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Evidence of Fibrinolytic Protease in the Latex of Synadenium Grantii Hook 'f'

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Abstract

Introduction: Enzymes catalyze the process of proteolysis are proteases, they are wide spread in nature, distributed in plant lattices, microorganisms, animals and humans. They perform various biological functions.

Objectives: The present research study purpose was an evaluation of in-vitro total proteolytic and fibrinolytic activity in the latex of Synadenium grantii. The proteolytic activity compared with plant latex enzyme Papain of Carica papaya, and also the percentage of fibrinolytic activity with the bacterial thrombolytic enzyme streptokinase from Streptococcus aureus.

Methods: The protease activity and associated fibrinolytic activity determined by using partially purified enzyme from the latex.

1. The total proteolytic activity determined in terms of caseinolytic activity on one percent casein substrate at temperature of 37^0C and pH 7.6 (0.2M Sodium Phosphate Buffer). The soluble casein fragments released during proteolysis were estimated by Lowry reaction.
2. The fibrinolytic activity exhibited by protease measured by plasma clot lysis at 37^0C in 24 hours.
3. The protease fraction from latex was isolated and purified by adapting few biochemical techniques such as freezing and thawing, D-Galactose Agarose gel chromatography, acetone fractionation and sephadex G-100 Gel filtration chromatography techniques. The purity of enzyme tested on discontinuous Gel gradient of SDS-PAGE.

Results: The experimental results of our study on proteolytic and fibrinolytic activities evaluated at each step of purification protocol were reported as

specific activity (activity per mg protein) and percentage of clot liquefaction. These activities were compared to well known thrombolytic enzyme streptokinase (100units/ml) and Papain (100units/ml). The main characteristic property of purified enzyme was thermo stability with an optimum temperature 50^0C; alkaline stability with an optimum pH 8.0 and completely inhibited by Phenyl Methane Sulphonyl Fluoride (PMSF) convinced the serine residue at the active site. The specific activity in crude latex was 1.75 with 50 percent of fibrinolytic activity and purified enzyme contained 15.8 with 85 percent fibrinolytic activity. Similarly, streptokinase and papain possess specific activity 45.0 and 40.0 respectively. The former was considered as standard fibrinolytic enzyme for comparison with 95 percent fibrinolytic activity. Although, papain has potent proteolytic activity and wide industrial and therapeutic application, it did not show any fibrinolytic activity like Synadenium grantii latex enzyme.

Conclusion: The conclusion drawn in Our study after testing the hypothesis by experimental procedures is that the in vitro fibrinolytic activity on plasma clot is the inherent property of protease (fibrinolytic protease) present in latex of Synadenium grantii Hook 'f' and its comparison with streptokinase and papain were new aspect in principal conclusion.

Key words: Protease, Streptokinase, Caseinolytic, Fibrinolytic, latex, Euphorbiaceae, Papain, Thrombin.

Introduction

The enzymes that cleave peptide bonds of protein are referred to as proteolytic enzymes or proteases. These are widely distributed in nature, present in animals and man [1-3], micro organisms [4-5] and in plant lattices [6-8]. The characteristic property of each protease through an irreversible cleavage is crucial for functional activation of proteins, proteins involved in blood coagulation, fibrinolysis, complement activation, and protein digestion in biological system. Therefore, they have become the focus of wide range of industrial and medical application. The information available on plant protease is vast; majority of these proteases have been subjected for isolation to homogeneity and characterized. The survey on review of literature revealed the availability of seldom information on protease activity from plant lattices. They are Papain (Carica papaya Lynn), Euphorbain (Euphorbia lathyris Willd), Curcain (Jatropa curcas Lynn), Calatropin (Calatropis gigantean Robert brown) and Eravatamin (Ervatomia coronaria Jacq.) etc.

Study area

The laticeferous perennial shrub Synadenium grantii belongs to family Euphorbiaceae not having any information on fibrinolytic protease in the latex and also this property of enzyme is not justified with any known fibrinolytic (thrombolytic) enzyme. Therefore, this prevailing research gap necessitates the present study. There are few

research evidences showed that the S. grantii latex had been subjected to study the active skin irritant principle[9], Esterase and Carboxy esterase activity [10-11], Protease activity [12] and also for Lectin activity [13]. Hence, subsequent study centered on evaluation of in-vitro fibrinolytic activity by protease enzyme and also its comparison with bacterial Streptokinase [14].

Materials and Methods

Plant material

The latex from S. grantii grown in outskirts of R. L. Jalappa Hospital and Research Centre, kolar was collected, processed and stored at 4^0C.

Chemicals: The fine research chemicals like Sephadex G-100, Bovine Serum Albumin used in the study were obtained from Sigma chemical company [USA]. Phenyl Methane Sulfonyl Fluoride (PMSF), Thrombin and Papain from Hi Media Bombay. Streptokinase borrowed from Jayadeva Institute of Cardiovascular science and Research Bangalore, Sodium Dodecyl Sulphate (SDS) from Bio-Rad laboratories. All other chemicals used were of Analytical Research grade obtained locally.

Methods:

1. Protein Estimation*:* Protein concentration in the enzyme extract was determined using Folin Ciocalteu reagent as per the procedure of Lowry et al [15], Crystalline Bovine Serum Albumin used as standard protein for preparation of standard curve for protein. The different aliquots of protein standard allowed reacting with Folin phenol reagent. The absorption of the blue colour developed was measured at 540nm in spectrophotometer [Thermo USA].

2. Protease assay*:* The protease assay was done according to the method of Kunitz [16a] , which was adapted in a research work described by Sumathi and Pattabiraman [16b]. The enzyme extract of processed latex was incubated with 1.0ml of 1% casein substrate prepared using 0.2M phosphate buffer at pH 7.6 for 20 minutes at 37^0C . The reaction was arrested by addition of 3.0ml of 5% Tri Chloro Acetic acid (TCA) [16]. The TCA soluble fragments and total protein of the enzyme extract estimated by the procedure of Lowry et al [15].

3. Determination of Fibrinolytic activity*:* The fibrinolytic activity determined on plasma clot as per the method described by Simon and Michalle [17] with slight modification. Where instead of measuring chromogen released after proteolysis the rate of clot liquefaction accounted.

Each 100µl of plasma diluted to 0.8ml with 0.15M sodium chloride (saline) and each one was clotted by addition of 0.1ml of 2.5 NIH units/ml thrombin, clot formation was confirmed, 0.1 ml of streptokinase (100units /ml), or purified latex enzyme (100units/ml) or Papain (100units/ml) overlayed on plasma clot respectively. Control tube received 0.1ml of saline. After 24 hours at 37^0C, the extent of lysis of fibrin clot was determined by aspirating all the fluid produced using tuberculin

syringe and measured the volume. Fibrinolytic activity expressed as volume percentage of 1.0ml clot that was liquefied in 24 hours.

4. Isolation and Purification of protease:

(a) Clarification of latex: The crude latex (68.75mg/ml) is diluted and subjected for freezing and thawing until it produce clear latex sera, that was separated by centrifugation at 10,000 g for 15 minutes at 4^0C.

(b) D-Galactose Agarose gel chromatography: The clear aqueous latex sera obtained from the above step passed through Agarose gel column (24cms x 1.4cms) pre equilibrated with sodium phosphate buffer (0.2M at pH 7.6) all the materials eluted by gravity flow collected and pooled.

(c) Acetone fractionation and Gel permeation chromatography: The pooled eluted fractions subjected for concentration of protein by acetone fractionation to 80 percent saturation at 4^0C. The precipitate obtained was centrifuged in refrigerated centrifuge at 10 000 g at 4^0C for 20 minutes. The precipitate separated was dissolved in minimal volume of sodium phosphate buffer and passed through Sephadex G-100 gel filtration column (7.5x1.6cms). All the materials collected, and active fractions pooled and freeze dried. This protein powder further dissolved in phosphate buffer that contains (6.25 mg/ml protein) which was checked for purity on SDS-PAGE. Assay of enzyme for proteolytic and fibrinolytic activities during purification protocol were tabulated in Table – 1.

Table 1: Comparison of total Proteolytic activity and fibrinolytic activity during purification protocol.

SL.No	Enzyme fractions	Total protein (mg/ml)	Total activity (units/ml)	Specific activity (activity/mg. protein)	fibrinolytic activity (percentage)
1	Crude latex	68.75	120	1.75	50
2	Latex Aqueous Extract	31.25	110	3.54	75
3	D-Galactose Agarose gel chromatography	18.75	100	5.6	85
4	Gel permeation chromatography (Sephadex-G-100)	6.25	95	15.8	90

(d) SDS-PAGE: Protein separation was carried out on SDS-PAGE as per the method of Laemmili [18]. A discontinuous gel gradient system consists of 8% separating gel on which proteins are separated and 5% stacking gel concentrates the protein [19]. The protein obtained from the purification step subjected to heating at 100^0C for 3-5minutes in a boiling water bath with SDS-Gel loading buffer containing 0.05M Tris-HCl buffer at pH 6.8, 0.1M Dithiothretol (DTT), 0.1% bromophenol blue tracking dye.10% glycerol and 2% Sodium Dodecyl sulphate [SDS]. The treated sample was placed in to pre-determined bottom of well for electrophoresis. The direct current of 8volts was applied until the dye front moves to the resolving gel and then increased to 15 volts until the tracking dye reaches the bottom of resolving gel. The separated proteins on gel were fixed and stained using coomassie brilliant blue R-250. The background stain was washed with distaining solution. The separated protein band was finally photographed and documented in Fig 1.

Figure 1: Electrophoretic pattern of purified fraction of S. grantii on SDS-PAGE.

(f) Effect of temperature on protease enzyme activity: The enzyme fraction was kept in water bath, at specific temperature interval; 0.1ml of aliquot was used for assay of protease activity on casein substrate. Enzyme activity against temperature presented in the Fig 2.

(g) Effect of pH on protease enzyme activity: The purified enzyme fraction dissolved in different buffer system such as pH 2.0 (0.2M Glycine–Hcl buffer), pH 5.0 (0.2M Sodium phosphate buffer), pH 7.0 (0.2M phosphate buffer), pH 9.0 (0.2M Tris buffer) and also pH 1.0 using 2M HCl and pH 13.0 using 1M sodium hydroxide. After incubating each sample for an hour at room temperature, 0.1ml of aliquot from each system was assayed for protease activity Fig 3.

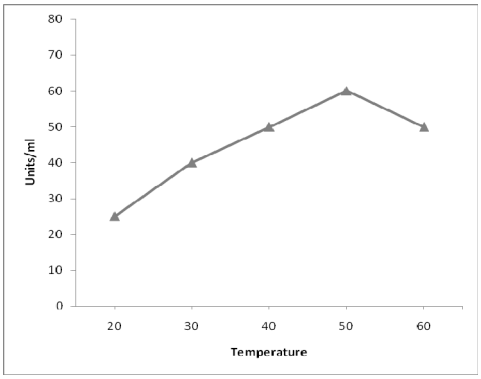

Figure 2: Effect of temperature on enzyme activity.

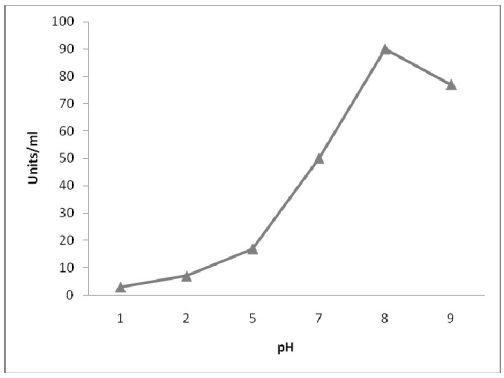

Figure 3: Effect of pH on enzyme activity.

(h) Effect of inhibitor on enzyme activity: The purified enzyme fraction dissolved in phosphate buffer (0.2M at pH 7.6)and was mixed with PMSF(1mM PMSF in 0.2M Methanol) and incubated at room temperature for one hour, 0.1ml of aliquot was used for proteolytic and fibrinolytic activity determination.

Results

The result of total proteolytic activity in terms of specific activity in latex of Synadenium grantii compared with Papain and streptokinase tabulated in Table 2

Protease activity and corresponding fibrinolytic activity at each step of purification protocol compared and presented. The factors that affect the proteolytic activity presented. The factors such as temperature, pH and inhibitor and their influence on purified enzyme activity presented. The partially purified enzyme checked for its purity on SDS-PAGE. The proteolytic activity and fibrinolytic activity of Synadenium grantii latex enzyme compared to streptokinase and these findings were documented.

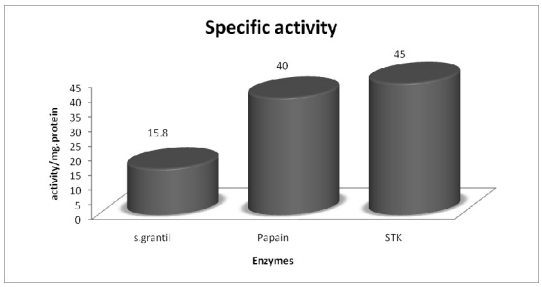

Figure 4: Comparison of total proteolyic activity of latex enzyme from S. grantii, papain and streptokinase (STK).

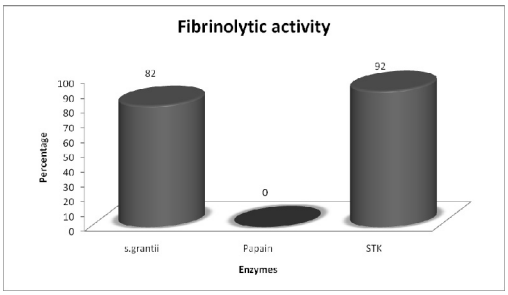

Figure 5: Comparison of percentage of Fibrinolytic activity of latex enzyme from S. grantii, papain and streptokinase (STK).

Table 2: Comparison of total proteolytic activity, fibrinolytic activity of latex enzyme of S. grantii, with papain and streptokinase.

SL.No	Enzyme fractions	Specific activity (activity/mg. protein)	Fibrinolytic activity (percentage)
1	Synadenium grantii latex enzyme	15.8	82
2	Papain (100U/ml)	40	Nil
3	Streptokinase (STK) (100U/ml)	45	92

Discussion

The protease activity in the latex of S. grantii belonging to family Euphorbiaceae is similar to few plant latex proteases studied by several workers. They are papain from Carica Papaya from Moraceae [20], Ficin from Ficus carica from Moraceae [8], Calatropin from Calatropis gigantean from Asclepiadaceae [21], Euphorbain from Euphorbia lathyris from Euphorbiaceae [6] and Eravatamin of Ervatomia coronaria [22].

Lynn Clevette-Radford in 1988, studied several species of Euphorbiaceae family [23] accordingly, few findings such as protease presence, temperature and pH stability and nature of enzyme are quite similar to plant proteases. These biochemical observations confirm the support to taxonomical study. Similarly, purified enzyme shows thermo stability up to 60^0C , pH stability between 5.0-9.0.

The chemical property of all crude plant lattices when released has pH 6.0 but the magnitude of protease activity was found high in alkaline pH, or pH dependent activity due to more solubilisation of protein. Unlike other lattices, the latex of S. grantii when extracted in to double distilled water than the buffer system and also on subjecting to freezing and thawing procedure yields clear latex aqueous extract. However, the buffer extract had milky tinge, therefore latex solubilised in water containing Sodium tetrathionate and EDTA during purification protocol.

The temperature stability of crude enzyme fraction facilitates to adapt purification procedure at room temperature. Thus, enzyme fraction shows increase in activity from 20 - 60^0C with an optimum temperature 37^0C which is similar to other protease of latex [24].

The effect of pH on protease activity gradually increases from 5.0-9.0 with the optimum pH of 8.0 this enzyme was inactive in pH 1.0 when used 0.2M Hydrochloric acid and at pH 13.0 when used 0.1M Sodium hydroxide [25].

During isolation and purification, freezing and thawing technique helps to remove gum, scum and white material completely without affecting enzyme activity. During

freezing, some proteins of less interest and white material with gum coagulated in to a lump while thawing some proteins redissolved in to aqueous media accounts for protease activity. In this study, the conventional technique found to be successful in removal of white material from the latex against the methods adapted such as celite-400 adsorption chromatography as used for papain, bromelain by Pere Clapes and his Co-workers [26] and also by Microfilatration as used by Whittington [27].

The latex aqueous extract, passed through Galactose-Agarose Column to remove any Agarose binding protein since, the latex contains the carbohydrate binding protein Lectin [28]. This technique removes nearly 50 percent of the proteins from the latex extract without altering enzyme activity. Eighty percent cold acetone fractionation of pooled elutant fractions of Agarose column chromatography concentrates the protein, acetone precipitation selected as priority over ammonium sulphate saturation since the latter imparts brownish colour to extract even after dialysis against 0.02M phosphate buffer at pH 7.6 overnight using Poly Vinyl Pyrrolidine (PVP).

The acetone precipitate obtained was redissolved in phosphate buffer and that was passed through Gel permeation chromatography using sephadex G-100 because the same technique used for purification of Euphorbain from Euphorbia lathyris of the same family [29]. The purity and complexity tested on SDS-PAGE.

The temperature stability of enzyme isolated from S. grantii was stable at 50^0C but loses activity at 90^0 C, this thermal stability is similar to other protease like Papain at 80^0C [24], Chymopapain at 75^0C [30], Ficin at 55^0C [31] and Calatropin at 55^0C [21].

Blood Coagulation and Fibrinolysis accomplished by serine proteases [32], Plasminogen activator, exogenous therapeutic activators or foreign activators are enzymes and enzyme activators used to accelerate clot [33] foreign activators are bacterial streptokinase, staphylokinase etc. catalyses fibrinolysis. Few plant sources reported for fibrinolytic activity are Allium cepa [34] Calatropis procera [35] similarly, the protease of S. grantii acts on Plasma clot obtained by thrombin.

In the present study, we are reporting an evidence of fibrinolytic protease by inhibiting enzyme by treating with 1mM PMSF. Thus, inhibited fraction did not show any proteolytic and fibrinolytic activity. As per the findings of the study, at purification protocol, fibrinolytic activity measured along with proteolytic activity at each step of purification showed gradual increase of both the activities. This experimental result indicated that Fibrinolytic activity is an inherent property of the protease.

Conclusion

The enzyme isolated and partially purified from the latex of Synadenium grantii Hook ’f’ is a thermo stable protease having optimum temperature 50c, stable in wide pH range 5.0-9.0 with optimum pH 8.0, serine protease acts on substrates like casein and fibrin clot, possess good fibrinolytic activity when compared to streptokinase and fibrinolytic activity is inherent property of latex protease. Therefore, this fibrinolytic protease from Synadenium grantii is similar to streptokinase in clearing the plasma clot obtained by thrombin action in vitro. However, further studies are needed to explore the utility as therapeutic agent by confirming in vivo studies.

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