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)

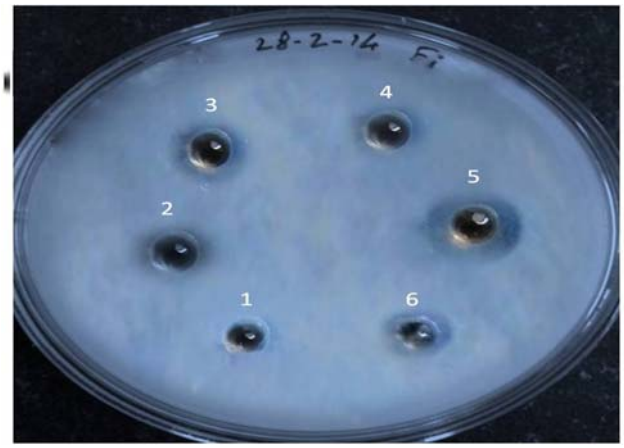


**Figure 1**: Fibrin Agarose Plate Assay
1-Control, 2-Crude Sample, 3-Fraction F6, 4- Fraction F8, 5-Fraction F11, 6-Fraction F1

**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 |

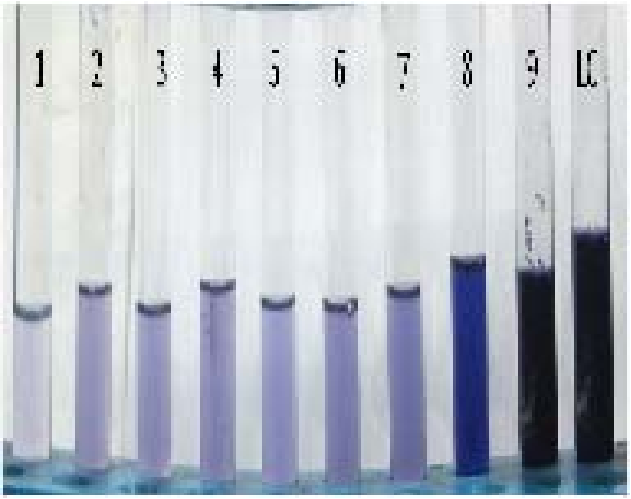


**Figure 2:** Fibrinolytic Assay by Spectrophotometric method
Standards - [1= 0.05mg/ml, 2= 0.10mg/ml, 3= 0.15mg/ml, 4=0.20mg/ml, 5= 0.25mg/ml], Samples - 6= Crude, 7= Fraction 6, 8= Fraction 8, 9= Fraction 11, 10-Fraction 13

**Table 2:** Determination of fibrinolytic activity by spectrophotometric method

| S.No | Sample | Conc.of Tyrosine (µ 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 | 0.4733 | 0.2 ±0.03 |
| 9 | Fraction 11 | 16252.30 | 1.0603 | 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.

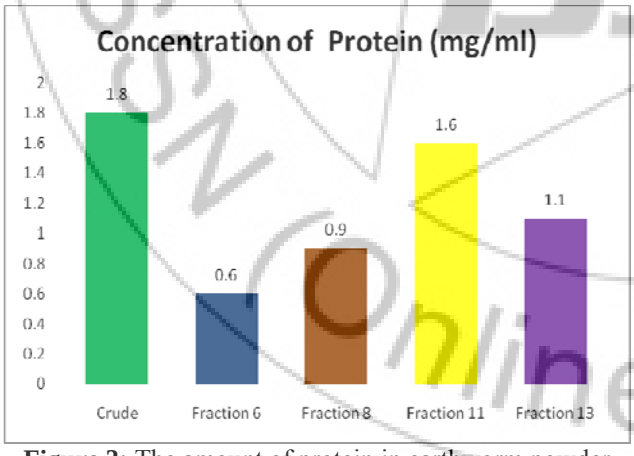


**Figure 3:** The amount of protein in earthworm powder crude and Fractions 6,8,11 &13

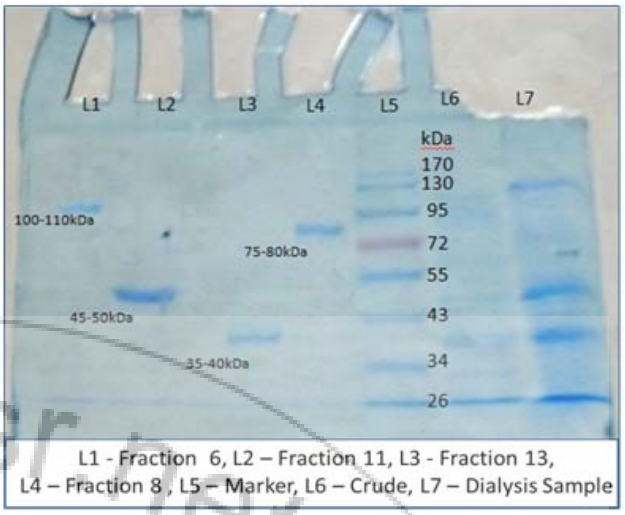


**Figure 4:** SDS-PAGE analysis of purified protease

**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 |

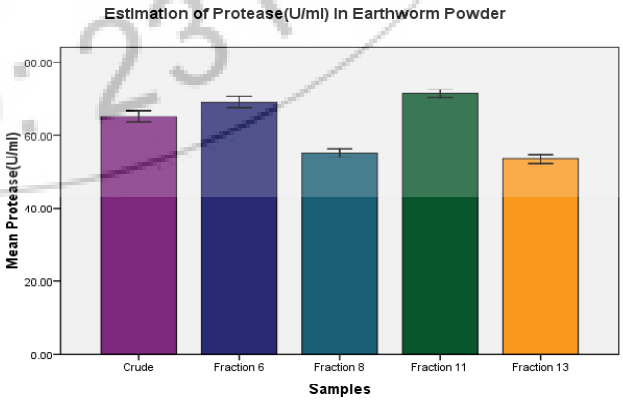


**Figure 5:** Estimation of Protease (U/ml) in Earthworm Powder. Each value represents mean ± SD, n = 6. The

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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