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Fibrinolytic Serine Protease Isolated from Earthworm *Lampito mauritii*

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Abstract: ***The four enzyme fractions (F 6, 8, 11, 13) with fibrinolytic activities were purified from earthworm Lampito mauritii using the procedures of autolysis, ammonium sulfate fractionation, and column chromatography. The proteolytic activities on the casein substrate of the four iso-enzymes ranged from 53.51 to 71.85 unit/mg. with the rank activity orders of F13 > F8 > F6 > F11. The fibrinolytic activities of the four fractions on the fibrin plates ranged from F11 > F6 > F13> F8. The molecular weights of each iso-enzyme, as estimated by SDS-PAGE, were 100-110 (F6), 75-80 (F8), 45-50 (F11), 35-40 (F13) kDa respectively. For convenience, the proteases are named on the basis of the earthworm species and the protein function, for instance, Lampito mauritii (LaP).Earthworm protease has been applied in several areas such as clinical treatment of clotting diseases, anti-tumor study, environmental protection and nutritional production.***

Keywords: Protease, Fibrinolytic, *Lampito mauritii*, proteolytic,fibrin plate.

1. Introduction

Hemostasis is a tightly regulated process for keeping an optimal balance between coagulation and anticoagulation. The maintenance of this process is essential for the prevention of bleeding and thrombosis, which occurs by the sequential and short-lived activation of a coagulation cascade series of enzymes. This process ultimately results in the production of an insoluble fibrin clot. Fibrin is the primary protein component of blood clots, which are formed from fibrinogen by thrombin [25]. The insoluble fibrin fiber is hydrolyzed into fibrin degradation products by plasmin, which is generated from plasminogen by plasminogen activators such as tissue plasminogen activator (t-PA), vascular plasminogen activator, blood plasminogen activator, urokinase, Hageman factor, and streptokinase plasminogen complex[4],[5]. Fibrin clot formation and fibrinolysis are normally well balanced in biological systems. However, when fibrin is not hydrolyzed, due to some disorder, thromboses can occur. Myocardial infarction is the most common form of such thromboses. The fibrinolytic agents available today for clinical use are mostly plasminogen activators such as the tissue-type plasminogen activator, the urokinase-type plasminogen activator and the streptokinase-type plasminogen activator. Despite their widespread use all these agents have undesired side effects, exhibit a low specificity for fibrin and are relatively expensive. Therefore, the search for other fibrinolytic enzymes from various sources continues. Recently, research progress has been made in developing medicines which prevent thrombus formation or which dissolve fibrin clots.Plasmin-like enzymes from the snake venom [14] and hirudine,an anticoagulant isolated from the leech have been well characterized and is now commercially available. With the advancement of technology, especially in molecular biology and proteomics in last few decades, numerous compounds have been isolated and characterized for their therapeutic applications from various species of earthworm [6]. Among these therapeutic potentials fibrinolytic property of earthworm proteases is explored significantly. Large-scale research about earthworm protease began in 1980. A group of proteases with fibrinolytic activity isolated from the earthworm *Lumbricus rubellus* [19]. More proteases have been obtained from different species, such as lumbrokinase [20] earthworm-tissue plasminogen activator[8], earthworm plasminogen activator [28]-[31] component A of EFE (EFEa) [24],[8] and biologically active glycolipoprotein complex (G-90) [7],[8][24].This was a breakthrough in earthworm based medicine and concurrently various species of earthworms are explored for isolation of earthworm fibrinolytic enzyme (EFE) by different researchers. Recent studies have confirmed clinical significance of EFE in cerebral ischemia and more emphasis is given to refine earthworm serine protease as external fibrinolytic agent, efficiently working in neuronal tissues [10]. Subsequently different purification methods were applied to isolate the enzymes, including gel filtration, affinity chromatography, ion exchanging chromatography and high-pressure liquid chromatography (HPLC). Experiments were conducted to understand the therapeutic properties such as anti-inflammatory, anti-oxidative, haematological and serum biochemical markers of earthworm paste (EP) derived from an indigenous species *Lampito mauritii* (Kinberg) [3]. The earthworm proteases are multicomponent. Because of various living environments, different species of earthworms have different resultant isozymes. The proteases are independently studied in research groups [17], [19], [33].

Thus, one isozyme may have multiple names. Here, we name the protease after the formal name of earthworm species and the protein function, for example, the protease from *Lampito mauritii* is called *L.mauritii* protease (*LmP*).To our knowledge there are no reports about the identification of fibrinolytic enzymes in *Lampito mauritii*. Therefore we have attempted to isolate and purify the fibrinolytic enzyme from Lampito *mauritii* powder.

2. Materials and Methods

2.1. Earthworms

Matured earthworms of *Lampito mauritii* obtained from paddy field were maintained in large pits that contained mixture of soil and cow-dung at 25 ± 2ºC. The worms were acclimatized for at least 15 days before pounded into powder.

2.2. Preparation of Earthworm Powder

Earthworms in the size ranging between 15 and 18 cm were alone chosen for this investigation. 500 sexually matured, clitellated (900 mg/worm) earthworms *Lampito mauritii* (Kinberg) were obtained from the container. They were washed with running tap water and then fed with wet blotting paper for 18-20 hours for gut clearance. The gut cleared worms were again washed with distilled water. The worms were kept in a plastic trough tightly covered with polythene cover and exposed to sunlight for three days to kill the earthworms. Mucus and coelomic fluid oozed out digests the dead worms forming a brown colored paste and this was freeze dried and vacuum dried into powder [3].

2.3. Isolation and purification of enzyme

Earthworm powder was purified with successive chromatographic purification which includes salt precipitation, dialysis and size based separations. Total protein content of the earthworm powder was estimated by Lowry's Method [16] also precipitated with ammonium sulfate and 80% salt employed to recover 100% of protein. Precipitated protein part was separated from supernatant by high speed centrifugation at 20 000 r/min for 30 min at 4 °C. The precipitate was suspended in 25mL of 20 mM phosphate buffer pH 7.0 and subjected to dialysis. Dialysis was performed to remove salt from crude protein by using dialyzing membrane of molecular weight cut off (MWCO) of 10 kDa. Dialysis was done against 20 mM phosphate buffer pH 7.0 for 12 h continuously with changing the phosphate buffer at an interval of 4hrs. After complete dialysis, dialyzed sample was carefully collected in a vial using sterile pipette and stored at 4^{o}C until further use.Sephadex-G100 (G E Healthcare) was used to prepare column for size based separation. Sephadex-G100 beads were allowed for complete swelling in 20mM phosphate buffer pH 7.5for 48 hours at room temperature with mild agitation and sonicated to remove entrapped air. Sephadex-G100 beads were loaded in glass column (60×1.5 cm) and allowed to pack under gravity for 3–4 h. 2 ml of dialyzed sample was loaded in the Sephadex G-100 column and eluted with excess of 20mMphosphate buffer pH 7.5 in fraction with 3ml per minute speed [19].

2.4. Determination of total protein content

Protein content of each fraction was determined by Lowry's method. Bovine serum albumin (BSA) was used as standard for the plot of standard graph between optical density and concentration. The crude and purified fractions, each of 100 µL mixed with0.5 mL Lowry's reagent and 5mL alkaline copper sulphate were incubated at room temperature for 30 min in dark, and optical density was measured at 660 nm.

2.5. Assay of enzyme activity

The proteolytic and fibrinolytic activities were determined using the casein hydrolysis,[1] fibrin plate [2],[9],[26]and spectrophotometric method[21] .

2.5.1. Determination of Proteolytic activity

Proteolytic activity was assayed using casein as substrate [1]. Since casein is the major protein source for measuring the activity of serine proteases. The casein method was prepared and assayed as follows: 0.5 ml of the Crude and fraction samples were mixed with 1.0 ml 0.05 M phosphate buffer (pH 7.4) containing 1% casein, and incubated for 30 min at 37 °C. The reaction was stopped using 6 ml of 15% trichloroacetic acid (TCA) for 30 min. The reactants were filtered with Whatman No. 1 filter paper. To this 1.0 ml of 0.5N NaOH solution and 1.0 ml of the folin ciocalteu reagent were added. Absorbance of the assays was measured at 660 nm. The assay was performed in triplicate and protease activity expressed in µmol. A standard curve was generated using solutions of 0–1000 µM/mL tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µM/mL tyrosine.

2.5.2. Determination of Fibrinolytic activity– Fibrin Agarose Plate Method

Fibrinolytic activity of crude and purified protein fractions was determined by using fibrin agarose plate method . For the preparation of fibrin plates, a 0.6% (w/v) agarose solution (21 mL), 5% (w/v) bovine fibrinogen 2 ml, 1.2 units/mL of bovine plasminogen 1 mL and 15 units/mL of thrombin 1 mL in 100 mM/L PBS buffer (pH 7.4) were mixed in a Petri dish (diameter, 90 mm). The solution was left for 1 h at room temperature to form a Fibrin clot layer[26]. Wells were punched into the plate using well borer and 200µl of crude and fractions were loaded into the wells and were incubated at 37^{o}C for 15 hr and mean diameter of hydrolyzed clear zones were measured[9].

2.5.3. Determination of Fibrinolytic activity - Spectrophotometric Method

The spectrophometric method of fibrinolytic activity was determined by measuring the liberated α-amino nitrogen.Enzyme was incubated with 100 mM sodium acetate buffer, pH 4.5, and 3% of the fibrin substrate.Assay was carried out at 37^{o}C for 1h and then stopped by addition of 20% (v/v) trichloroacetic acid. After centrifugation, supernatant was added to ninhydrin reagent and boiled for 10 minutes.At an absorbance of 570 nm increase in free amino groups was determined [21].Fibrinolytic activity was calculated using the following formula,

Activity = Micromoles of Tyrosine liberated / Incubation Time x Volume of Enzyme used

2.6. Measurement of molecular weights

The four fractions shown fibrinolytic and proteolytic activity collected for its molecular weight. SDS-PAGE was run according to the method of Laemmli using 12% polyacrylamide gel [15]. The samples were stained with Coomassie brilliant blue R-250. The molecular weights were calculated according to known molecular weight standards.

3. Results and Discussion

Crude extract of earthworm powder was taken and subjected to a cascade of purification techniques. Salt precipitation recovered 100% crude protein with use of 80% ammonium sulphate and excess salt was removed by dialysis. Dialysed samples were loaded in the Sephadex G-100 column. Approximately 40 fractions were collected and each fractions was assessed for proteolytic,fibrinolytic and total protein content.

The earthworm protease functions in the fibrinolysis and plasminogen activation, therefore they have been used to treat the thrombosis [12],[13],[18]. The proteases during oral administration experiment, both in animals and clinics show significant fibrinolytic efficacy. In addition, the proteases are stable during a long-term storage at room temperature [23], in the form of oral capsule. So far, the earthworm proteases have been used as an orally administered fibrinolytic agent to prevent and treat clotting diseases, such as myocardial infarction and cerebral thrombus [11]. In the present study the four fractions showed fibrinolytic activities on the fibrin plate and spectrophotometric method (fig 1&2)(Tab 1&2).Fibrinolytic enzyme assay by spectrophotometric method and fibrin agarose plate method clearly shows strong fibrinolytic activity in fraction 11 but less in other fractions. Thus we concluded that purified protein in selected fractions, F11 was responsible for fibrinolytic activity of earthworm sample. These results are similar to the results shown in the reports of Mihara [20] and Nakajima[22] wherein they found six fractions and they had proteolytic activities. The rank order of the fibrinolytic activity of the four fractions on the fibrin plate was F11 > F6 > F13> F8. Current study demonstrated that the whole earthworm powder of *L.mauritii* has active principles involved in strong fibrinolytic activity. Fibrinolytic activity assay of crude and fractions by Spectrophotometric method shows fibrinolytic activity of the *Lm*P. Highest activity was shown by fraction 11 (5.42units/min) and crude (4.56units/min) whereas other fractions shows very less fibrinolytic activity(tab. 2)

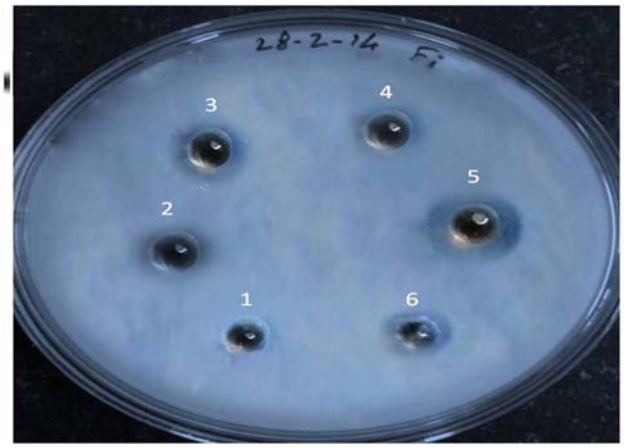

Figure 1: Fibrin Agarose Plate Assay
1-Control, 2-Crude Sample, 3-Fraction F6, 4- Fraction F8, 5-Fraction F11, 6-Fraction F1

Table 1: Mean diameters of the hydrolysed clear zone

Well No.	*Sample*	*Diameter of clear zone(mm) Mean±SD*
1	Control	0
2	Crude	10 ± 0.09
3	Fraction 6	10 ± 0.08
4	Fraction 8	9 ± 0.06
5	Fraction 11	16 ± 0.03
6	Fraction 13	9.5 ± 0.05

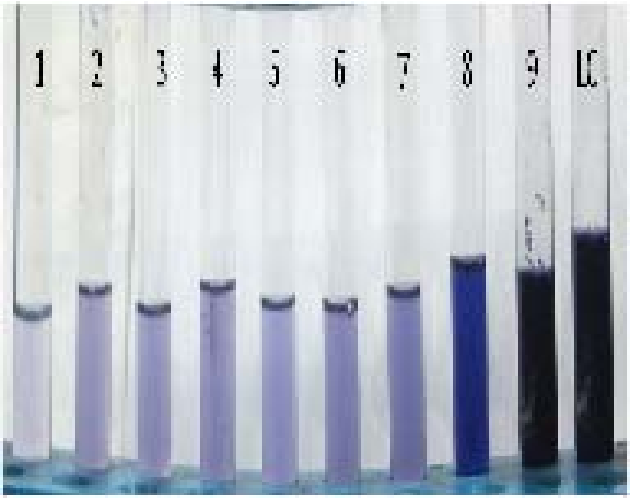

Figure 2: Fibrinolytic Assay by Spectrophotometric method
Standards - [1= 0.05mg/ml, 2= 0.10mg/ml, 3= 0.15mg/ml, 4=0.20mg/ml, 5= 0.25mg/ml], Samples - 6= Crude, 7= Fraction 6, 8= Fraction 8, 9= Fraction 11, 10-Fraction 13

Table 2: Determination of fibrinolytic activity by spectrophotometric method

S.No	Sample	Conc.of Tyrosine (moles)	Absorbance at 570nm	Activity (units/min)
1	Std(0.05mg/ml)	275	0.37	-
2	Std(0.05mg/ml)	550	0.39	-
3	Std(0.05mg/ml)	825	0.43	-
4	Std(0.05mg/ml)	1100	0.46	-
5	Std(0.05mg/ml)	1375	0.47	-
6	Crude	13672.8	1.712	4.56 ± 0.06
7	Fraction 6	474.85	0.389	0.16 ± 0.09
8	Fraction 8	608.55	0.4023	0.2 ±0.01
9	Fraction 11	16252.30	1.0608	5.42± 0.04
10	Fraction 13	1755.67	0.5166	0.59± 0.01

The isoenzymes were shown to be homogenous on 12%SDS –PAGE.(Fig 4).The enzymes migrated as only one band on SDS PAGE. However, the molecular weights that were reported by Nakajima were from 24 to43kDa which differs from these findings [22]. This means that although the isoenzymes were showing similar fibrinolytic activity, their sources and molecular weights were different. Based on these results, each enzyme was considered to consist of a single polypeptide chain. The amount of protein in crude and fractions F6, F8, F11 and F 13 were calculated as 1.8 mg/ml, 0.6 mg/ml, 0.9 mg/ml, 1.6 mg/ml and 1.1 mg/ml respectively (Fig.3).The molecular weights of four fractions F6, F8, F11, F13 from gel chromatography were 100-110kDa, 75-80kDa, 45-50kDa and 35-40kDa respectively (Table. 3 & Fig.4).Crude sample shows multiple bands with varying intensity.

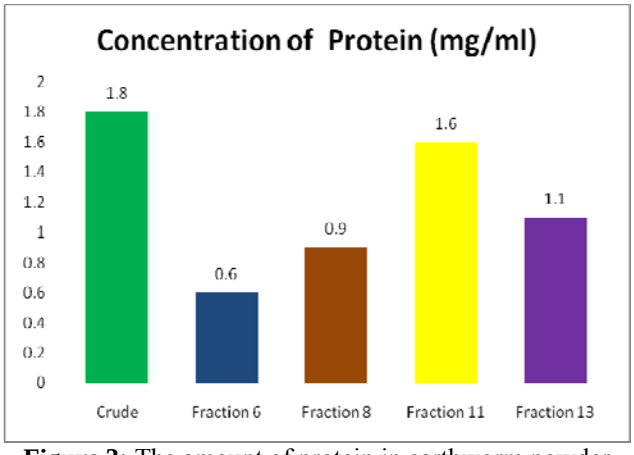

Figure 3: The amount of protein in earthworm powder crude and Fractions 6,8,11 &13

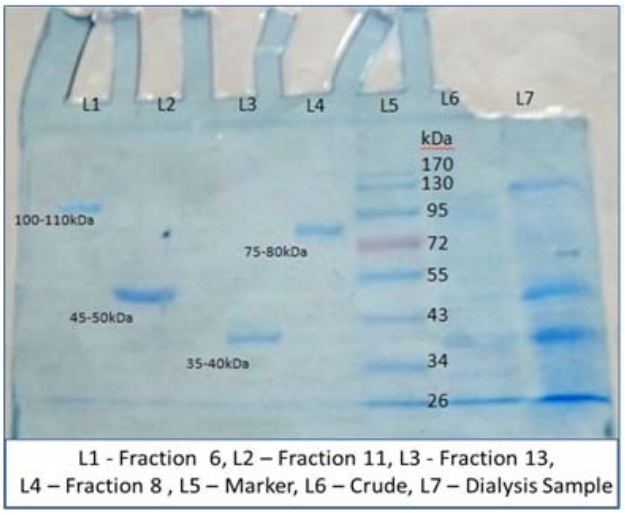

Figure 4: SDS-PAGE analysis of purified protease

Table 3. The Molecular Weight of crude and Fractions 6, 8, 11&13

Samples	*Molecular Weight (kDa)*
Fraction 6	100-110
Fraction 8	75-80
Fraction 11	45-50
Fraction 13	35-40

The activity of casein degradation by the fractions shown in Table. 4 proteolytic activity ranged from 53.5 to 71.4 units per ml with rank order of the activity being F11>F6>F13>F8 showed the highest degradation activity (71.4 U/ml) on the casein substrate. The crude extract of *L.mauritii* powder had 65.1unit/ml specific activities on the casein substrate and contained 1.8 mg/ml of protein. Fractions 6, 8, 11&13 had 69.1, 53.5, 71.4 & 55.1 U/ml of specific activities on the casein substrate and contained 0.6, 0.9, 1.6 &1.1 mg/ml of protein respectively.

Table 4: Proteolytic activity of Earthworm powder (Crude, Fractions 6, 8, 11&13)

Samples	*Proteolyticactivity(U/ml) Mean ± SD*
Crude	65.1733 ± 0.62820
Fraction 6	69.1133 ± 0.63760
Fraction 8	53.5000 ± 0.50000
Fraction 11	71.4500 ± 0.42720
Fraction 13	55.1767 ± 0.43294

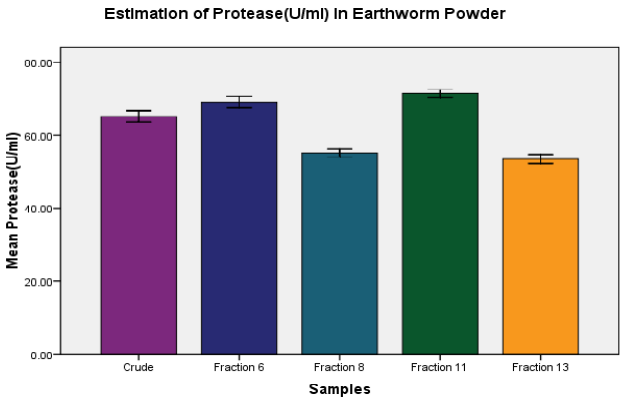

Figure 5: Estimation of Protease (U/ml) in Earthworm Powder. Each value represents mean ± SD, n = 6. The

statistical significance was tested between and within groups of each sample at 0.05 level using one-way analysis of variance (ANOVA)

The earthworm protease blocks the increase in the light scattering intensity, because the enzyme hydrolyzes both fibrinogen and fibrin. The amount of the protease is inversely proportional to the intensity. The proteases have the abilities not only to hydrolyze fibrin and other protein, but also activate proenzymes such as plasminogen and prothrombin. We attempted to isolate and purify four distinct protein fractions from the crude extract, of which 45-50 kDa fraction(F -11) in this study has been found to possess strong protease function. In conclusion, the protease enzyme we obtained from the earthworm *Lampito mauritii* (*La*P) exhibited profound fibrinolytic and protease activity. It may be useful for thrombolytic therapy similar to other potent fibrinolytic enzymes such as nattokinase and Lumbrikinase. This enzyme could provide an adjunct to the costly fibrinolytic enzymes that are currently used for managing heart disease since large quantities can be conveniently and efficiently produced. Therefore, *L.mauritii* powder may become a new source of fibrinolytic enzymes for future applications. Lastly, further studies are necessary to elucidate the characterization of fibrinolytic enzyme from earthworm with emphasis on the aminoacid sequence similarity to those of other trypsin like serine proteases.

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