Isolation, Screening, Characterization and Production of Fibrinolytic enzyme from marine microorganism

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ABSTRACT

Fibrinolytic enzymes are agents that dissolve fibrin clots. These fibrinolytic agents have potential use to treat cardiovascular diseases, such as heart attack and stroke. The present study aimed at the production, purification and characterization of fibrinolytic enzyme from the bacteria isolated from sea water. The isolated organism was cultured in the Luria Bertani broth (LB broth) at 37ºC for 24 hrs. The extracellular enzyme was extracted and the assays like fibrinolytic, caesinolytic, albuminolytic activity was done. The fibrinolytic activity of that enzyme was twofold higher than that of caesinolytic activity and 10 fold higher than that of albuminolytic. The activity of the enzyme was also checked on RBC and on the animal tissue and no damage was occurred. The optimum pH and temperature were 7 and 45°C. Enzyme was purified using differential ammonium sulphate precipitation. The 75% saturation was found to be more active and it was further purified by using Affinity chromatography run in SDS-Polyacrylamide Gel Electrophoresis. It was characterized as metalloprotease by using protease inhibitors. The biochemical characterization of the organism was carried out and compared with the PIB (Probability Index of Bacteria)win matrix and finally it was identified as Vibrio campbelli.

Keyword : - *Fibrin, Fibrinolytic activity, PIB, Affinity chromatography.*

1 INTRODUCTION

Blood clot in the blood vessel is called **thrombus.** . Thrombus is formed when the body has been injured or the blood vessels are damaged by arteriosclerosis or chronic bed rest it leads to blood stagnates. The block occurs in the greater portions of the blood vessel and causes the cardiovascular diseases like heart attack and cerebrovascular diseases like stroke **[43]**.

The plasmin produced through the streptokinase mediated activation of plasminogen breaks down streptokinase. This limits the in vivo half-life of streptokinase to about 30 min. Although streptokinase survives in circulation significantly longer than does tPA (a half-life of about 5 min), this is still short for efficient therapy [45]. Unlike microbial streptokinase, human urokinase is not antigenic or pyrogenic, but its recovery from urine is expensive and supply is limited for any extensive use in therapy [44]. Like streptokinase, urokinase activates both the circulating plasminogen and the clot-bound plasminogen. Because plasminogen activation by uPA and streptokinase is not specific to the clot-bound plasminogen, the use of these activators is associated with a serious risk of hemorrhage [44].

tPA has been developed for the treatment of thrombosis because of its efficacy and stronger affinity to fibrin [26]. In this paper we report the production and purification of fibrinolytic enzyme from Marine microorganism which particularly targeting on Fibrin.

2. MATERIALS AND METHODS

2.1 ISOLATION OF MICROORGANISM:

Preparation of Fibrin

The blood was collected from the chicken in a container containing citrate buffer. Then the blood was centrifuged at 6,000 to 7,000 rpm for 10 minutes until it becomes clear solution. Then the plasma was collected and poured it in to the petriplate. To that 200µl of Cacl2 was added and it was allowed to clot. It was then washed three times with water to remove the soluble proteins like albumins and globulins and it was dried and powdered. The dried powder is the fibrin.

Isolation of Microbe

The broth was prepared with the minimal media and fibrin and inoculated it with 10ml of raw seawater. It was incubated at 37°C for overnight.

2.2 INOCULATION IN ENRICHMENT MEDIA

The grown colonies in the minimal broth containing fibrin were enriched by spread plated on nutrient agar. It was then incubated at 37°C for 24 hrs. White and yellow colonies were formed and it was named as fl17 and fl18.

2.3 SCREENING OF THE COLONIES:

2.3. a FIBRINOLYTIC ASSAY BY FIBRIN AGAR PLATE[6]:

The colonies grown on the nutrient agar Plate was patched over the fibrin agar. The plate was then incubated in 37º for 24 hours. The zone was measured and the yellow colony (fl18) showing larger zone and in the tube method also fl18 is showed more activity was taken for further study.

Fig.1 : Enzymes dissolving the clot. Fl18 is showing more activity

2.4 ENZYME PREPARATION [1]

The fl18 was grown in peptone broth for 24hrs at 37°C and it was centrifuged at 6,000 to 7,000 rpm for 10 minutes. Then 20µl of chloroform was added to the supernatant and this crude enzyme was subject to further analysis.

2.5 ENZYME ASSAY

The comparison of Enyme activity was done by Fibrinolytic activity, Caesinolytic activity and albuminolytic activity to find out the specificity of the enzyme.

Table- 1: Fibrinolytic Activity

Control	Test	Zero Control
0.5gFibrin + 500 µl of tris+50µl water	0.5gFibrin + 500µl of tris+50µl crude enzyme	0.5gFibrin + 50µl crude enzyme (no Incubation)

Table- 2: Caesinolytic Activity

Control	Test	Zero Control
50µl milk+ 500µl of tris+50µl water	50µl milk+ 500µl of tris+50µl crude enzyme	50µl milk+ 500µl of tris+50µl crude enzyme +0.6ml TCA (no incubation)

Table- 3: Albuminolytic Activity

Control	Test	Zero Control
50µl BSA+ 500µl of tris+50µl water	50µl BSA+ 500µl of tris+50µl crude enzyme	50µl BSA+ 500µl of tris+50µl crude enzyme +0.6ml TCA (no incubation)

This set up was kept for 3 hours incubation. It was stored in 4°C for overnight and centrifuged at 10,000 rpm for 10min.the pellet was dissolved in 0.6ml of 0.1M NaOH. It was subject to protein estimation (Lowery et.al method). Then the activity of the enzyme was calculated

2.6 ENZYME PURIFICATION

10ml of crude enzyme was taken in 4 test tubes. To each test tube different concentration of $(NH_4)_2SO_4$ was added (25%, 50%, 75%, and 100%). It was dissolved properly and overnight incubation at 4°C and centrifuged at 7,000 rpm for 20 mins. The pellet was collected and suspended in 1ml of 10mM Tris and gone for dialysis. The enzyme further purified by affinity chromatography. The fibrin was taken and it was equilibrate with 0.1M Tris buffer pH 7 and it was washed thrice with Tris buffer and packed in to the glass column (5 by 60 cm) and it was eluted by NaCl

gradient at a rate of 2.0ml/min at 4°C. the fractions were mixed with acetone and allowed to stand at 4°C for 16 hours and the precipitate was taken and run it on the SDS-PAGE.

2.7 EFFECT OF ENZYME ACTIVITY AT DIFFERENT pH [46]

The optimum pH of the enzyme was identified by dissolving pure enzyme in Citrate buffer 0.1M pH – 6 and 7, Tris buffer 0.1M pH – 8 and 9, Phosphoric acid buffer 0.1M pH – 10 and 11.then the mixture was added to the fibrin and it was incubated for 3 hours and it was centrifuged at 10,000 rpm and the pellet was dissolved in 0.1M NaCl and this was taken protein estimation to find out the activity.

2.8 ENZYME ACTIVITY ON RBC [41]

The specificity of the enzyme only on fibrin and not on RBC was checked .

Table -4: Enzyme Activity on RBC

TEST fl_{18}	CONTROL
RBC from 1ml blood	RBC from 1ml blood
500µl Saline	500µl Saline
100µl Enzyme	100µl Saline

suspend it and kept for 3hrs incubation. Then it was centrifuged at 3,000 rpm for 10mins. The supernatant was taken and mixed with 2ml of saline. The O.D. values were measured at 520 and 540 nm

2.9 ENZYME ACTIVITY ON ANIMAL TISSUE:

The enzyme used to treat blood clot should not damage or pierce the vein. The enzyme is injected along with the dye. If any damage causes to vein the dye will come out in to the solution. Hepatic portal vein was collected from the goat. It was then injected with 125µl of dye to the control. For test the enzyme and dye is mixed well (1:2) then 125µl of this was injected to the vein. It was tied at the ends using the thread and placed in the eppendrofs containing saline. It was kept for 3hrs incubation.

Fig.2 : Enzyme activity on Animal tissue

2.10 ENZYME CHARACTERISATION

Purified enzyme was dissolved in 10 mM Tris buffer (pH 10.0) and mixed with each salt solution to give a final inhibitor concentration of 0.1 or 1.0 mM. Enzyme samples were separately incubated at 37°C for 10 min with each of the following inhibitors phenylmethylsulfonyl fluoride (a serine protease inhibitor), EDTA(a metalloprotease inhibitor), e-aminocaproic acid, E64 (cysteine protease inhibitor) and 2,4-dinitrophenol. Residual activity was then determined.

3. RESULTS

3.1 ENZYME ASSAY:

Enzyme activity was found to be two times more Fibrinolytic than Caesinolytic activity and 10 times more Fibrinolytic than the Albuminolytic.

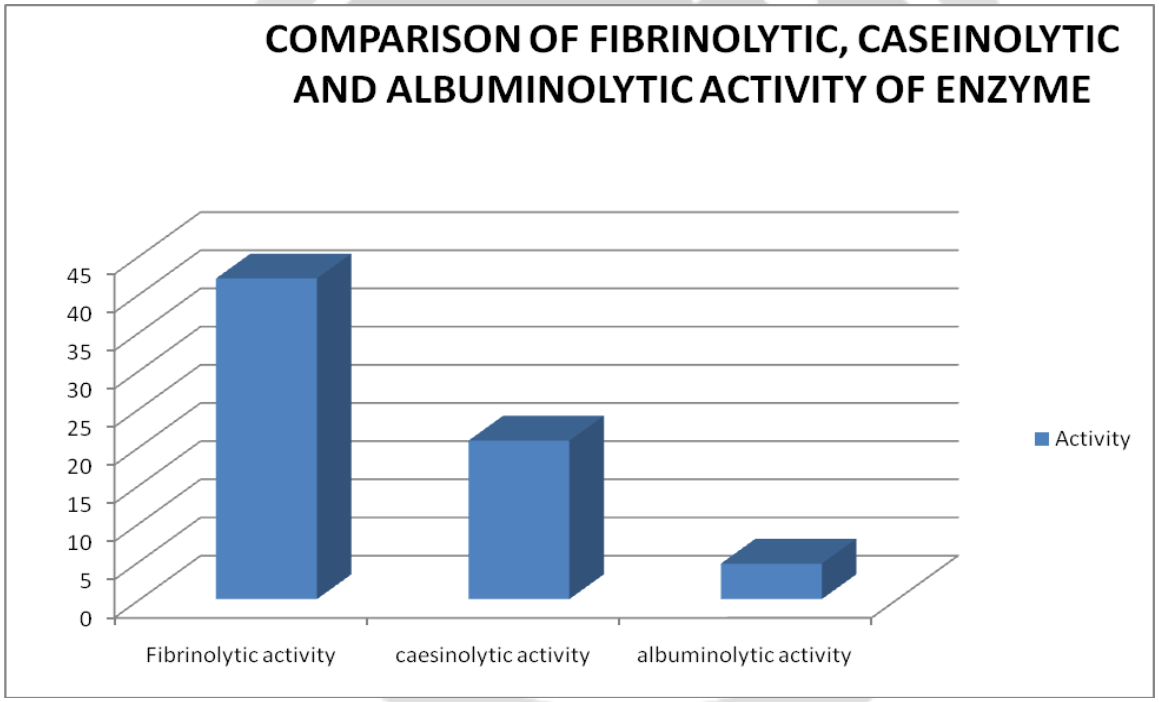

CHART-1:Specificity of Enzyme activity

3.2 ENZYME PURIFICATION:

The enzyme purified from the Affinity chromatography and it was run on the SDS-PAGE

3.3 EFFECT OF ENZYME ON DIFFERENT pH:

The activity was found to be more in the case of pH 7

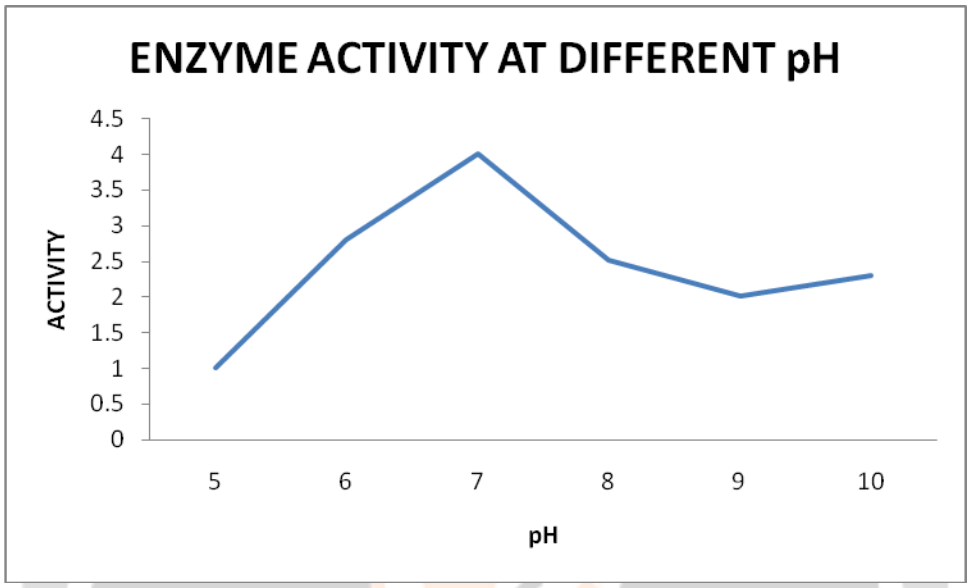

CHART-2: Enzyme activity at different pH

3.4 ENZYME ACTIVITY ON RBC:

Table-5: Enzyme activity on RBC

Nanometer	**520nm**	**540nm**
TEST fl_{18}	0.12	0.11
CONTROL	0.11	0.10

No activity because OD values are not negligible

3.5 ENZYME ACTIVITY ON ANIMAL TISSUE

No colour change was observed in the eppendrof containing saline. It indicates there is no activity of the enzyme on animal tissue

3.6 ENZYME CHARACTERIZATION

The activity of the Enzyme is totally arrested in the case of EDTA added enzyme so the enzyme could be characterised as metalloprotease enzyme

3.7 BIOCHEMICAL CHARACTERIZATION:

TABLE -6: BIOCHEMICAL CHARACTERIZATION

TEST	RESULT
Glucose Utilization	+
Citrate utilization	+
Oxidase	+
Catalase	+
Anaerobic test	+
Amylolytic activity	+
TCBS	+
Lysine Decarboxylase	-
Arginine Decarboxylase	-
Ornithine Decarboxylase	-
Nutrition requirement	-
Nitrogen Requirement	+
Nitrate reduction	+
D-Mannitol	-
Na requirement	+
Swarming	-
Voges Proskauer	-
Ampicillin resistance	+

By comparing those results with PIB win matrix the ID score is 0.96, so most probably the organism is *Vibrio Campbelli* [2]. It shows the characteristic feature of C*hromobacterium fluviatile* also but it does not grow in TCBS agar. ID score is 0.[14]..

4. DISCUSSION

Streptokinase, Urokinase and tissue plasminogen activators are the general enzymes that are used to dissolve blood clots in our body. They trigger the production of plasmin from plasminogen and there by cleave the blood clot at the lysine and arginine residues. These enzymes have a low specificity to fibrin and are expensiv[34]. In this present

study the enzyme extracted from *vibrio campbelli* acts directly on the blood clot or fibrin itself. It is highly specific to fibrin.

The enzyme extracted from the bacillus natto (Nattokinase) is specific to fibrin, casein, albumin and it shows broad specificity. The Vibrio Extracellular protein extracted from Vibrio vulnificus also shows broad specific activity on fibrin, casein, albumin [1]. In this present study fibrinolytic, caseinolytic, albuminolytic activity is done. It shows less caesinolytic and albuminolytic and more fibrinolytic. The albuminolytic activity is ten times less than that of fibrinolytic activity. It is concluded by Lowry et al., method.

The nattokinase was stable within the pH of 6 to 10 and at a temperature of 40ºC. The activity was maximum at pH 8. The Vibrio extracellular enzyme extracted from Vibrio vulnificus shows optimal activity at 7.5 -8. [1]. In this present attempt, enzyme is stable at the pH 5 – 11, the activity was more at pH 7 and it was stable at 40°C as like that of nattokinase. When the nattokinase was dropped over the fibrin clot, it dissolves the whole fibrin clot in 18hrs at 37 ºC [34]. In present study the extracellular enzyme from Vibrio campbelli dissolves the clot completely in 24hrs at 37 ºC.

The activity of nattokinase on RBC was checked and it is found to be less aggregation of RBC and no lysis of RBC was occur [41]. Here the RBC lysis test is performed and the enzymes did not lyse the RBC thereby indicating that the enzyme does not act on RBC and lyse only the fibrin, so the specificity towards fibrin is more for this enzyme. The activity of the enzyme is checked in animal (goat) vein, the enzyme does not cause any damage to the vein. It is traced with the help of inert dye.

The metalloprotease enzyme from Vibrio vulnificus was purified by differential ammonium sulphate precipitation. It showed more activity at 70% [1]. The purified enzyme by differential ammonium sulphate precipitation gives the result at 75% the enzyme is more fibrinolytic activity, so the purification is more at 75%. This forms first step in purification.

The enzyme from the bacillus strain CK -114 was characterized by treatment with protease inhibitors like EDTA, PMSF, EGTA, 2,4 dinitrophenol. The EDTA, EGTA, 2,4 dinitrophenol does not show any inhibition. The enzyme activity was inhibited with PMSF. So it was characterized as alkaline serine protease [42]. The enzyme is treated with protease inhibitors like EDTA and PMSF, the fibrinolytic assay is carried out. The results yield that EDTA treatment inhibits the activity of the enzyme. The treatment with PMSF does not alter the characteristics of the enzyme. So the enzyme could be or most probably a metalloprotease. And these results suggest that the enzyme may not be a Serine protease. Then, enzyme can be purified further by affinity column chromatography and run it on SDS-PAGE

5.CONCLUSION

The present study shows that the production, purification and characterization of fibrinolytic enzyme from the bacteria isolated from sea water. The fibrinolytic activity of that enzyme was found to be twofold higher than that of caesinolytic activity and 10 fold higher than that of albuminolytic. The activity of the enzyme was also checked on RBC and on the animal tissue. These results indicate the specificity of the enzyme activity only on the fibrin and it also shows the tissues are not damaged. If we go for further purification and more clinical trials are required. If it shows positive results then the enzyme can gain more importance in the treatment of cardio-vascular disease and stroke.

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