS-PAGE under non-reduced condition. Plasma protein (100 µg) alone (1), plasma protein treated with 5 µg (2), 10 µg (3), 20 µg (4), 30 µg (5), 40 µg (6), 50 µg (7) of AqSEJ of for 12 hours, (F) 20 µg of fibrinogen (positive control).

AqSEJ specifically hydrolyzed fibrinogen and fibrin but not other plasma proteins

AqSEJ hydrolyzed human fibrinogen and fibrin clot. AqSEJ at the concentration of 0-20 µg for the incubation period of 4 hours preferentially degraded Aα chain followed by Bβ chain of fibrinogen in a dose dependent manner (Figure 3a). However, when the incubation period was extended up to 24 hours it hydrolyzed Aα, Bβ and γ chains of fibrinogen (Figure 3b). In both the cases (4 hours and 24 hours) the activity of AqSEJ was authenticated by the progressive decreased intensity of the susceptible fibrinogen chains and appearance of new low molecular weight peptides as degradation products on SDS-PAGE under reduced condition. The fibrinogenolytic activity was completely neutralized by PMSF and IAA (Figure 3c). Furthermore, AqSEJ hydrolyzed washed plasma/fibrin clot and the specific activity was found to be 2.62 units/mg/

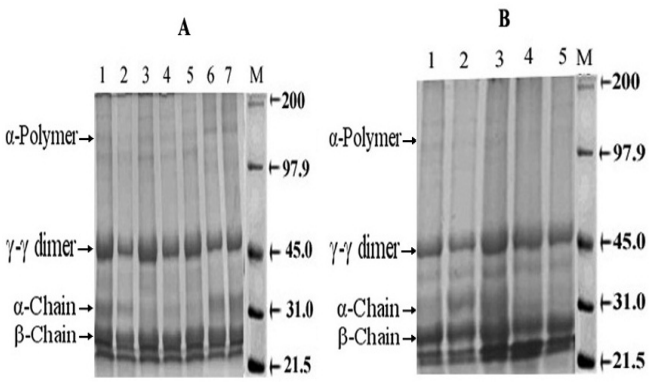

Figure 4: Fibrinolytic activity

(a) Dose dependent effect: plasma clot alone (1) and plasma clot treated with 5 µg (2), 10 µg (3), 20 µg (4), 30 µg (5) of AqSEJ respectively, 30 µg of AqSEJ was preincubated with 5 mM PMSF for 30 min at 37°C (6) and 30 µg of AqSEJ was preincubated with 5 mM IAA (7) for 8 hours at 37°C; **(b) Time dependent effect:** AqSEJ (30 µg) was incubated with plasma clot for 0 hour (1), 2 hours (2), 4 hours (3), 8 hours (4), 12 hours (5).

min. The plasma/fibrin clot hydrolyzed pattern of AqSEJ was also analyzed on the SDS-PAGE. The (Figure 4a & b) represents both dose and time dependent fibrinolytic activity of AqSEJ. AqSEJ preferentially degraded α polymer and α chain of partially cross linked human fibrin clot, while γ-γ dimer and β chain remained resistant to proteolysis over the incubation period of 12 hours. These results suggested that AqSEJ preferentially cleaves the clot and might be used as clot dissolving factor. Since AqSEJ exhibits proteolytic activity and degrades fibrinogen, experiments were carried out to rule out the possibility that AqSEJ exerts its proteolytic activity on other plasma protein. AqSEJ did not hydrolyze any of the plasma protein other than fibrinogen (Figure 5). The intensity of the fibrinogen band was progressively vanished with increase in concentration of AqSEJ (0-50 µg) which is compared with positive control fibrinogen alone.

AqSEJ is devoid of plasminogen activation ability

AqSEJ degraded azocasien but did not activate plasminogen, while streptokinase a positive control activated the plasminogen. These results suggest that AqSEJ specifically acts on fibrinogen and directly dissolves fibrin clot without activating plasminogen to plasmin.

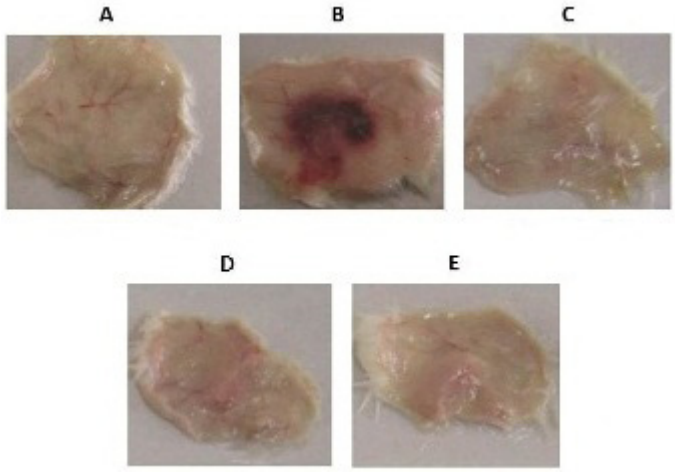

Figure 6: Dose dependent hemorrhagic activity of AqSEJ.

A: saline, B: positive control 2 MDH venom, C: 50 µg, D: 100 µg and E: 200 µg of AqSEJ was injected independently in to mice in a total volume of 50 µL intradermally..

AqSEJ do not cause hemolysis, edema and hemorrhage

AqSEJ did not hydrolyze RBC. Moreover, it did not cause hemorrhage and edema in experimental mice up to the concentration of 200 µg (Figure 6) while positive control Daboia russelli venom induced hemorrhage and edema in experimental mice. These results suggest that the AqSEJ are relatively non-toxic at the tested dose.

DISCUSSION

In recent time, the therapeutic usage of proteolytic enzymes from ticks, earth worm, leach, venom of snake, spider, bee, scorpion and latex of plants have been gaining much importance.[24,27-29] Despite anticancer, antihypertensive, antiulcer, antioxidant, antifungal and antimicrobial activities of Jackfruit,[9,11] its potential role of fibrino (geno) lysis was not explored. Thus, the current study demonstrates the fibrinogen and fibrin clot hydrolyzing activities of AqSEJ.

AqSEJ showed dissimilar protein banding pattern under reducing and non-reducing conditions, suggesting the presence of oligomeric proteins. AqSEJ was positive to PAS staining only at the low molecular weight region, suggesting proteases were devoid of carbohydrate moities. AqSEJ hydrolyzed casein, gelatin, fibrinogen and fibrin suggesting proteolytic activity. Proteolytic activity was also confirmed using casein and gelatin zymography, AqSEJ exhibited two translucent activity bands of higher molecular weight region, suggests the presence of two isoforms of proteases. Interestingly, proteolytic activity of the AqSEJ was completely neutralized only by PMSF and IAA indicating the presence of serine and cystein proteases respectively.

Fibrinogen (factor I) is a large plasma glycoprotein having the molecular mass of 340 kDa, comprises of three polypeptide chains Aα, Bβ and γ, play an important role in fibrin clot formation. Physiologically thrombin specifically degrades Aα and Bβ chains of fibrinogen from N-terminal end and generates fibrinopeptide A and B that facilitates the generation of fibrin.[27,30] The proteolytic enzymes may degrade fibringogen either from N-terminal end or C terminal end. The proteolytic enzymes (serine, metallo and cystein) degrade fibrinogen similar to thrombin and are considered to be thrombin like enzymes.[30] *Artocarpus heterophyllus* latex completely degraded α chain, while partially degraded β and γ chains of human fibrinogen.[15] AqSEJ non-specifically hydrolyzed all the chains (Aα, Bβ andγ) of fibrinogen; therefore it is too premature to say the site of action of proteolytic enzyme on the fibrinogen.

Fibrinolysis is an important event in preventing blood clot, wound healing and normal circulation of the blood. Physiologically plasmin hydrolyzes the fibrin.[27-29] Interestingly, AqSEJ preferentially hydrolyzed α polymer and α chain of human fibrin, thus it may serve as a clot dissolving factor in thrombotic disorder. It is to emphasis that, AqSEJ degraded all the chains of fibrinogen, α polymer and α chain of human fibrin but did not hydrolyze other human plasma proteins revealed its specificity towards only fibrinogen and fibrin. The fibrino(geno)lytic activity was completely abolished by PMSF, IAA but did not EDTA and 1,10-Phenanthroline revealed the role of serine and cysteine proteases in the fibrino(geno)lytris. Moreover, AqSEJ did not activate plasminogen to plasmin revealed its direct action on fibrin.

Interestingly, AqSEJ was non-toxic to experimental mice as it did not show indirect hemolytic activity, hemorrhage and edema in experimental mice.

CONCLUSION

This study reports on the fibrino(geno)lytic activities of AqSEJ. Hence, further identification and biochemical characterization of active fibrino(geno)lytic enzyme from the AqSEJ may present a promising alternative in the treatment of thrombotic disorders.

CONFLICT OF INTEREST

No competing financial interest exists.

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