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Isolation and Purification of Thrombolytic Enzyme Extracted from Earthworm Punjab, Pakistan

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Abstract

The Cardiovascular disease due to thrombus (clot) formation is the major factor of death throughout the world. Earthworms being the eco engineers has thrombolytic enzyme that can be used for thrombolysis. The thrombolytic enzyme was isolated and purified from supernatant of earthworm *Apporectodea longa* by column chromatography. Six Strain BKT 11, BKT 15, BKT 17, BKT 26, BKT 27 and BKT 28 shows the thrombolytic activity 791.64 U/mg, 1362.39 U/mg, 1205.4 U/mg, 710.63 U/mg, 529.66 U/mg and 625.00 U/mg respectively. Thrombolytic activity was confirmed by blood clot lysis method. Different concentrations 50 µl,100 µl, 150 µl, 200 µl and 250 µl of extracted enzyme were applied on 25mg of wet blood clot along with control where distill water used. These fractions of extracted enzymes represent the dissolution of clot (thrombolysis). The molecular weight 32 KDa was determined by sodium dodecyl sulphate gel electrophoresis (SDS-PAGE). Results show that extracted elute have potential of fibrinolytic activity in this specie of earthworm and it can serve as a suitable therapeutic agent.

Key words: Thrombolytic activity, Casein plate assay, Blood clot lysis, spectrophotometry, Gel electrophoresis.

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1. INTRODUCTION

Thrombolytic enzymes among various organisms were identified like snakes, bacteria, and fungi 1. Various foods also used in thrombolytic therapy in managing cardiovascular diseases 2.

Various blood clot dissolving agents has been used for thrombolysis like urokinase, streptokinase and tissue plasminogen activator (t-PA). Major Key factor is the balance between blood flow and thrombus formation that prevent the clot formation. When this balance is disturbed thrombus formation occurs 3. Many enzymes having thrombolytic activity were reported, and they are being used for thrombolysis but due to expensiveness, and undesirable side effects they are not economical 4.

Earthworms are responsible for physical as well as chemical changes in soil and ultimately increase the soil fertility. Earthworm has diverse habitat. They mostly occur at those sites that are rich in organic matter like manures, litter, compost etc. They are also present in fresh and brackish water. They are also found in hydrophilic environment close to both fresh and brackish water, some species can survive under snow 5.

There is large number of microorganisms present in the gut of earthworm. In addition to the microbes, they also contain wide variety of enzymes and hormones. After short period of time the semi digested waste later on converted into vermicompost 6.

Economically earthworms are very important they are called ecological engineers. They increase the fertility of soil. Being a soil dweller, they are helpful in maintaining balance in ecosystem regarding soil fertility 7.

Immunoglobulin recognition is one of the fascinating zones to use earthworm in medicine. They produce various types of leucocytes and immune protective molecules. They also have innate and adaptive immunity 8. Various species of earthworm has fibrinolytic activities such as Lumbricus rubellus, Esnia anderia and Pirenx excavates. There is potential to used them against thrombosis9. The earthworm proteases having fibrinolytic activity preferred to use as it is easy to raise earthworm, inexpensive and orally administered 10.

In addition to thrombosis antitumor activity is also reported in earthworm. It is reported that the fibrinolytic enzyme obtained from E. foetida show antitumor activity against human hepatoma cell both in vivo and in vitro 11. It also founded that earthworm fibrinolytic enzyme can induce apoptosis in hepatocellular carcinoma that is the third leading cause of cancer 12.

Thrombosis is the major cause of various heart disorders leading to death. This project aimed to isolate and purify the thrombolytic enzyme from Apporectodea longa specie of earthworm from district Gujrat – Pakistan.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of Earthworm

Earthworm were collected from different localities that are 20-25 km apart of District Gujrat by simple hand sorting method and identified by their morphological characters. The identified earthworms were preserved in 90% ethanol solution.

2.2 Screening of Thrombolytic Enzyme

For screening of thrombolytic enzyme partial purification followed by complete purification was done by ammonium sulphate precipitation and column chromatography respectively.

2.3 Partial Purification

Ammonium sulphates was weighted and added in 10 ml of the sample under 60% saturation at room temperature and left overnight at 4 ◦C. Next day the sample was loaded in the membrane at room temperature. The sample along with membrane was placed inside the beaker containing Tric-HCl buffer and left-over night for dialyzing. After 24 hours sample was removed from the membrane and poured inside the sterile falcon tube and stored at 4 ◦C.

2.4 Complete Purification

For complete purification of enzyme Column (Sephadex G-75 column) was assembled for sample loading then spectrophotometry was done by using spectrophotometer (Humas Korea Model # Think Hs 3300).

2.5 Enzyme Assay

Casein plate assay and blood clot lysis was done for enzyme assay. Enzyme activity was determined by the blood clot Holmstrom method 19.

a) Caseinolysis

Casein plate method was used for enzyme assay 13,14. For preparation of casein solution 0.5 g of casein along with 1.5 g of bacterial agarose dissolved in 10 ml of distill water at pH 9. The solution was placed in microwave for 30 sec to mix gently and make homogenized solution. Casein solution along with petri plates was autoclaved. The autoclaved petri plates were placed inside the laminar flow to avoid microbial contamination. The ager-casein solution was poured in petri plates under sterile condition inside laminar flow. 200 µl of enzyme were poured on the prepared plates and then packed with the help of tape. After 10-15 min of pouring the enzyme plates were placed inside the incubator at 37 ◦c and left overnight.

b) Blood Clot Lysis Method

Blood samples were taken from NSMC (Nawaz Sharif medical college)- UOG diagnostic lab. Serum removed and blood clot was used to check thrombolytic activity. Blood clot was taken in micro centrifuge tube. 200µl of enzyme was added in experimental tubes. While equal volume of distal water was added in the control. Enzyme activity was checked on different intervals of time.

2.6 Molecular Weight Determination

For determination of molecular weight SDS –PAGE was done with suitable marker. Elutes obtained from column chromatography were further analyzed by SDS-PAGE. 12% polyacrylamide gel was used for SDS-PAGE. Laemmli protocol was followed for SDS PAGE 15.

3. RESULTS AND DISCUSSIONS

After identification of Apporectodea longa species isolation of enzyme was performed. Extracted enzyme activity was later measured by casein plate method which represent the fibrinolytic activity. Various fractions of extracted enzyme were used which lyse the casein media lead to the formation of halo whose diameter varies indicate lysis occurred. To further confirm the fibrinolytic activity blood clot lysis was performed that proved the activity of enzyme. 200 µl of enzyme can lyse the 25 mg of blood clot efficiently. SDS-PAGE reveals protein has molecular weight 32 KDa.

Collection of samples

Preserved earthworms were identified to be Aporrectodea longa after screening. The general morphological features of this species are described in table 1.

Table 1. - Morphological Characters of Aporrectodea longa

Characters	Aporrectodea longa
Length	90-170 mm
Diameter	4.0-9.0 mm
Saddle	27/28-35/36
Body color	Brown–purple on upper side, pale on underside. Head end is darker then tail end.
Glandular area	9, 10 and 11 segment under side

50 elutes were obtained after partial and complete purification that were further proceeded by spectrophotometry.

3.1 Spectrophotometric Analysis

The purified crude enzyme from sephadex G 75 obtained. Absorbance values of 50 fractions at 595nm were measured and enzyme concentration was measured by using Bradford standard curve. The standard curve

indicates that fractions BKT 11, BKT15, BKT17, BKT 26, BKT 27 and BKT 28 have highest concentration of enzyme.

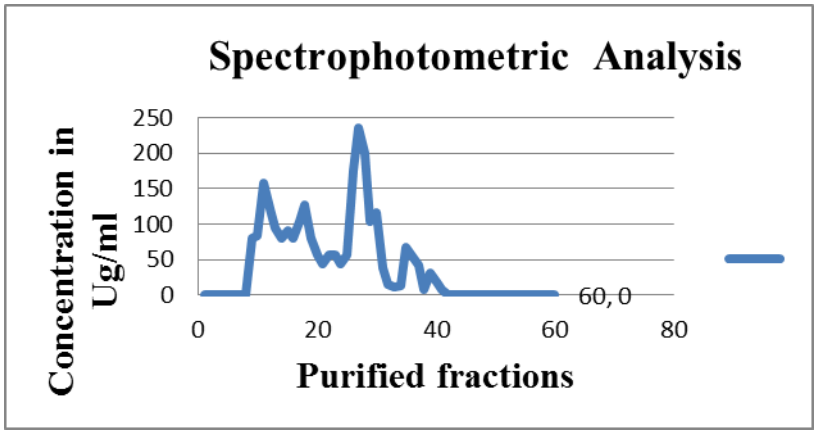

Fig.1. Relative concentration of enzymes purification

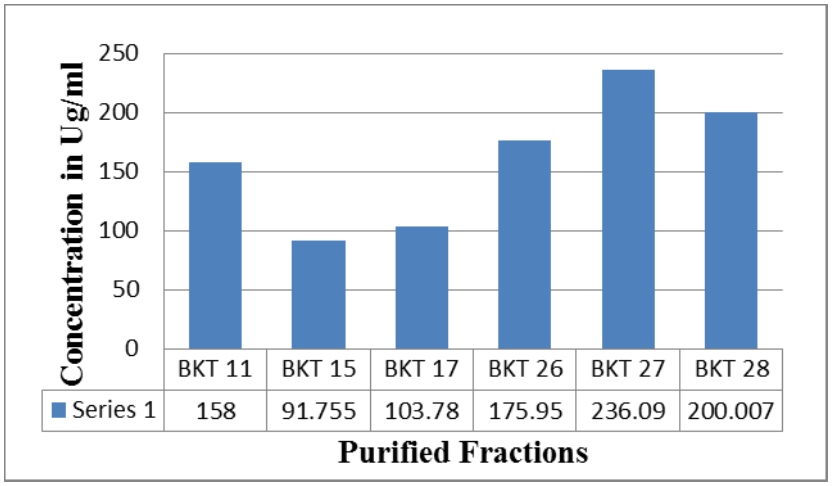

Fig. 2. Amount of enzyme extracted from sample BKT 11, BKT 15, BKT 17, BKT 26, BKT 27 and BKT 28.

3.2 Enzyme Assay

a) Caseinolysis

Casein –agar plate assay used to find out the fibrinolytic activity in different studies. The hydrolyzed region or area of halo basically indicates the trend of lysis ability 16. In case of some bacteria the trend of lysis is very low while in others large halo was appeared 17. It is also reported that in some cases certain developing agents are required to detect the lysed region. In the present study the large area of lysed region was detected, and it is visible without any dye.

Six fractions of the enzyme after spectrophotometry showed maximum peak so these six fractions were subjected to the casein plate assay. Of these three BKT 26, BKT 27 and BKT 28 showed the enzyme activity that was indicated by halo formation. Mean diameter was 2.8cm, 3.6cm and 3.5cm respectively.

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Table 2.- Elute Fractions Having Caseinolytic Activity

Elute Fraction	Halo Formation	Diameter in cm
BKT 11	-	-
BKT 15	-	-
BKT 17	-	-
BKT 26	+	2.8cm
BKT 27	+	3.6cm
BKT 28	+	3.5cm

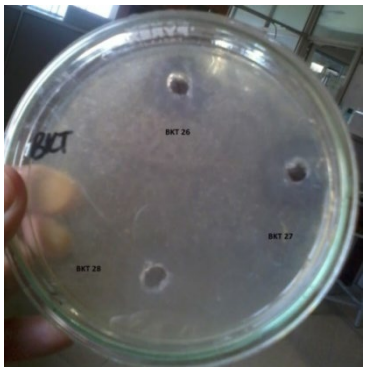

Fig. 3. The elute BKT 26, BKT 27 and BKT 28 lysed the casein plate confirmed the enzyme activity.

a) Blood Clot Lysis

Different volumes of enzyme extract were poured on the 25mg wet blood clots. After incubation period of 24h at 37ºC the clot lytic activity was observed and is described in the table 3.

Table 3. Hydrolytic Activity of Enzyme For 25 Mg of Clot For 24h Incubation At 37 ºc.

Volume of enzyme poured (µl)	Lysis of blood clot (25 mg wet)
50	No lysis
100	Partly hydrolyzed
150	Partly hydrolyzed
200	Complete hydrolysis
250	Complete hydrolysis
50	No lysis

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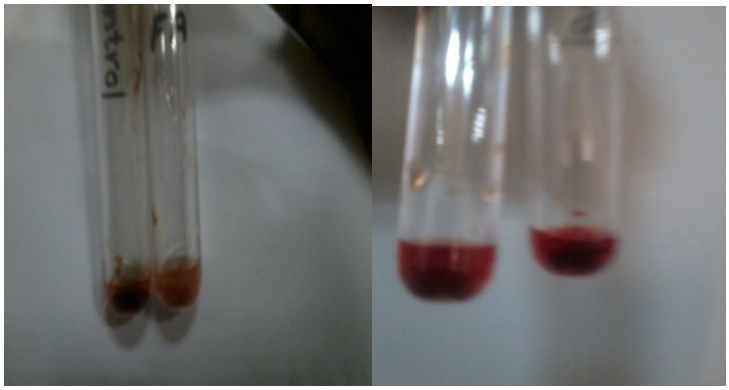

Fig. IV. (a) **Fig. IV. (b)**

Fig. 4. (a) Control with no lysis of blood clot and sample contain 200 µl of enzyme showing lysis. (b) indicated the clot lysis with 250 µl of enzyme.

The results showed that 250 µl of enzyme completely solubilized the clot. We consider that the amount of enzyme which hydrolyses 1 mg of clot is equal to 1Unit enzyme activity.

1 mg blood clot solubilized = 1 unit enzyme activity

It is concluded that 250 µl of enzyme completely solubilized the 25mg of clot it means that 10 ml of enzyme extract has 25Units in each 250 µl of extract. The enzyme activity is measured in enzyme unit. 1 ml of blood clot that is lysed is consider as 1 unit enzyme activity. The results indicate that samples have fibrinolytic activity. Enzymes activity is in range of 40-100 µl. The least concentration that completely lyses the 1ml of clotted blood is 40 enzyme units.

Table 4. - Least Quantity of Enzyme Required for Clot Lysis from Earthworm Samples.

Sample	10 µl	20 µl	30µl	40µl	50µl	60µl	70µl	80µl	90µl	100µl	Control
BKT 11	-	-	-	-	+	+	+	+	+	+	-
BKT 15	-	-	-	-	-	-	+	+	+	+	-
BKT 17	-	-	-	-	-	+	+	+	+	+	-
BKT 26	-	-	-	+	+	+	+	+	+	+	-
BKT 27	-	-	-	-	+	+	+	+	+	+	-
BKT 28	-	-	-	-	+	+	+	+	+	+	-

Table 5. - Least concentration that give complete lysis of 1ml of clotted blood at 37 ◦c.

Samples	**BKT 11**	**BKT 15**	**BKT 17**	**BKT 26**	**BKT 27**	**BKT 28**
Enzyme (µl)	50	70	60	40	50	50

These results indicate there is a strong fibrinolytic activity in earthworm Aporrectodea longa.

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3.4 Molecular Weight Determination

SDS PAGE represent those various proteins having low molecular weight are present in extracted solution. The specific enzyme having fibrinolytic activity from Aporrectodea longa has molecular weight of about 34 KDa indicated in figure as band. According to Phan. B. T. T *et a*l the molecular weight of fibrinolytic enzymes was in the range of 28-35 KDa from earthworm specie Perionyx excavates18.

These results indicate there is a strong fibrinolytic activity in earthworm *Aporrectodea longa.*

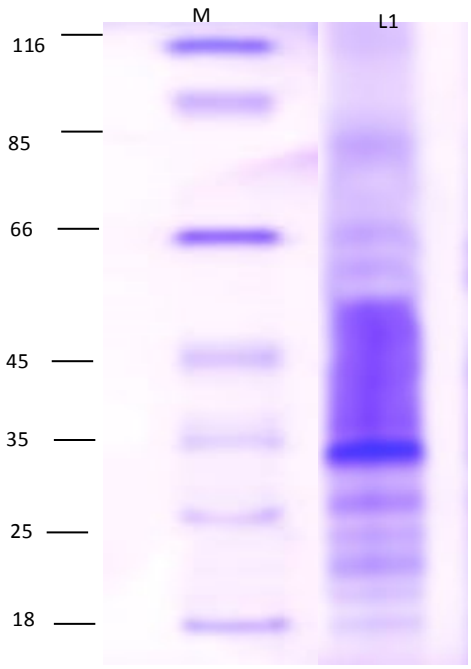

Fig. 5. SDS-PAGE of purified enzyme with Coomassie blue staining (Lane 1: Enzyme fraction and Lane M contains marker. The numbers on left side correspond to the positions of molecular weight markers).

This article describes the isolation and purification of fibrinolytic enzyme from earthworm (Apporectodea longa) for the assessment of its use as a thrombolytic agent. Sumi et al reported that strain extracted from bacteria were orally administered did not have a strong impact on thrombolysis.

Earthworm have vermicultured for disposal of waste as well as for increasing the fertility of fields in 1899 it was reported that certain enzymes were secreted from the gut of earthworm that could dissolve fibrin.20

Earthworm fibrinolytic enzyme component A (EFEa) from Eisenia fetida is a strong fibrinolytic enzyme that not only directly degrades fibrin, but also activates plasminogen.21 Likewise fibrinolytic activity was found in Apporectodea Longa in present study. This proves that earthworm can be the potential source for thrombolysis and it could be economical for the patients if extracted from this cheap source.

4. CONCLUSION

Among the collected specimens of, during the study, from different habitats of Tehsil Gujrat Punjab, Pakistan Aporrectodea longa was the most abundant specie. Results indicate elute this specie has potential thrombolytic activity. Further studies needed to find out the way to formulate the drug to get benefit from this specie in control of cardiovascular disorders.

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CONFLICT OF INTEREST

Authors have declared no conflict of interest.

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