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Purification and characterization of a 34-kDa, heat stable glycoprotein from *Synadenium grantii* latex: action on human fibrinogen and fibrin clot

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Abstract

Latex glycoprotein (LGP) from *Synadenium grantii* latex was purified by the combination of heat precipitation and gel permeation chromatography. LGP is a heat stable protein even at 80 °C showed a sharp single band both in SDS-PAGE as well as in native (acidic) PAGE. LGP is a monomeric protein appears as single band under reducing condition. It is a less hydrophobic protein showed sharp single peak in RP-HPLC with retention time of 13.3 m. The relative molecular mass of LGP is 34.4 kDa. CD spectrum of LGP explains less content of α-helix (7%), and high content of β-pleated sheets (48%) and random coils (46%). The N-terminal sequence of LGP is D-F-P-S-D-W-Y-A-Y-E-G-Y-V-I-D-R-P-F-S. Purified LGP is a fibrinogen degrading protease hydrolyses all the three subunits in the order of Aα, Bβ and γ. The hydrolytic pattern is totally different from plasmin as well as thrombin. LGP reduces recalcification time from 165 to 30 s with citrated human plasma but did not show thrombin like as well as factor Xa-like activity. Although LGP induces procoagulant activity, it hydrolyses partially cross-linked fibrin clot. It hydrolyses all the subunits of partially cross-linked fibrin clot (α- chains, β-chain and γ-γ dimer). LGP is a serine protease, inhibited by PMSF. Other serine protease inhibitors, aprotinin and leupeptin did not inhibit the caseinolytic activity as well as fibrinogenolytic activity. We report purification and characterization of a glycoprotein from *Synadenium grantii* latex with human fibrino(geno)lytic activity.

Keywords: *Synadenium grantii*; Latex glycoprotein (LGP); Hemostasis; fibrinogenolytic activity; Fibrin clot hydrolyzing activity

1. Introduction

Proteases are the group of hydrolytic enzymes, play an important role in many regulatory processes. Generally mammalian proteases involved in the activation of blood coagulation and fibrinolysis [1], degrade useless proteins [2], zymogens activation [3], angiogenesis, tissue proliferation and atherosclerosis [4]. Coagulation and fibrinolysis are the two important proteolytic events associated with wound healing [4] and myocardial infarction [5]. Coagulation is a stepwise activation of several factors, results in the conversion of fibrinogen to fibrin through the action of thrombin, facilitating the rapid response of the body to injury [6]. Fibrinolysis dissolves thrombi and haemostatic plugs through the action of plasmin on fibrin, facilitates wound healing and easy flow of blood. Several proteases affecting blood coagulation and fibrinolysis have been isolated and well characterized from snake venoms [7,8], leeches [9], annelids [10,11], insects [12], caterpillar [13], algae [14] and other microbial sources [15]. So far proteases from plant latex, which affect coagulation and fibrinolysis, have not been isolated and well studied.

Plant latex is a complex mixture of organic and inorganic compounds [16], waxy materials and hydrolytic enzymes [17]. Among hydrolytic enzymes, protease is the major fraction and plays a very important role in plant physiology [3], host pathogen interaction [18] and exerts several pharmacological actions, interfering in hemostasis [19]. Recently we have shown the involvement of *Calotropis gigantea* latex proteases in coagulation and fibrino(geno)lysis [20].

Abbreviations: °C, degree Celsius; cm, centimeter; EDTA, ethylene diaminetetraaceticacid; EGTA, ethylene glycol *NNN′N′*-tetraaceticacid; h, hour(s); IAA, iodoacetic acid; μg, microgram; m, minute(s); mg, milligram; ml, milliliter; mM, millimolar; nm, nanometer; %, percentage; PMSF, phenylmethylsulphonyl fluoride; s, second(s); SDS, sodiumdodecylsulfate; S.E.M., standard error of the mean; TCA, trichloroaceticacid; TFA, triflouroaceticacid.

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Synadenium grantii is one of the important latex bearing plants belonging to the family Euphorbiaceae. Like other plant latex *S. grantii* latex is also rich in proteolytic enzymes. Menon et al. [3] isolated and characterized two serine proteases from *S. grantii* latex extract. However, the involvement of *S. grantii* latex proteases in coagulation and fibrino(geno)lysis has not been reported. In the present study we have purified and characterized a fibrino(geno)lytic glycoprotein (LGP) from the latex of *S. grantii*.

2. Materials and methods

Synadenium grantii latex was collected in local area of Mysore, India. Sephadex G-75, casein, human fibrinogen, human plasmin, thrombin, PMSF, aprotinin, leupeptin and IAA were purchased from Sigma chemicals (St Louis, MO, USA). EDTA, EGTA, 1,10-phenanthroline and TFA were purchased from SRL chemicals, India. Congenital Factor Xa deficient human plasma was purchased from Hart Biologicals, Hartle Pool, UK. Prothrombin kit was purchased from Tulip Diagnostics (P) Ltd., Goa, India. Protein molecular weight markers were purchased from Genei Pvt. Ltd., Bangalore, India. All other chemicals are of analytical grade and solvents were redistilled before use. Fresh human blood sample was collected from healthy donors.

2.1. Collection and preliminary processing of latex

The latex was collected in a clean glass beaker by breaking tender parts of the plant. This latex was diluted with three volumes of 10 mM phosphate buffer (pH 7.0) and kept overnight at –20 °C. Then the sample was allowed to thaw and supernatant was decanted and centrifuged at 12,000 rpm for 20 min at room temperature. The clear supernatant was decanted and dialyzed against 10 mM phosphate buffer (pH 7.0). The protein concentration in the supernatant was estimated according to the method of Lowry et al. [21] and used as crude enzyme source.

2.2. Purification of a glycoprotein from S. grantii latex

2.2.1. Sephadex G-75 column chromatography

Glycoprotein from *Synadenium grantii* latex was purified by using Sephadex G-75 column chromatography. Latex was boiled on water bath by maintaining the temperature at 80 °C for 3 min. The coagulated proteins were removed by centrifugation at 12,000 rpm and supernatant was used for purification. 70 mg of boiled latex protein sample was loaded onto the Sephadex G-75 column (148 × 0.8 cm^2) previously equilibrated with 10 mM Sodium phosphate buffer pH 7.0 in presence of 200 mM NaCl. Elution was carried out using the same buffer with flow rate of 20 ml/h and 2 ml fractions were collected at room temperature. Protein elution was monitored at 280 nm using Shimadzu spectrophotometer (1601A). Peak that showed fibrinogenolytic activity was pooled separately and rechromatographed and stored at 4 °C for further use.

2.2.2. Electrophoresis

SDS-PAGE was carried out according to the method of Laemmli et al. [22] for Crude and boiled latex, Sephadex G-75 fractions and purified glycoptorein on 12.5% polyacrylamide gel containing 0.1% SDS using Tris–glycine buffer pH 8.8. The bands were visualized by staining with Coomassie brilliant blue R-250.

Glycoprotein staining was performed by periodic acid Schiffs (PAS) staining according to the method of Leach et al. [23].

Native PAGE was carried out according to the method of Davis [24] for crude and boiled latex and purified glycoprotein under acidic (pH 4.3) condition using β-alanine acetic acid buffer. The bands were visualized by staining with Coomassie brilliant blue R-250.

2.2.3. High performance liquid chromatography

Purified glycoprotein (LGP) was subjected to RP-HPLC in a Vydac C_4 column (10 μm, 4.6 × 250 mm) with dual wavelength detector. The column was equilibrated with 0.1% TFA and eluted using linear gradient of 100% acetonitrile in 0.1% TFA.

2.3. Biophysical characterization

2.3.1. Mass spectrometry

The molecular mass of LGP was determined by matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (Voyager Spec # 1 MC) in the + ve ionization mode. A-cyano-4-hydroxycinnamic acid was used as MALDI matrix.

2.3.2. UV and fluorescence spectroscopy

UV spectrum of LGP was analyzed using Shimadzu Spectrophotometer (1601 A) ranging from 200 to 300 nm. Fluorescence spectrum was analyzed using Shimadzu Spectroflourimeter (RF 5301 PC). Spectrum was recorded at 300–390 nm and excited at 280 nm.

2.3.3. Circular dichroism of LGP

The CD spectrum of native protein was obtained in a Jasco J715 Spectropolarimeter. LGP (0.4 mg/ml) in 10 mM Tris–HCl buffer pH 7.0 was taken in quartz cuvette with path length of 1 cm. Far UV-CD spectra were recorded at room temperature between 195–250 nm. The bandwidth was 1 nm and response time was 2s. The final spectrum was cumulative of three scans.

2.3.4. N-terminal sequence

N-terminal sequence of LGP was analyzed by automated Edman degradation method using PROCISE-cLC sequencer connected to HPLC. N-terminal sequence homology was analyzed using BLAST database search.

2.4. Biochemical characterization

2.4.1. Caseinolytic activity

Caseinolytic activity was assayed according to the method of Murata et al. [25]. Casein 0.4 ml (2% in 0.2 M Tris–HCl buffer pH 8.5) was incubated with different concentrations of crude, boiled latex and purified LGP separately for 2 h. The reaction was stopped by adding 1.5 ml of 0.44 M TCA and allowed to stand for 30 min. The 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 1:2 diluted Folin reagent and the color developed was read at 660 nm. One unit of enzyme activity was defined as the amount of enzyme required to cause an increase in absorbance of 0.01 at 660 nm/h at 37 °C. Activity was expressed as units/m at 37 °C.

For inhibition studies LGP was preincubated with and without effective concentrations of specific protease inhibitors for 15 min at 37 °C. The reaction was initiated by adding 0.4 ml of 2% casein and further assay was carried out as explained earlier.

2.4.2. Human fibrinogenolytic activity

Fibrinogenolytic activity was measured according to the method of Ouyang and Teng [26]. The reaction mixture 40 μl contained 50 μg of human fibrinogen in 10 mM Tris–HCl buffer pH 7.6 was incubated at 37 °C with different concentration of purified LGP separately. Similar conditions were maintained for time-dependent assay by varying incubation period. The reaction was terminated by adding 20 μl of denaturing buffer containing 1 M urea, 4% SDS and 4% β-mercaptoethanol. The hydrolyzed products were analyzed using 10% SDS-PAGE and protein pattern was visualized by staining with Coomassie brilliant blue R-250.

For inhibition studies LGP was pre-incubated with and without specific protease inhibitors in presence of 10 mM Tris–HCl buffer pH 7.6 for 20 m at 37 °C. The reaction was initiated by adding 50 μg fibrinogen and further assay was carried out as explained earlier.

For comparison studies, different concentration of LGP and human plasmin were incubated separately with 50 μg fibrinogen in presence of 10 mM Tris–HCl buffer pH 7.6. Further assay and degradative products of fibrinogen were analyzed as explained earlier.

2.4.3. Clotting activity

Clotting activity of LGP was studied using citrated human plasma. Fresh human blood was mixed with 0.11 M Tri-sodium citrate in the ratio nine parts to one. The mixture was centrifuged for 15 min at 500 g. The supernatant was used as platelet poor plasma (PPP). To 300 μl of pre warmed PPP, different concentrations of LGP in 0.01 M Tris–HCl buffer (pH 7.4) was added. The time taken for visible clot to appear from the time of addition of LGP was recorded. For control experiments Tris–HCl buffer alone was added instead of the enzyme source.

2.4.4. Recalcification time

Re-calcification time was determined according to the procedure described by Condrea et al. [27]. Fresh human blood was mixed with 0.11 M Tri-sodium citrate in the ratio nine parts to one. The mixture was centrifuged for 15 min at 500 g. The supernatant was used as platelet poor plasma (PPP). To 300 μl of pre warmed PPP, different concentrations of LGP in 0.01 M Tris–HCl buffer (pH 7.4) was added and incubated for one m. The clot formation was initiated by adding 30 μl of 0.25 M $CaCl_2$. The time taken for visible clot to appear from the time of addition of $CaCl_2$ was recorded. For control experiments Tris–HCl buffer alone was added instead of the enzyme source.

2.4.5. Prothrombin time

Prothrombin time was determined according to the method of Quick [28]. Fresh citrated human plasma (0.1 ml) was incubated separately with various concentrations of LGP and the clotting time was recorded by adding 0.2 ml of brain thromboplastin. For control experiments brain thromboplastin alone was added to citrated plasma.

2.4.6. Factor Xa like activity

Factor Xa like activity was assayed using the congenital factor X deficient plasma. Deficient plasma was incubated with different doses of LGP at 37 °C and the clot formation was observed for the factor Xa like activity. For the control experiments 10 μl of normal plasma with 30 μl of 0.25 M $CaCl_2$ was supplemented with out the LGP.

2.4.7. Thrombin like activity

Thrombin like activity was determined according to the method explained by Denson [29]. The clotting mixture contained 0.4 ml of 0.5% human fibrinogen and the clotting time was recorded upon addition of different concentration of LGP. For control experiments fibrinogen was incubated with thrombin.

2.4.8. Fibrin clot hydrolyzing activity

Partially cross-linked fibrin was prepared according to the method of Pizzo et al. [30] with slight modification. 150 μl (1 mg/ml) fibrinogen was incubated with 5 μl of 0.5 M $CaCl_2$ and 10 μl (0.25 NIH U) of thrombin. After 20 min the fibrin clot was transferred to new Eppendorf centrifuge tube and incubated with different concentrations of LGP for 150 min in presence of 10 mM Tris–HCl buffer pH 7.6 in 40 μl reaction volume. The reaction was terminated by adding 20 μl of denaturing buffer containing 1 M urea, 4% SDS and 4% β-mercaptoethanol. The fibrinopeptides were analyzed using 7.5% SDS-PAGE and protein pattern was visualized by staining with Coomassie brilliant blue R-250.

3. Results

Dialyzed crude latex of *S. grantii* latex was fractionated on Sephadex G-75 column, which resolved into four peaks (data not shown). Among these four peaks, 2nd and 3rd peaks con-

stitute 50–60% of the protein and showed potent human fibrinogenolytic activity. However, in this method proteins were resolved poorly. To overcome this problem, latex was boiled at 80 °C for 3 min, which removes 30% of the total proteins by coagulation. Coagulated proteins were removed by centrifugation and supernatant is rich in fibrinogenolytic activity. Further, the resultant supernatant was concentrated and applied on to Sephadex G-75 column chromatography, which resolved into four peaks Sg I, Sg II, Sg III and Sg IV (Fig. 1). Unlike in the first case, Sg II and Sg III comprise 20% of protein, with potent fibrinogenolytic activity. In this step Sg II alone comprises 10% of the protein and contains a single fibrinogen degrading protein with little contamination. Rechromatography of Sg II yields homogenous fraction with fibrinogenolytic activity. Proteolytic activity of purified protein on casein was comparatively less in boiled sample. However, fibrinogenolytic activity increases onefold from crude to boiled sample and twofold to purified protein. Purification and proteolytic activity with respect to casein are summarized in Table 1.

Fig. 2 shows the electrophoretic pattern of crude, boiled and purified protein of latex under SDS and on native acidic

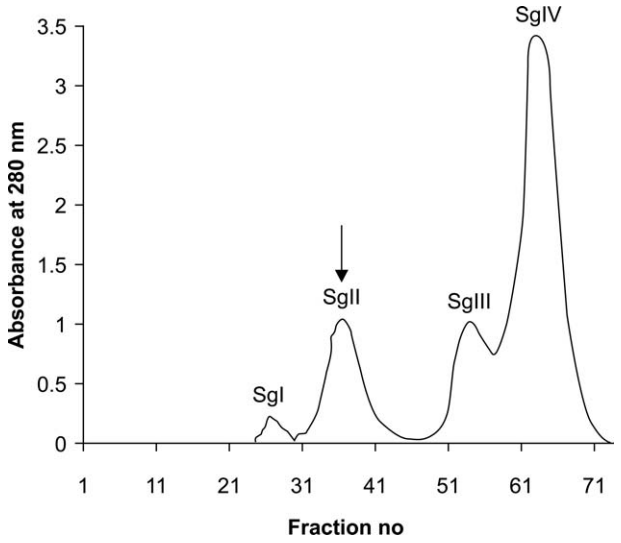

Fig. 1. Sephadex G-75 elution profile of *S. grantii* latex extract.
70 mg of boiled latex protein sample was loaded onto the Sephadex G-75 column (148 × 0.8 cm^2) equilibrated with 10 mM Sodium phosphate buffer pH 7.0 in presence of 200 mM NaCl. Elution was carried out using the same buffer with flow rate of 20 ml/h and 2 ml fractions were collected. Protein elution was monitored at 280 nm using Shimadzu spectrophotometer (1601A). Arrow mark shows the protein peak exhibiting fibrinogenolytic activity.

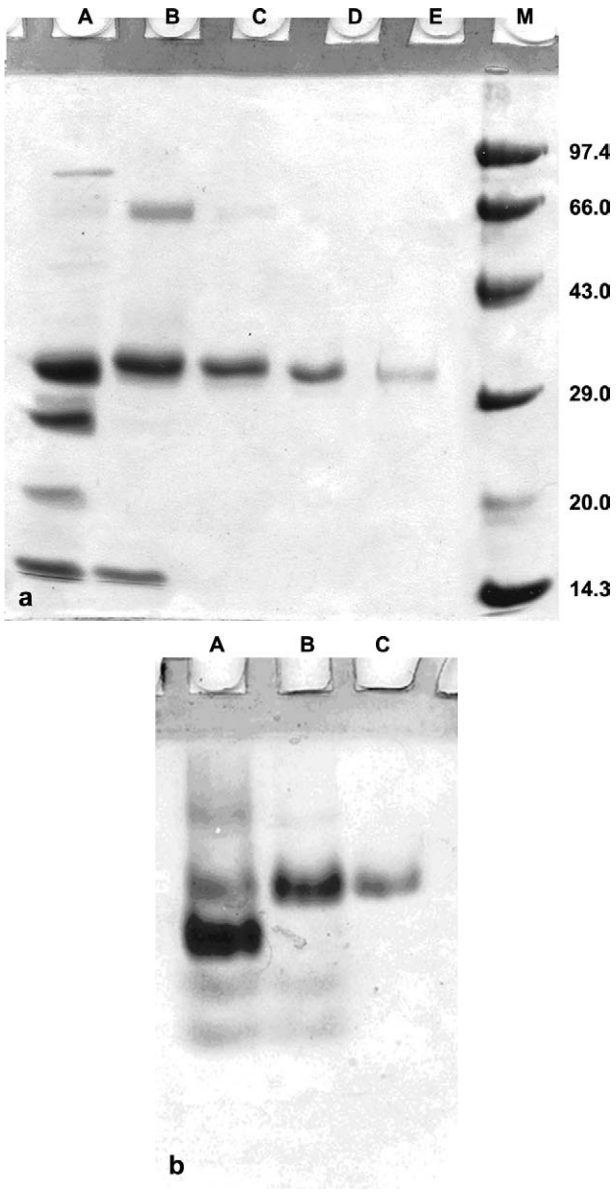

Fig. 2. Electrophoretic pattern of crude, boiled latex and LGP.
a. SDS-PAGE of crude (75 μg): A, boiled (50 μg): B, SgII (25 μg): C, rechromatographed (25 μg): D, LGP under non-reducing (20 μg): E and reducing condition (20 μg): F.M represents the molecular weight markers ranging from 14.3 to 97.4 kDa. (Lysozyme: 14.3, Soybean trypsin inhibitor: 20.0, Carbonic anhydrase: 29.0, Ovalbumin: 43.0, Bovine serum albumin: 66.0 and phosphorylase b: 97.4.)
b. Native PAGE of crude (75 μg): A, boiled (50 μg): B and LGP (25 μg): C under acidic condition using β-alanine and acetic acid buffer system. Gel was stained with 0.1% of Coomassie brilliant blue R-250.

Table 1
Summary of purification of latex glycoprotein (LGP) from *synadenium grantii* latex

Step	Fraction	Total protein (mg)	Total activity [a] (units/min)	Specific activity (units/mg/min)	Yield [b]	
					Protein	Activity
Boiling	Crude	100	2500	25	100	100
	Boiled	70	1250	17.8	70	50
Sephadex	Boiled	50	892.8	17.8	100	100
G-75	SgII	13.2	277	20.9	26.4	31
Rechromagraphy	LGP	9.3	202	21.7	18.6	22.6

[a] One unit of enzyme activity is defined as a amount of enzyme required to increase an absorbance at 660 nm.
[b] protein contribution to the total protein and enzyme activity processed in each step.

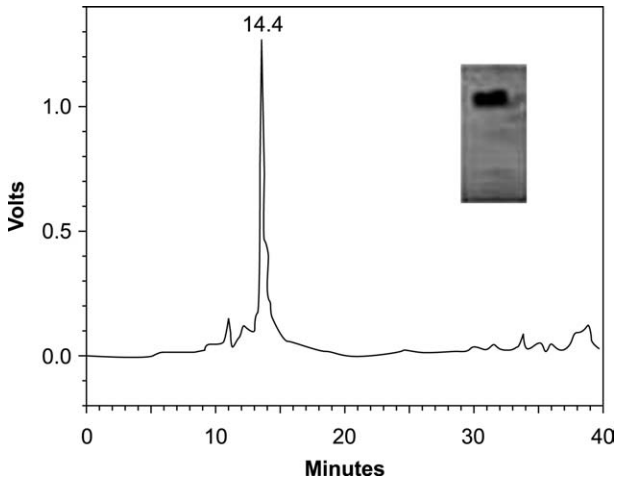

Fig. 3. RP-HPLC profile of LGP.
20 μg of LGP was subjected to RP-HPLC in a Vydac C_4 column (10 μm, 4.6 × 250 mm) with dual wavelength detector. The column was equilibrated with 0.1% TFA and eluted using linear gradient of 100% acetonitrile in 0.1% TFA. Inset figure shows the PAS staining of LGP.

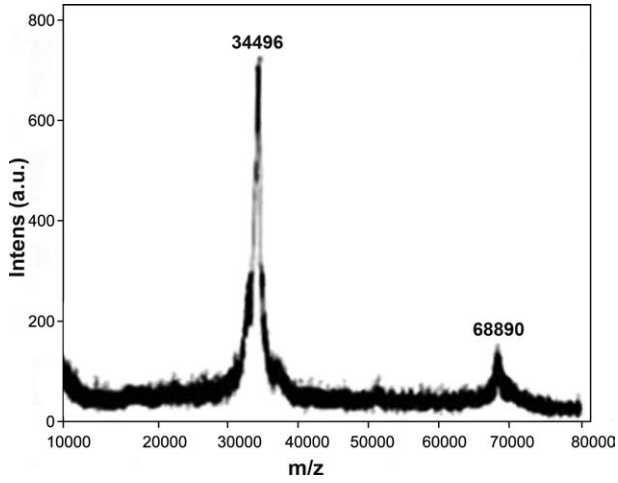

Fig. 4. MALDI-TOF of LGP.
LGP was subjected to matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (Voyager Spec # 1 MC) in the + ve ionization mode. A-Cyano-4- hydroxycinnamic acid was used as MALDI matrix.

PAGE. In presence of SDS crude latex exhibits 4–5 prominent protein bands; where as boiled sample results in the elimination of two major protein bands without affecting fibrinogen degrading activity. The purified protein showed single band both under non-reducing and reducing conditions (Fig. 2a). Similarly crude latex exhibited four major protein bands in native PAGE under acidic condition. One of the major bands is removed while boiling without affecting fibrinogen-degrading activity (Fig. 2b). Purified protein is a glycoprotein, as evident by PAS staining (Inset Fig. 3). This purified glycoprotein was termed as latex glycoprotein (LGP). The purity of protein was assessed by RP-HPLC using Vydac C4 column, which gave a single sharp peak with retention time of 13.3 m (Fig. 3).

Further the biophysical characterization of LGP was analyzed by Mass Spectrometry, UV and fluorescence spectroscopy, Circular dichroism and N-terminal amino acid sequence. The approximate relative molecular mass of the protein according to SDS-PAGE is 30–35 kDa. The exact molecular mass determined by mass spectrometry (MALDI-TOF) is 34.4 kDa (Fig. 4). LGP exhibited the characteristic protein spectrum at 220 and 280 nm. (data not shown). The fluorescence spectrum explained the presence of high amount of tyrosine and tryptophan residues, quenches at 315–340 nm (data not shown). The secondary structure of LGP was analyzed by CD spectrum using K2D program. Secondary structure of CD spectrum (200–260 nm) showed 7% of α-helical content, 48% β pleated sheets and 46% random coils (Fig. 5). N-terminal sequence of protein confirms its purity and it is a single protein. N-terminal sequence of glycoprotein is D-F-P-S-D-W-Y-A-Y-E-G-Y-V-I-D-R-P-F-S.

Biochemical and pharmacological actions of Latex glycoprotein was studied using human fibrinogen and partially cross-linked fibrin. Fig. 6 shows the human fibrinogenolytic activity of LGP as a function of protein concentration, time and its inhibition studies. LGP hydrolyzed all the three subunits (Aα, Bβ and γ) of human fibrinogen in a dose-dependent manner. LGP preferentially acts on Aα subunit of fibrinogen and also hydrolyzes Bβ subunit. Aα and Bβ subunits were hydrolyzed completely even at low concentration of LGP. As the concentration increases γ subunit also hydrolyzed as shown in Fig. 6a. Fig. 6b shows the time-dependent fibrinogenolysis at 1.5 μg LGP. It hydrolyzed Aα subunit completely within 5 min incubation time by releasing protein fragments in the range of 37.0 and 29.0 kDa. At 15 min time of incubation, Bβ subunit starts hydrolyzed with decreasing the band intensity of 37.0 kDa and increasing the intensity of 29.0 kDa protein band. Over 30 min incubation, 75% of the Bβ chain was hydrolyzed with disappearance of 37.0 kDa proteins as well as decrease in the intensity of 29.0 kDa protein fragments.

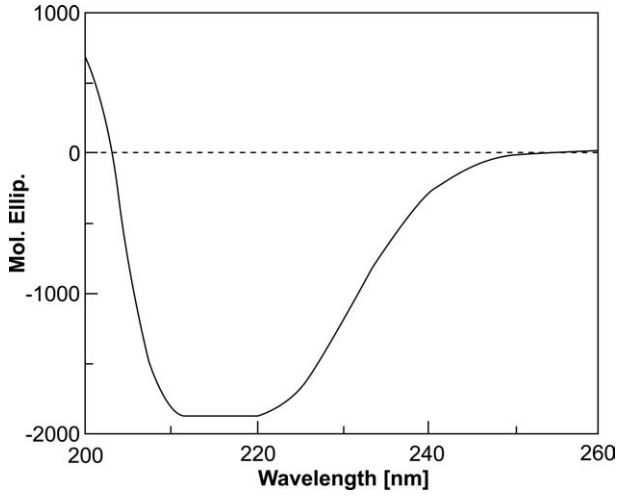

Fig. 5. Circular Dichroism spectra of LGP.
LGP (0.4 mg/ml) in 10 mM Tris–HCl buffer pH 7.0 was taken in quartz cuvette with path length of 1 cm. Far UV-CD spectra were recorded at room temperature between 195–250 nm using a Jasco J715 Spectropolarimeter.

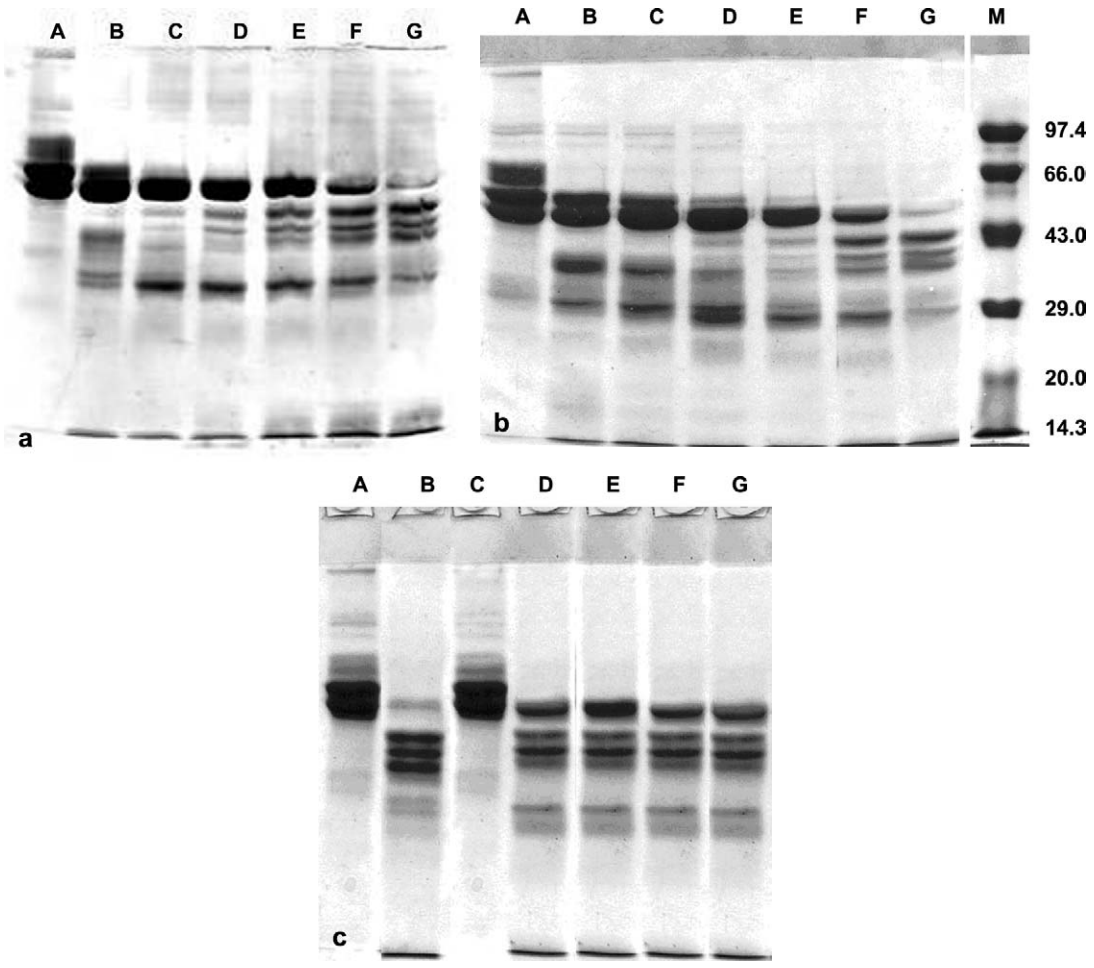

Fig. 6. Fibrinogenolytic activity of LGP.
a Dose-dependent activity: 50 μg of fibrinogen was incubated with 0 μg: A, 0.5 μg: B, 1 μg: C, 2 μg: D, 3 μg: E, 4 μg: F and 5 μg: G of LGP for 2 h at 37 °C.
b Time-dependent activity: 50 μg of human fibrinogen was incubated with 2 μg of LGP for 0 m: A, 5 m: B, 15 m: C, 30 m: D, 60 m: E, 120 m: F and 240 m: G at 37 °C. M represents the molecular weight markers ranging from 14.3–97.4 kDa.
c Inhibition of fibrinogenolytic activity: 1.5 μg of LGP was pre-incubated with and without specific protease inhibitors for 15 min at 370C. Further reaction was initiated by adding 50 μg fibrinogen and incubated for 2 h.
A: 50 μg fibrinogen, B: 50 μg fibrinogen with 1.5 μg LGP (Rx), C: Rx with 5 mM PMSF, D: Rx with 100 μM IAA, E, F and G: Rx with 5 mM of EDTA, EGTA and Phenantharoline, respectively. 10% SDS-PAGE shows the hydrolyzing pattern of human fibrinogen.

This hydrolysis results in the generation of new protein fragments of 27.0 kDa. As the time increases Bβ chain hydrolyzed completely with appearance of 43.0 and 39.0 kDa protein fragments, where as 29.0 kDa proteins fragment disappears with increasing intensity of 27.0 kDa protein bands. Over 120 min time of incubation Bβ chain hydrolyzed completely with accumulation of 43.0 and 39.0 kDa with reappearance of 37.0 kDa protein bands due to the hydrolysis of γ subunit. At maximum time of incubation period (240 m) 75–80% hydrolysis of γ subunit was seen with increased intensity of 43.0, 39.0, 37.0 and appearance of 14.0 kDa bands. Further increase in the time did not alter the pattern and intensity of protein bands (data not shown). In order to know the nature of LGP to which class it belongs, inhibition study was carried out on fibrinogenolytic activity using specific protease inhibitors. Fig. 6c shows the inhibition of fibrinogenolytic activity of LGP. The fibrinogenolytic activity is completely inhibited by PMSF, a serine protease inhibitor. Other protease inhibitors, aprotinin and leupeptin did not inhibit the proteolytic activity of LGP. The proteolytic activity was not altered by any other metallo and cysteine protease inhibitors (EDTA, EGTA, phenanthroline and iodoacetic acid).

Fibrinogenases so far isolated will either induce or inhibit the clot formation by acting on fibrinogen. To check the coagulant (pro/anti) nature of the protein, the thrombin like activity on purified fibrinogen, factor Xa like activity using congenital factor X deficient plasma, recalcification time and prothrombin time on citrated human plasma were studied. LGP did not induce clot formation with purified fibrinogen even at high concentration (20 μg) of protein and over increased incubation period (36 h). Similarly, LGP did not exhibit factor Xa like activity with congenital factor X deficient plasma. However, the LGP induces clot formation with citrated human plasma and did not alter the pro-thrombin time. LGP reduces the recalcification time from 165 to 30 s (Fig. 7). In absence of $CaCl_2$, LGP failed to induce clot with citrated human plasma.

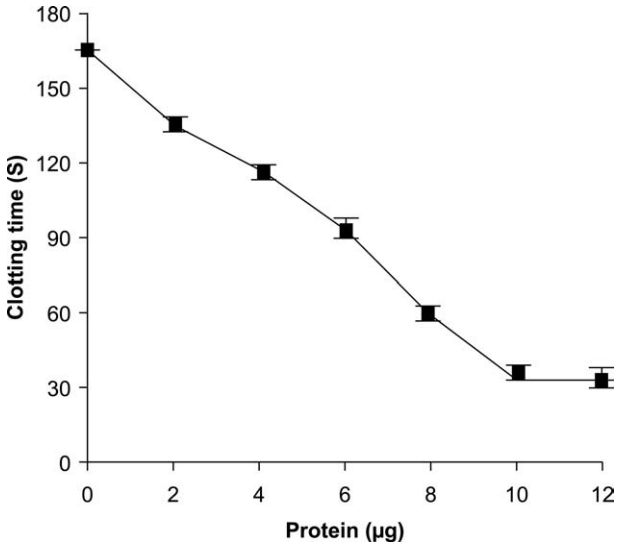

Fig. 7. Effect of LGP on recalcification time of citrated human plasma.
LGP ranging from 2 µg to 12 µg was pre incubated with 300 µl of citrated human plasma in the presence of 30 µl Tris–HCl buffer (10 mM, pH 7.6) for 1 min at 37 °C. 30 µl of 0.25 M $CaCl_2$ was added to the pre-incubated mixture and clotting time was recorded.

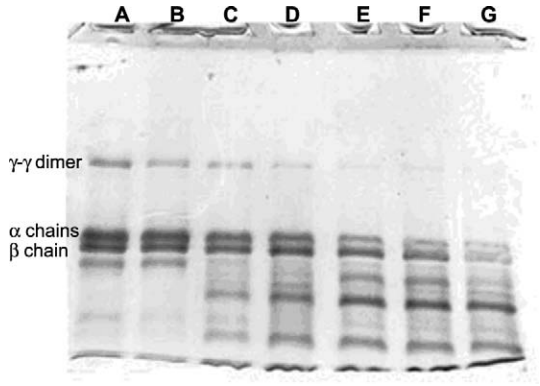

Fig. 8. Thrombin induced fibrin clot hydrolyzing activity.
Thrombin induced fibrin clot was incubated with 1 µg to 10 µg LGP in presence of Tris–HCl buffer (10 mM; pH 7.6) for 150 min at 37 °C. SDS-PAGE (7.5%) shows plasma clot cleavage pattern by the action of LGP. A: control (fibrin clot), B: fibrin clot + 1 µg LGP, C: fibrin clot + 2 µg LGP, D: fibrin clot + 4 µg LGP, E: fibrin clot + 6 µg LGP, F: fibrin clot + 8 µg LGP, G: fibrin + 10 µg LGP.

Although, LGP induces clot formation on human citrated plasma, it also showed unusual fibrin clot hydrolyzing activity. It hydrolyzed all the subunits (α-chains, β chain and γ–γ dimer) of partially cross-linked fibrin dose dependently (Fig. 8). However, LGP did not prolong clot formation (anticoagulant) even at higher concentration of protein. Plasmin, the mammalian fibrinolytic enzyme involved in hemostasis, and wound healing where it hydrolyses all the three subunits of fibrinogen and fibrin, a unique character. Further, to analyze the pattern of fibrinogenolysis of glycoprotein, a comparative fibrinogenolytic study was carried out with human plasmin. Hydrolysis of fibrinogen by plasmin generates specific medium and low molecular weight proteins. The ultimate hydrolysis of human fibrinogen by plasmin results in the generation of 44.5, 37.0, 25.0 and 15.0 kDa molecular mass species. Similarly, fibrinogenolysis of LGP also generates the protein and peptides in the range of approximately 43.0, 41.0 kDa, and 29.0–33.0 and below 15.0 kDa (Fig. 9).

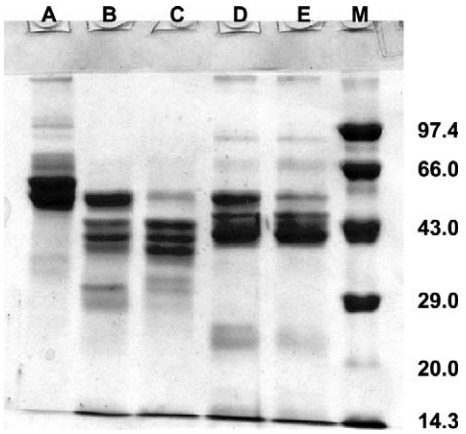

Fig. 9. Comparative fibrinogenolysis of LGP and human plasmin.
50 µg human fibrinogen incubated with LGP (1 µg and 2 µg) and human plasmin (0.5 µg and 1 µg) in presence of Tris–HCl buffer (10 mM; pH 7.6) for 2 h at 37 °C. 10% SDS-PAGE shows the fibrinogenolytic activity of LGP and plasmin. A: 50 µg fibrinogen, B: fibrinogen + 1 µg LGP, C: fibrinogen + 2 µg LGP, D: fibrinogen + 0.5 µg plasmin, E: fibrinogen + 1 µg plasmin, M-represents protein molecular weight markers ranging from 14.3 to 97.4 kDa.

4. Discussion

Hemostasis involves the two important proteolytic systems, coagulation and fibrinolysis [4]. Coagulation system involves the action of series of serine proteases results in the formation of hemostatic plug [6]. Fibrinolysis is a process which operates to the opposite of coagulation where in, the formed clot is hydrolysed by the action of plasmin [5]. Number of proteases interfering in hemostasis have been isolated and characterized from several sources. Though, several proteases from plant latex have been isolated their interference in hemostasis has not been studied except from the latex of *Ficus carica* [19]. From *S. grantii* latex two high molecular weight serine proteases have been purified [3]. However, their role in hemostasis has not been studied. The present study reporting the purification and characterization of a glycoprotein involved in hemostasis with respect to human fibrinogen and fibrin clot hydrolyzing activity.

Purification of protease from *S. grantii* latex involved the combination of heat precipitation and gel permeation chromatography. *S. grantii* latex upon Sephadex G-75 column chromatography did not resolve into well-separated peaks due to narrow difference in the molecular mass of the two major proteins. Some of the proteins in *S. grantii* latex were coagulated at high temperature without affecting fibrinogenolytic activity. Heat precipitation has been employed in the purification of PLA_2 enzyme in serum, plasma and extra cellular exudates such as synovial and pleural fluids [31,32]. Similarly, heat precipitation also employed in the purification of clotting factor VIII and hemoglobin [33,34]. Most of the proteases isolated from plant latex are heat labile and autocatalytic in nature

[35–39]. However, the glycoprotein purified from *S. grantii* latex is a heat stable and intact even at 80 °C. This data clearly suggests that purified glycoprotein is different from other plant latex proteases characterized so far.

Upon boiling the caseinolytic activity of latex comparatively decreased 2.5-fold than crude latex. Where as the fibrinogenolytic activity was increased twice to that of crude latex. This data indicates that latex is a mixture of specific and non-specific proteases and most of them are heat labile. The non specific casein degrading proteases are heat labile in *S. grantii* latex. The fibrinogen degrading enzymes are heat stable and remain in the solution. This result is further substantiated by SDS-PAGE, where upon heating three thermo stable proteins remain in the solution at the molecular mass of 66 kDa, 35 kDa and 16 kDa. The RP-HPLC data of glycoprotein suggests that the enzyme is less hydrophobic in nature although, it is in the hydrophobic (waxy) environment in the latex.

So far the protease isolated from latex are in the range of 50–80 kDa [40–42] and 20–35 kDa [43]. The molecular mass of the two casein-degrading proteases isolated from *S. grantiii* latex is in the range of 76 kDa [3]. Unlike latex proteases purified by Menon et al. [3] the LGP is a 34.4-kDa protease, which correlates with medium molecular weight (20–35 kDa) proteases isolated from other latex source. A few reports explain the presence of proteases in plant latex with glycoprotein in nature [44–46]. LGP isolated from *S. grantii* latex is a glycoprotein without autocatalytic activity. Large molecular weight proteases isolated from *S. grantii* latex by Menon et al. [3] is shown to be acidic in nature. In contrast, LGP is found to be basic in nature as resolved in native gel under acidic condition. LGP has a compact secondary structure with high content of β pleated sheets and random coils. The presence of high content of β pleated sheets and random coils suggesting the stability even at 80 °C for 3 min. The N-terminal sequence of the glycoprotein did not match with any plant or animal serine proteases and did not show any sequence homology. The amino acid sequence even did not match with latex proteases, which have been well characterized so far. This data confirms it is a novel proteolytic enzyme efficiently acting on human fibrinogen.

Blood coagulation cascade is a tightly regulated pathway involves several coagulation factors with and without proteolytic activity. Numbers of proteases, which interfere in hemostasis, have been isolated and well characterized from snake venoms. Among these proteases, fibrinogenolytic enzymes are predominant which either activate or inhibit blood coagulation. Most of the fibrinogenases isolated from snake venoms are either α or β fibrinogenases or both. So far only one protease cerestase isolated from *Cerestase cerestase* snake venom able to hydrolyse γ subunit of fibrinogen [47]. Very few γ subunit hydrolyzing fibrinogenolytic enzymes have been isolated from insects, algae and caterpillar [12–14]. However, LGP isolated from *S. grantii* latex is a non-specific fibrinogenases, hydrolyses all the 3 subunits of fibrinogen, which is a unique property of plasmin [30]. The LGP preferentially hydrolyzes Aα subunit, which is not a glycoprotein over Bβ and γ subunits. However, Bβ and γ subunits are glycoproteins also acted upon by LGP at high concentration and on prolonged incubation period. Thrombin, a specific fibrinogenolytic enzyme hydrolyses Aα and Bβ chains and forms fibrin clot by releasing fibrinopeptide A and B. Although LGP hydrolyzes fibrinogen, unlike thrombin it did not induce clot formation with purified fibrinogen. This data indicates LGP lacks thrombin like activity and it appears that its site of action on fibrinogen is totally different from that of thrombin. Since, LGP has no thrombin like activity; it did not induce clot formation with citrated plasma without $CaCl_2$. However, it induces clot formation in citrated plasma recalcification time and did not alter the prothrombin time suggesting the involvement of LGP in the intrinsic pathway of blood coagulation. In this line of studies a factor Xa like activity was performed using congenital factor Xa deficient plasma. It failed to induce clot formation in factor Xa deficient plasma. This study clearly suggests that LGP has no Factor Xa like activity and confirms its involvement in the intrinsic pathway of blood coagulation cascade. In addition to fibrinogenolytic and clot inducing activity LGP also exhibited clot-dissolving property. It efficiently hydrolyzes all the subunits of partially cross-linked fibrin clot induced by thrombin. Plasmin is the key enzyme, which hydrolyses fibrin clot, and hemostatic plug facilitating wound healing [4]. Since purified LGP exhibited clot-inducing and dissolving properties, this might be the basis of plant latex used in folk medicine by tribal people to stop bleeding and wound healing.

Most of the fibrinogenases so far isolated from snake venoms are either serine or metalloproteases [47]. Mammalian fibrinogenases belong to serine proteases [48]. Fibrinogenases isolated from caterpillar, earthworms, insects and microbial source belong to serine class of proteases [11–14]. Since, the fibrinogenolytic activity of LGP is inhibited by PMSF; it belongs to the serine class of proteases. However, other serine protease inhibitors, aprotinin and leupeptin did not inhibit the fibrinogenolytic activity of LGP. There are some serine proteases that are not inhibited by aprotinin, such as thrombin and factor Xa of the blood coagulation cascade [49]. Several latex proteases exhibited the same properties which are not inhibited by aprotinin and leupeptin [3]. Earlier Richter et al. [19], reported the involvement of a cysteine protease, ficin isolated from *Ficus carica* in the activation of factor X. Recently we reported the involvement of cysteine proteases from *C. gigantea* latex in hemostasis as fibrino(geno)lytic enzymes [20]. However, LGP is a serine protease and the involvement of this class of plant latex protease in hemosatasis reporting for the first time.

Although purified LGP and human plasmin hydrolyze all the three subunits of fibrinogen, the hydrolyzing pattern is different. Comparatively, LGP generates more degradative products approximately in the range of 43.0, 41.0, 35.0, 27.0–29.0 and 14.0 kDa molecular mass. These results suggested that LGP has multiple site of action on fibrinogen and different from plasmin. Further, the exact site of action in

blood coagulation cascade in sight its therapeutic application in stop bleeding and wound healing can be explored.

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